Applying UV absorbance and fluorescence indices to estimate inactivation of bacteria and formation of bromate during ozonation of water and wastewater effluent

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Abstract

Ozone is an effective oxidant and disinfectant commonly used for elimination of micropollutants and inactivation of resistant microbes. However, undesirable oxidation/disinfection byproducts such as bromate might form during ozonation. In this study, the UV absorbance and fluorescence indices were applied as surrogate indicators for predicting the inactivation of bacteria and formation of bromate during ozonation of water and wastewater effluents. The inactivation efficiencies of lab-cultured *Escherichia coli* (*E. coli*) and autochthonous bacteria were measured by plating (for *E. coli* only) and flow cytometry with fluorescence staining. During ozonation of *E. coli* spiked into wastewater effluents (~10⁶ cell/mL), the priority of inactivation efficiency determined by different cell viability methods were in the order of CFU > membrane damage > DNA damage. Approximately, 99% membrane damage and/or 90% DNA damage are conservatively supposed as an indicator for sufficient bacterial inactivation as well as degradation of antibiotic resistance genes. The related required O₃ dosing thresholds for sufficient inactivation of *E. coli* and autochthonous bacteria refer to ~0.6 O₃/DOC (g/g), ~50% decrease of UVA254, ~60% decrease of UVA280, or ~80% decrease of humic-like fluorescence. Within the range of 10⁶-10⁸ cell/mL, the bacterial concentration did not have significant effects on the required thresholds of the specific O₃ doses or spectroscopic indicators required for bacterial inactivation. The addition of 50 mM *tert*-BuOH as ·OH scavenger increased the required specific ozone doses but decreased the losses of spectroscopic indicators necessary for sufficient bacterial inactivation, and also suggested that the membrane/DNA damages for bacterial inactivation were mainly attributed to the direct O₃ attacks. The bromate concentration was determined using ion chromatography with MS/MS detection. The results showed that when O₃ was dosed at the required thresholds for sufficient bacterial inactivation, bromate formation could usually be suppressed below 10 µg/L. The present work supports that it is possible to reach a balance between bacