Oxidation of Microcystis aeruginosa and Anabaena flos-aquae by ozone: Impacts on cell integrity and chlorination by-product formation

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A B S T R A C T

Pre-ozonation of cyanobacterial (CB) cells in raw water and inter-ozonation of settled water can cause CB cell damage. However, there is limited information about the level of lysis or changes in cell properties after ozonation, release of intracellular compounds and their contribution to the formation of disinfection by-products (DBPs). This study aims to: (1) assess the extent of the pre-ozonation effects on CB cell properties; (2) determine the CT (ozone concentration × detention time) values required for complete loss of cell viability; and (3) study the DBPs formation associated with the pre-ozonation of cyanobacterial cells in laboratorial suspensions. To these ends, both Microcystis aeruginosa and Anabaena flos-aquae suspensions were prepared at concentrations of 250,000 cells mL−1 and 1,500,000 cells mL−1 and were subjected to ozone dosages of 0.5, 2.0 and 4.0 mg L−1 at pH 6 and pH 8. A quick and complete loss of viability was achieved for both CB species after exposure (CT) to ozone of <0.2 mg min L−1, although no significant decrease in total cell numbers was observed. Maximum dissolved organic carbon (DOC) releases of 0.96 mg L−1 and 1.63 mg L−1 were measured after ozonation of 250,000 cells mL−1 of M. aeruginosa and A. flos-aquae, respectively. DOC release was found to be pH and ozone dose dependent. Ozonation of CB cells increased formation of trihalomethanes (THM) and haloacetic acids (HAA), mainly for suspensions of A. flos-aquae at pH 8 (by 174% and 65% for THM and HAA respectively). Utilities considering using ozone for oxidising CB cells should weigh out the benefit of CB control with the potential increased formation of chlorinated DBPs.

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1. Introduction

The presence of cyanobacteria is ubiquitous in the environment. Cyanobacteria has been reported in water sources, such as lakes and reservoirs (Oberholster et al., 2006; Carvalho et al., 2008; Sömek et al., 2008; Graham et al., 2010), where they may cause drastic changes in turbidity, pH, dissolved oxygen and taste and odour. These variations in water quality can negatively impact treatment processes used for drinking water supply. Consequently, cyanobacterial blooms pose a great challenge for drinking water production (Merel et al., 2010; Zamyadi et al., 2012a).

Increases in cell concentrations and metabolic substances produced by cyanobacteria, referred to as algalogenic organic matter (AOM), are responsible for a series of disturbances in water treatment processes (Henderson et al., 2008b). AOM includes extracellular organic matter (EOM) and intracellular organic matter (IOM). EOM results from the metabolic activity of cells during exponential and stationary growth phases whereas IOM can be released in the aquatic environment following cell lysis. The latter can be induced by aging of the algae population (Pivokonsky et al., 2006; Henderson et al., 2008a) and by water treatment processes such as oxidation (Plummer and Edzwald, 2002; Huang et al., 2009).

Although conventional treatment processes (coagulation/flocculation, sedimentation/flocculation and sand filtration) are effective in removing intact cyanobacteria cells (Chow et al., 1999; Teixeira and Rosa, 2006), the massive presence of algae and cyanobacteria may reduce cell-removal efficiency (Zamyadi et al., 2012b). Pre-oxidation, especially with chlorine, and cyanobacteria may reduce cell-removal efficiency, and other intracellular metabolites (Plummer and Edzwald, 2001, 2002; Chen et al., 2009; Ma et al., 2012a). However, the use of oxidants can also cause damage to cyanobacteria cells and promote the release of IOM, including intracellular metabolites such as MB (2-methyl isoborneol), geosmin or cyanotoxins (Daly et al., 2007; Lin et al., 2009; Miao and Tao, 2009; Zamyadi et al., 2010, 2012a; Ma et al., 2012b). The release of IOM increases dissolved organic carbon (DOC) which may negatively impact the coagulation process (Ma et al., 2012b) and contribute to DBPs formation (Plummer and Edzwald, 2001; Nguyen et al., 2005; Hong et al., 2008; Huang et al., 2009; Fang et al., 2010a,b; Li et al., 2012; Zamyadi et al., 2012a).

Previous studies have shown that pre-ozonation, similarly to other oxidants, may cause changes in cell surface characteristics, increases in DOC concentrations and the release of other intracellular metabolites (Plummer and Edzwald, 2001, 2002; Hoefer et al., 2002; Huang et al., 2008; Miao and Tao, 2009). However, information about the extent of ozone damage to various cyanobacterial cells under variable oxidation conditions (i.e., contact time, ozone exposure (CT), and pH) remains limited. Such information is necessary to evaluate whether pre-ozonation is a viable option for controlling cyanobacterial blooms. This is especially important given recent evidence showing that CBs cells can accumulate in various processes within treatment plants (especially clarifiers) and that CB species influence the efficacy of coagulation (Zamyadi et al., 2012b). Recent studies in the literature demonstrate the effectiveness of ozone in different applications aiming at the removal of algae and cyanobacteria (Cheng et al., 2010; Li et al., 2011; Wu et al., 2012). For this reason, it is interesting to evaluate the ozone damages in cellular structure of these microorganisms and the cell response to the oxidant application.

The overall objective of this study is to assess the oxidation kinetics of cyanobacterial cells by ozone and the contribution of algal organic matter to DBPs formation under various ozone operational conditions. More specifically, this research (1) develops a kinetic model to describe the reactivity of Microcystis aeruginosa and Anabaena flos-aquae with ozone, (2) assesses the impact of ozonation on cell characteristics (morphology and membrane integrity) and the release of IOM for increasing ozone exposures (CT), and (3) evaluates the release of THM and HAA precursors after pre-ozonation of cyanobacterial cells.

2. Experimental procedures

2.1. Cell culture and harvesting

A toxic strain of M. aeruginosa (CPCC 299) and a nontoxic strain of A. flos-aquae (CPCC 64), which were obtained from the Canadian Phycological Culture Centre, were cultured in ASM-1 (Gorham et al., 1964) and BG-11 (Rippka et al., 1979) inorganic growth media, respectively. The cultures were incubated at 26 ± 1 °C with sufficient aeration under 12 h of light-dark cycles at a light intensity of 70 μmol s m⁻². Algae cells in the stationary phase were separated by filtration (1.2 μm and 10 μm), re-suspended in borate buffer at pH 7 and enumerated by 400× microscopy, using a Sedgewick-Rafter counting chamber.

2.2. Cell characteristics: lysis, integrity and morphology

To determine cell lysis, cell integrity and cell wall morphology, three types of microscope techniques were used: (1) cell lysis was assessed by evaluating the decline in total cell counts using optical microscopy in a Sedgewick-Rafter counting chamber after preservation in a lugol solution (duplicate enumeration/sample), (2) cell integrity was determined using the BacLight™ test (Boulos et al., 1999; Stocks, 2004; Berney et al., 2007), and (3) cell wall morphology was qualitatively evaluated using a scanning electron microscope (SEM, JSM-7600F, JEOL, Japan).

The cell volume for each cyanobacteria was estimated from cell measures by optical microscopy considering diameter of M. aeruginosa or the width and length of A. flos-aquae. Measurements revealed an average diameter of 4.5 μm for M. aeruginosa (approximately 48 μm³ of cell volume) and an average width and length of 4 and 6.5 μm for A. flos-aquae, respectively (approximately 75 μm³ of cell volume). Measurements were performed on 30 cells were repeated on several occasions to yield the average values.

2.3. Ozone experiments

For experiments investigating ozone decay and its impact on cyanobacterial viability, stock ozone solutions (60–70 mg O₃ L⁻¹) were prepared by diffusing gaseous ozone that was produced
with a bench-top ozone generator (TG-10, Ozone Solutions, Inc, USA) through a glass flask containing ultrapure water chilled at 4 °C. The concentration of the ozone stock solution and the dissolved ozone residual in samples were determined according to the standard colorimetric method 4500-O3 (APHA, 2005) using indigo trisulfonate (C00000M = 20,000 M⁻¹ cm⁻³). Samples were analysed at 600 nm with a Varian spectrophotometer (Cary 100, Varian) in a 1-cm or 2-cm quartz cell. Ozone performance was evaluated for both M. aeruginosa and A. flos-aquae using three different applied doses (0.5, 2.0 or 4.0 mg L⁻¹), two initial cell densities (250,000 and 1,500,000 cell mL⁻¹) and two pH conditions (6 or 8). This experimental design yielded a total of 24 ozonation assays, to which 6 additional assays, in the absence of algae cells, were added as controls. Previous experiments with CB cells without ozone at two pH conditions (6 and 8) showed there was no difference on cell viability. All experimental procedures were performed at room temperature of 20 °C.

The pH values were adjusted using a borate buffer (ionic strength: 0.05 M). An ozone decay curve was monitored in a true-batch reactor for each of the conditions described above (ozone dose, cell density and pH). The applied ozone doses were administered by injecting an aliquot of the ozone stock solution via a syringe into a continuously stirred 2-L glass reactor containing the sample (initial sample volume between 500 and 1200 mL) and equipped with a floating Teflon lid to prevent ozone degassing. Ozone residuals were measured over a 10 min period by collecting 5 mL samples that were dispensed into 20 mL of indigo (0.02, 1 or 3%) solution. In parallel experiments, samples were collected for DOC measurements, cell viability and cell counts at predetermined contact times (0.5, 1.5, 3, 5 and 10 min). The samples (sampling volume of 150 mL) were immediately quenched with sodium bisulfite (NaHSO₃ – 2.2 mg/mg O₃) to stop the ozone reaction. To quantify *OH radical formation, a parallel reactor was always spiked with 200 μg L⁻¹ (1.28 μM) of para-chlorobenzoic acid (pCBA). A sub-sample of 1.5 mL was filtered using 0.45 μm pore filter (Millex-HV) and analysed using HPLC. The column characteristics, mobile phase and detection were the same as the column characteristics, mobile phase and detection described by Vincent et al. (2010).

2.4. DOC quantification

DOC samples were filtered through pre-rinsed 0.45 μm hydrophilic polyethersulfone membranes (Supor™-450, PALL, USA). DOC analysis measurements were conducted on a 5310C laboratory total organic carbon analyser (Sievers Instruments, Inc., GE, USA). Extracellular DOC samples were obtained by filtration immediately after ozone exposure. For the measurements of total DOC values (extracellular and intracellular DOC), the samples were subjected to three cycles of freezing and thawing to achieve a complete disruption of cells and the release of intracellular substances (Moreno et al., 2004; Qian et al., 2010) Preliminary microscopic observations of disrupted cells confirmed the efficiency of the freeze-thaw procedure in releasing cell-bound toxins. DOC release during ozonation results were normalised by dividing the DOC values by the ultimate filterable DOC released (DOC extracellular), which was observed at the highest pH and ozone dosage.

2.5. Formation of DBPs precursors

The potential for pre-ozonation to increase DBPs formation was evaluated by chlorinating raw and pre-ozonated water samples. An amount of free chlorine that was sufficient to achieve a free chlorine residual of 1.0 ± 0.2 mg L⁻¹ after a 24-hr chlorine contact time at 22 °C was added to the samples. At the end of the incubation period, samples for THM and HAA were quenched with excess of ammonium sulphate. Measurements of free chlorine residuals were performed using the DPD colorimetric method 4500C1-G (APHA, 2005). Samples were analysed for four THMs (chloroform, bromoform, bromodichloromethane, dibromochloromethane) referred as total THM (TTTH) and six HAA (monochloroacetic acid, dichloroacetic acid, trichloroacetic acid, monobromoacetic acid, dibromoacetic acid and bromochloroacetic acid) referred as HAAs. THMs were extracted by liquid/liquid extraction with hexane as described in Standard Methods 6232B (APHA, 1998). HAA compounds were extracted by liquid/liquid extraction with methyl tert-butyl ether (MtBE) followed by derivation with acidic methanol in accordance with USEPA Method 552.2 (USEPA, 1995). All DBPs were analysed on a gas chromatograph (CP-3800, Varian) equipped with an electron capture detector (GC/ECD) (Column characteristics: capillary column RTX-5MS with 30 m × 0.25 mm ID and 0.25 μm film thickness).

2.6. CT calculations: molecular and free radical exposures

For each specific contact time, the molecular ozone exposure (CT value – ozone concentration × detention time), expressed as mg min L⁻¹, was calculated by numerical integration using the trapezoidal method, which calculates the area under the decay curve. To validate the estimated numerical CT, they were compared to modelled CT values, which were obtained by assuming a pseudo-first-order decay kinetic (k’) in (Eq. (1))

\[
CT_{D3} = \frac{C_0}{k'} \left[1 - \exp(-k' \cdot t)\right]
\]

where \(C_0\) = initial ozone residual (mg L⁻¹) determined from the exponential fit of the relationship between the ozone residual and time (min); and \(k' = \) the ozone pseudo-first-order decay constant (min⁻¹).

*OH radical exposure (CT_{OH}) was estimated using the reaction-rate constant for *OH radical (k_{OH,pCBA} = 5.2 \times 10^9 M⁻¹ s⁻¹) and a second-order kinetic such that

\[
\frac{d[pCBA]}{dt} = -k_{OH,pCBA} \cdot [pCBA] \cdot [OH]
\]

where \(k_{OH,pCBA}\) is the pCBA rate constant; [pCBA] is the pCBA concentration; and [OH] is the free hydroxyl radical concentration.

Subsequently, *OH exposure is defined as:

\[
CT_{OH} = \int OH \cdot dt = \frac{\ln\left(\frac{[pCBA]}{[pCBA]_{0}}\right)}{k_{pCBA}}
\]
2.7. Kinetic modelling

Ozone decay was modelled as a two-step process: immediate demand followed by an n-th order kinetic. The immediate ozone demand characterises the amount of ozone consumed in the first stage of the oxidation process (measured after a few seconds) (Park et al., 2001). In this study, the immediate ozone demand was evaluated as the ozone consumed after 15 s of contact time (our earliest measurement). A step-forward general regression model was developed to predict ozone demand as a function of ozone dose, pH, organism type and DOC. Ozone decay after immediate demand was modelled using two alternative strategies. Initially, pseudo-first-order kinetics were fitted successively to each individual modelled using two alternative strategies. Initially, pseudo-first-order kinetics were fitted successively to each individual decay curve (Eq. (4)) as

\[ \frac{C}{C_0} = \exp(-k \cdot t) \]  

(4)

where \( C \) and \( C_0 \) are the final and initial concentrations of the target compound (mg L\(^{-1}\)), respectively; \( k \) is the pseudo-first-order reaction rate constant (min\(^{-1}\)); and \( t \) is the time (min).

As an alternative approach, a generalised empirical kinetic rate law (Eq. (5)) was developed as followed:

\[ \frac{dO_3}{dt} = -k \cdot (O_3)^n \cdot (OH^-)^m \cdot (DOC_T)^p \]  

(5)

where \( O_3 \) is the ozone residual; \( t \) is time, \( OH^- \) is the hydroxide concentration, \( DOC_T \) is the total concentration of organic carbon; and \( n, m, p \) and \( k \) are fitting parameters.

Assuming that pH and \( DOC_T \) are constant during ozonation, Eq. (5) can be integrated into Eq. (6). Because no concentration measurement is available to represent the reactivity of DOC according to time, \( DOC_T \) is assumed to be constant to simplify the calculations. In addition, DOC represents a series of organic compounds rather than a single solute.

\[ O_3 = \left[ \frac{1}{(O_3)_0^{-1}} + k \cdot t \cdot (OH^-)^m \cdot (DOC_T)^p \right]^{1-n} \]  

(6)

Equation (6) was fitted separately for the ozone assays that were conducted for each of the two organisms as well as for the entire dataset (i.e., combined data for both organisms).

3. Results and discussion

3.1. DOC release

Ozonation of CB cell suspensions led to a substantial increase in DOC. For example, Fig. 1 shows increases in DOC (0.1–1.5 mg C L\(^{-1}\)) for \( M. \) aeruginosa and \( A. \) flos-aquae after ozonation using 0.5, 2.0 or 4.0 mg L\(^{-1}\) of ozone for a cell density of 250,000 cells mL\(^{-1}\). The large amount of extracellular DOC immediately after ozone exposure (0.5 min) can be related to two mechanisms: a rapid reaction between ozone and cell biomass, with a direct formation of DOC; and/or an indirect reaction from cell lysis, leading the release of intracellular compounds (Müller et al., 2003; Hammes et al., 2007; Ramseier et al., 2011).

As shown in Fig. 1, higher ozone doses increased the proportion of DOC release. After 10 min of contact time, an increase from 0.39 mg L\(^{-1}\) of DOC with 0.5 mg O\(_3\) L\(^{-1}\) to 0.96 mg L\(^{-1}\) after 4 mg O\(_3\) L\(^{-1}\), and from 0.67 mg L\(^{-1}\) of DOC with 0.5 mg O\(_3\) L\(^{-1}\) to 1.58 mg L\(^{-1}\) after 4 mg O\(_3\) L\(^{-1}\) were observed for \( M. \) aeruginosa and \( A. \) flos-aquae, respectively. Miao and Tao (2009) investigated DOC changes following ozone oxidation of \( M. \) aeruginosa cultures (undisclosed cell concentrations). They observed that the release of cell associated DOC increased from 0.34 to 2.56 mg L\(^{-1}\) with increasing ozone dosages from 0 to 5 mg O\(_3\) L\(^{-1}\).

A greater release of DOC while ozonating \( A. \) flos-aquae can be linked to cell size, which resulted in a greater release of intracellular substances or a greater quantity of cell biomass available for oxidation. Also, it can be observed on some occasions (cf. \( M. \) aeruginosa at pH 6), that increasing the dosage of ozone from 0.5 to 2 mg L\(^{-1}\) did not result in an increase in DOC concentration. This may indicate that the internal contents of cells would have already been completely released at the lowest ozone dosage. Thus, the greater concentration of DOC obtained with the higher ozone dosage (4 mg L\(^{-1}\)) would be related to the oxidation of the organic constituents in the cell structure. Similarly, for \( A. \) flos-aquae at pH 8, the increase in ozone dose from 2 to 4 mg L\(^{-1}\) resulted in no increase in DOC concentration, suggesting that all the organic material of cell would have been completely released or oxidized with an ozone dose of 2 mg O\(_3\) L\(^{-1}\).

The increase in extracellular DOC can also be correlated with the pH values. Our results indicate that the amount of DOC released at pH 8 was greater than at pH 6. Fig. 2 presents the normalised DOC release (expressed in % of the maximal DOC released) for both organisms as a function of molecular ozone exposure (CT\(_{O3}\)) and/or hydroxyl radical exposures (CT\(_{OH}\)). In Fig. 2 a, there is no obvious difference in the normalised DOC release between \( M. \) aeruginosa and \( A. \) flos-aquae. The higher DOC release, as observed in Fig. 1, was essentially caused by the higher biomass load of the larger \( A. \) flos-aquae. However, for both organisms, there is an important difference caused by the pH. DOC release at pH 6 plateaued at 30–50% of the ultimate DOC release observed at pH 8. Interestingly, plotting CT release with respect to CT\(_{OH}\) (Fig. 2b) provided a better description of the entire dataset. We infer that free radical plays an important role in the oxidation of algae cells. CT\(_{OH}\) is correlated with CT\(_{O3}\); higher CT\(_{O3}\) also leads to a higher CT\(_{OH}\). However, it also accounts for the effect of pH which explains its superiority over CT\(_{O3}\) in predicting DOC release. Huang et al. (2008) also observed an increase in DOC when pH was increased from 5.5 to 9.0. The authors suggested that this increase was caused by the increased liberation of intracellular organic matter and cell-wall polysaccharides during cell ozonation and that the action of pH on DOC release was regardless of ozone dosages. At alkaline pH, the higher concentration of hydroxide ion (OH\(^-\)) promotes ozone decomposition and the formation of hydroxyl free radicals (OH) (Widrig et al., 1996; Von Gunten, 2003). According to Widrig et al. (1996), OH radicals oxidation mechanisms are more prone to degrade saturated aliphatic molecules, which are largely present in algal-derived organic substances. Thus, higher concentrations of OH radicals due to alkaline pH or higher O\(_3\) doses may lead to increased cell damage or
reactions with organic material in the cell and, consequently, a greater amount of extracellular DOC.

3.2. Impact of CB cells and DOC on ozone decay

A total of 30 ozone decay curves were generated, including 24 reactors with algae and 6 reactors without cells, which served as controls. Immediate ozone demands are presented first. In a second step, the ozone decay kinetic was characterized using a classical pseudo-first-order model. Finally, a generalisation of ozone kinetic data was accomplished by the development of an n-th order model.

3.2.1. Immediate ozone demand

Immediate ozone demands varied from 0.00 to 0.82 mg L\(^{-1}\); higher demands were observed at higher ozone dosages, pH and DOC (Table 1). Interestingly, when all other variables were equivalent, immediate ozone demand was not statistically different \((p < 0.05)\) for \(M. aeruginosa\) and \(A. flos-aquae\). Consequently, it was possible to predict immediate ozone for both organisms with a high correlation \((R^2 = 0.90)\) using Eq. (7):

\[
D_0 = a + b \times O_3\text{Dose} + c \times \text{pH} + d \times \text{DOC} \times O_3\text{Dose} \tag{7}
\]

where \(D_0\) is the immediate ozone demand; and \(a, b, c\) and \(d\) are regression coefficients.

An assessment of the ANOVA table for this regression (data not shown) revealed that the interaction between DOC and ozone dose was the most important predictor in the equation. This interaction reflects the basic fact that an increase in ozone dosage produces large immediate demands in DOC-rich waters. As for pH, its increase led to higher immediate demands, which is a direct consequence of the increased free radicals activity (as will be shown later).

3.2.2. Pseudo-first-order kinetic model

Fig. 3 presents ozone decay for the various ozonation conditions that were investigated. For each individual ozone decay curve, a pseudo-first-order model was fitted by linear regression. Table 2 summarises the regression parameters derived from this analysis. Although a pseudo-first order model generally provided a good fit to the experimental data (typical \(R^2 > 0.95)\), some conditions clearly exhibited two kinetic

![Fig. 1 - DOC release for (a) M. aeruginosa and (b) A. flos-aquae for a density of 250,000 cells mL\(^{-1}\); ozone dose of 0.5, 2.0 and 4.0 mg L\(^{-1}\) and pH of 6 and 8 (MIC: M. aeruginosa, ANA: A. flos-aquae).](image)

![Fig. 2 - Normalised DOC release: (a) as a function of CT\(_{O3}\) and (b) as a function of CT\(_{OH}\).](image)
regimes (e.g., Fig. 3c). Half-life values varied from as low as 0.21 min to as high as 64 min (Table 2). As expected, higher pH and initial cell densities led to lower half-lifes.

Under identical ozonation conditions, higher ozone decay were always observed for *A. flos-aquae* than for *M. aeruginosa*. This effect may be due to the fact that the latter algae is larger (6.5 μm in length × 4 μm in width) than the former algae (4.5 μm in diameter). Because the initial cell density was equivalent for both organisms, *A. flos-aquae* brought a higher organic load. On average, DOC concentrations were 90% higher during assays performed with *A. flos-aquae*. To better distinguish the impacts of pH, organic carbon concentration and ozone residual on ozone decay kinetics, a generalised empirical rate law was fitted to the entire dataset. This will be the topic of the next section.

### 3.2.3. N-th order model

Equation (7) was adjusted for the four datasets described in Table 3: the ultrapure water dataset, *M. aeruginosa* dataset, *A. flos-aquae* dataset and the combination of *M. aeruginosa* and *A. flos-aquae* into one dataset. The models provided a good fit to the data ($R^2 = 0.87−0.92$). Ozone decomposition in ultrapure water proved to be second-order with respect to dissolved ozone, whereas reaction order for the hydroxide concentration was 0.53. Mizuno et al. (2007) developed a similar kinetic model to describe ozone decay in ultrapure water. They also observed second-order kinetics with respect to dissolved ozone. However, the order of reaction for hydroxide (0.73) was slightly higher than that obtained in this study, which is most likely due to the fact that the Mizuno et al. model was developed for a wider range of pH (4.0−7.8).

In the presence of algal organic matter (AOM), ozone decay is always second-order for O$_3$ and almost first-order for DOC. As for pH, the orders of reaction (0.18−0.34) were lower than in ultrapure waters, which indicates that the impact of pH on O$_3$ decay was reduced in the presence of AOM. The reactivity of *M. aeruginosa* ($k = 37.6 M^{0.4} (mg C/L)^{-1.2} min^{-1}$) was also higher than the reactivity for *A. flos-aquae* ($k = 2.46 M^{0.3} (mg C/L)^{-1} min^{-1}$). Such results indicates that the lower half-life values observed while oxidizing *A. flos-aquae* were caused by increased DOC concentrations rather than increased DOC reactivity. As discussed earlier, higher DOC was promoted by the larger biovolume of *A. flos-aquae* as compared with *M. aeruginosa*.

To directly compare ozone reactivity of both algae, the predictive models for immediate ozone demand and ozone decay were used to predict ozone decay profiles for a common ozonation condition (DOC$_T = 2 mg L^{-1}$, dose = 2 mg L$^{-1}$) and

<table>
<thead>
<tr>
<th>pH 6</th>
<th>Immediate demand (mg L$^{-1}$)</th>
<th>Immediate demand (mg L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ozone dose (mg L$^{-1}$)</td>
<td>0.5</td>
<td>0.00</td>
</tr>
<tr>
<td>2.0</td>
<td>0.22</td>
<td>2.0</td>
</tr>
<tr>
<td>4.0</td>
<td>0.50</td>
<td>4.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH 8</th>
<th>Immediate demand (mg L$^{-1}$)</th>
<th>Immediate demand (mg L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ozone dose (mg L$^{-1}$)</td>
<td>0.5</td>
<td>0.00</td>
</tr>
<tr>
<td>2.0</td>
<td>0.22</td>
<td>2.0</td>
</tr>
<tr>
<td>4.0</td>
<td>0.50</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Fig. 3 – Ozone decay curves in Milli-Q water without CB cells and in the presence of *M. aeruginosa* (MIC) and *A. flos-aquae* (ANA).
variable pH conditions (Fig. 4). Although ozone kinetics of *A. flos-aquae* are slightly faster than the ozone kinetics for *M. aeruginosa*, pooling the data for both organisms provides good predictions of ozone decay under equivalent ozonation conditions (pH, applied dose and DOC, T), as illustrated in Fig. 4.

### 3.3 Effect of ozone on the integrity of *M. aeruginosa* and *A. flos-aquae* cells

The impact of ozonation on cellular morphology and cell integrity was verified by SEM and BacLight methods, respectively. No loss of cell integrity was observed due to the direct addition of cells in buffered water. However, as shown in Fig. 5a–d, an immediate loss of integrity after exposure to ozone was observed for both tested species. All cells turned red/orange, even for the lowest contact time (30 ± 5 s) and ozone dosage of 0.5 mg L⁻¹. These results indicate immediate cell membrane damage at CT values ≤0.2 mg min L⁻¹ for all ozonation experiments. While carbohydrates and fatty acids react only slightly with ozone, amino acids, proteins and nucleic acids all react very quickly with this oxidant (Bablon et al., 1991). Both DNA and RNA can be degraded by ozone attack (molecular ozone or free radicals), even with the cell remaining intact (Theruvathu et al., 2001; Cataldo, 2005). With the increase in ozone dosages and contact time, propidium iodide binding was progressively lost as the target binding sites were oxidised.

Like other oxidants agents, ozone first attacks the cell wall and plasma membrane glycoproteins, glycolipids or certain amino acids. Rapid cell death after ozonation can be attributed to changes in cellular permeability followed by cell lysis (Greene et al., 1993; Morató et al., 2003). However, due to the high oxidation potential of ozone and because ozone further reacted with the nuclear material, lysis is not the primary inactivation mechanism; rather it is a consequence of high applied oxidant dosages (Greene et al., 1993). The cell counts performed during this study indicated that no lysis of *A. flos-aquae* occurred for all the ozonation conditions investigated. No lysis was observed at pH 8 for *M. aeruginosa*; lysis was only significant at pH 6 for the two highest ozone dosages. A total of 32% and 41% of cells were lysed when ozone dosages increased from 2 mg L⁻¹ and 4 mg L⁻¹, respectively. The higher ozone residual concentrations at pH 6 may have favoured cell lysis, although a more visible disruption of external cell

### Table 2 – First-order apparent rate constants (k) for ozone decay.

<table>
<thead>
<tr>
<th>CB specie</th>
<th>pH</th>
<th>O₃ dose (mg L⁻¹)</th>
<th>250,000 cells mL⁻¹</th>
<th>1,500,000 cells mL⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k</td>
<td>Half-life (min)</td>
<td>k</td>
<td>Half-life (min)</td>
</tr>
<tr>
<td>No cell</td>
<td>6</td>
<td>3.52 × 10⁻⁴ (0.95)</td>
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<td>3.52 × 10⁻⁴ (0.95)</td>
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<td>1.53 × 10⁻³ (0.94)</td>
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a Correlation coefficients (R²) presented in parentheses.

### Table 3 – Estimated parameters to determine the behaviour of ozone decay.

<table>
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<th>Estimated parameters</th>
<th>Ultrapure water</th>
<th>MIC</th>
<th>ANA</th>
<th>MIC + ANA</th>
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<tr>
<td>O₂</td>
<td>2.00</td>
<td>2.02</td>
<td>2.08</td>
<td>2.04</td>
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<tr>
<td>Rate constant</td>
<td>131.50</td>
<td>37.6⁺</td>
<td>2.46⁺</td>
<td>13.85⁺</td>
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<tr>
<td>pH effect</td>
<td>0.53</td>
<td>0.34</td>
<td>0.18</td>
<td>0.28</td>
</tr>
<tr>
<td>DOC, T effect</td>
<td>1.19</td>
<td>1.03</td>
<td>1.03</td>
<td>1.03</td>
</tr>
</tbody>
</table>

MIC: M. aeruginosa, ANA: A. flos-aquae.

* a M⁰⁻¹ (mg C/L)⁻¹ min⁻¹.
* b M⁰⁻¹ (mg C/L)⁻¹ min⁻¹.
* c M⁰⁻¹ (mg C/L)⁻¹ min⁻¹.
morphology was observed at pH 8 by SEM. We suggest that \(\cdot\)OH radicals have larger impacts on the cell. However, the higher concentration of extracellular DOC at pH 8 also acts as scavengers of \(\cdot\)OH radicals, reducing the availability of \(\cdot\)OH radicals to attack the target, as observed by Onstad et al. (2007) that verified a reduced efficiency of MC-LR lysis due to high concentration of DOC. In Fig. 5, SEM microphotographs are provided for conditions before (Fig. 5e and g) and after (Fig. 5f and h) exposure to ozone. Although there was no cell lysis verified for \textit{M. aeruginosa} after 10 min of contact time and 4.0 mg L\(^{-1}\) of ozone at pH 8 (Fig. 5f), a visible alteration in cell wall morphology was observed. In the other ozonation conditions, changes in cell wall morphology were less notable. After only 30 s of contact time with 2 mg L\(^{-1}\) of ozone, \textit{A. flos-aquae} cells appeared more impacted by ozone than \textit{M. aeruginosa} with visible changes (Fig. 5h) being noted in cell wall morphology. However, no reduction in cell number was observed.

Miao and Tao (2009) also evaluated the effects of ozone on \textit{M. aeruginosa} cells morphology. Doses of 1, 3 and 5 mg L\(^{-1}\) of ozone were applied to a cyanobacteria suspension. Removals up to 91% of Chl-a were obtained for an ozone dose of 5 mg L\(^{-1}\) within 60 min of contact time. The authors verified that ozone dose in the range of 1–5 mg L\(^{-1}\) resulted in cell wall alterations, with complete cell lysis after treatment with 3 mg L\(^{-1}\) of ozone, an opposed to the results observed in this study for a similar ozone dosage (4 mg O3 L\(^{-1}\)). Lin et al. (2009) also observed a greater susceptibility of the \textit{Anabaena} genus to oxidation by chlorine as compared with \textit{Microcystis}. In comparison with chlorine experiments, ozone exhibited a more effective impact on the integrity of CB cells. For example, Zamyadi et al. (2012a) verified the loss of integrity for several cyanobacteria species after the chlorination experiments at 2 and 5 mg Cl\(_2\) L\(^{-1}\) using higher contact times. A CT higher than 31 mg min L\(^{-1}\) was needed to disrupt the integrity (measured with the FDA method) of more than 99% of \textit{M. aeruginosa} cells, whereas a CT of approximately 8 mg min L\(^{-1}\) had an equivalent impact on \textit{Anabaena circinalis}, \textit{Cylindrospermopsis raciborskii}, and \textit{Anabaena} issatschenkia cells. The same behaviour was observed by Daly et al. (2007) while chlorinating \textit{M. aeruginosa} cells. Under the conditions evaluated in this study, a similar ozone dose (4 mg O3 L\(^{-1}\)) was needed to lyse only 41% of \textit{M. aeruginosa} cells, which implies that ozone is more effective than chlorine to render CB cells non viable at an equivalent dose.
oxidant exposure (CT). The results from this study are consistent with the results in the literature: release of DOC and internal metabolites precede cell lysis (Plummer and Edzwald, 2001; Miao and Tao, 2009). Our results indicate that very low ozone exposure may be sufficient to induce cell damage and loss of integrity.

3.4. Formation of disinfection by-products from CB cells

Several studies have quantitatively evaluated the reactivity of algal and cyanobacteria cells and their metabolites as precursors of chlorinated DBPs (Plummer and Edzwald, 2001; Hong et al., 2008; El-Aty et al., 2009; Huang et al., 2009; Zamyadi et al., 2012a). Pre-ozonation of cyanobacteria cells release intracellular compounds which may contribute to the pool of chlorinated DBPs precursors (assuming that post-chlorination is used as a final treatment). To evaluate their reactivity, ozonated samples spiked with the lower concentration of algae cells (250,000 cell mL\(^{-1}\)) were chlorinated and subjected to a THM and HAA\(_6\) analyses.

Our results indicated that the predominant chlorinated DBPs formed were chloroform (CHCl\(_3\)) and dichloroacetic acid (DCAA) for \(M.\ aeruginosa\), and CHCl\(_3\), DCAA and trichloroacetic acid (TCAA) for \(A. flos-aquae\) (Supplementary Data – Table SD-1). Fig 6 presents the total THM (TTHM) and HAA (THAA) concentrations measured for increasing ozone dosages. Higher concentrations of THM were measured when compared with HAA. A study from Hong et al. (2008) indicated that THM and HAA formation is directly related to cyanobacteria biochemical cellular composition. The formation of THM tends to be higher than the formation of HAA when there is a greater proportion of lipids in the cell composition. The formation of HAA, in turn, is related to the amount of proteins. According to Hong et al. (2008) the amount of proteins is proportionally higher in \(M.\ aeruginosa\) than in \(A.\ flos-aquae\) sp. cells, but Anaibaena sp. cells present a higher amount of lipids than \(M.\ aeruginosa\) cells. This finding could explain the higher \(A. flos-aquae\) THM formation observed in this study.

Higher ozone dosages resulted in higher DBPs formation, except for \(M.\ aeruginosa\) at pH 6 for which no THM could be detected even after 4 mg L\(^{-1}\) of ozone. Higher THM and HAA formation were observed for \(A. flos-aquae\), a conclusion that is consistent with the higher organic carbon extracellular after ozonation due to their larger cellular structure. Although higher DOC generally led to a higher DBPs formation, DBPs reactivity was both pH and organism-dependant. The following maximum yields were obtained for \(M.\ aeruginosa\) at pH 8: 11 µg/ mg C (TTHM = 38 µg L\(^{-1}\)) and 4 µg/mg C (HAA\(_6\) = 14 µg L\(^{-1}\)). For \(A. flos-aquae\), maximum yields of 16 µg/mg C (TTHM = 63 µg L\(^{-1}\)) and 6 µg/mg C (HAA\(_6\) = 24 µg L\(^{-1}\)) were calculated for TTHM and THAA at pH 8, respectively. Ratios of TCAA/DCAA for \(A. flos-aquae\) ranged from 0.14 to 0.21 at pH 6 and from 0.30 to 0.48 at pH 8, with lower ratios being achieved for higher ozone doses. These results indicate that the DOC produced by \(A. flos-aquae\) was more prone to DCAA than TCAA formation (Nguyen et al., 2005; El-Aty et al., 2009), which can be related to the lower level of aromatic carbon found in CB EOM (Hong et al., 2008, 2009). Due to the higher proportion of proteinaceous material in EOM, the dominance of DCAA is intensified (Huang et al., 2009). As noted in Table SD-1 (Supplementary Data), increasing ozone dose resulted in a slight increase of DCAA and decrease of TCAA at pH 8. This behaviour is most likely related to changes in hydrophobicity following their oxidation by ozone which increases the hydrophilic fraction (Szwitlik et al., 2004) more prone to DCAA formation. Hong et al. (2013) observed in their study that hydrophobic organic molecule fractions tended to be more important as precursors of THM and TCAA, while the hydrophilic fractions resulted in higher concentrations of DCAA.

The influence of pH on THM formation is the result of two mechanisms. In one mechanism, ozonation at a higher pH led to the release of increased DOC (as shown in Fig. 1). In addition, it is well-known that a higher pH increases free chlorine reactivity with natural organic matter. In a study that assesses the formation of DBPs from carbohydrates, Navalon et al. (2008) observed that basic pH values favour all chlorine reactivity pathways. Higher pH values favour THM formation (Liang and Singer, 2003; Navalon et al., 2008; Fang et al., 2010a) but can reduce the formation of TCAA (Fang et al., 2010a). DCAA formation is normally independent of pH (Liang and Singer, 2003). This explains why the impact of basic pH on HAA formation was less than the impact of pH on THM formation. In that case,

![Fig. 6 – TTHM and HAA\(_6\) formation following ozonation of (a) M. aeruginosa (b) A. flos-aquae at pH 6 and 8 using 10 min of contact time with 0.5, 2.0, and 4.0 mg O\(_3\) L\(^{-1}\) for 250,000 cells mL\(^{-1}\) (chlorination conditions: chlorine residual of 1.0 ± 0.2 mg L\(^{-1}\) after a 24-hr of contact time at 22 °C).](image-url)
higher HAA formation at pH 8 can be considered a direct result of the increase in the availability of DBPs (DOC and other intracellular compounds) precursors under this ozonation condition.

A comparative evaluation of the potential formation of DBPs from EOM and CB cells was not evaluated in this study. However, some of the observations on EOM were made on the accumulated by products during long term cell culture which raises the representativity of these findings in a real bloom (2012a). Other results obtained by Liu et al. (2011) in chlorination studies with M. aeruginosa indicate that only 22% of total THM formed was derived from EOM in a mixture including cells and EOM. In a study with M. aeruginosa cells, Fang et al. (2010b) evaluated the contribution of EOM, IOM and cells to the DBPs formation. They observed that EOM formed lower concentrations of chloroform and DCAA than IOM and CB cells, which presented a similar contribution to the final total concentration of DBPs formed. All these findings confirm that pre-oxidation of CB cells can impact significantly the formation of chlorination by-products in certain conditions by increasing the precursor’s pool, especially when cell concentrations are elevated. However, in many cases, background DBPs precursors level have been shown to be the main factor affecting the overall formation of DBPs in natural waters and the key factor limiting the use of pre-chlorination (2012a). In the case of ozonation, treatment conditions can enhance the reactivity and DBPs yield of cell-bound and cell-released precursors. When considering pre-ozonation, the contribution of CB cells to the pool of THM and HAA precursor should be considered carefully, especially if no additional treatment is capable of removing the released DBPs precursors after pre-ozonation.

4. Conclusion

A low-ozone dose (0.5 mg L⁻¹) was sufficient to cause a complete loss of integrity of M. aeruginosa and A. flos-aquae cells, for both cell densities and pH values investigated. Although no significant cell lysis was observed for most ozonation conditions, the damage on cell structure after ozonation resulted in an increase in DOC. Higher DOC release was observed for A. flos-aquae, an observation which was attributed to its differences in biovolume. DOC release increased with ozone dose and pH. The greater DOC release at pH 8 suggests that the attack of free radicals is an important contributor to this process. Kinetic analysis indicated that presence of CB cells in water can result in a considerable immediate ozone demand and this behaviour is dependent on ozone dose, DOC concentration and pH, but was independent of the type of CB species tested. Ozone decay was influenced by CB species and pH values, with the fastest ozone decay observed for A. flos-aquae cells at pH 8.

DOC release from CB cells was confirmed to contribute to DBPs formation. The ozone dose, pH and CB species were the most important predictors of DBPs formation. A greater amount of THM and HAA was quantified for A. flos-aquae and at pH 8, which may be related to the greater amount of DOC release and the cell composition of this algae. When higher ozone doses were applied, greater concentrations of THM and HAA were quantified.

Pre-ozonation is an attractive solution for CB impacted source waters. It can prevent CB cell accumulation within the plant (clarifiers and filters) thus reducing the risk of toxin release during treatment and facilitating the disposal of sludge. However, it is important to take into consideration the effect of pre-ozonation on DOC and DBPs formation which can be significant under certain ozonation conditions (high pH, high CB cell concentrations). Thus, the benefits of CBs control must be weighed against potential significant increases in DBPs formation considering the water quality at a given site.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2013.03.012.

References


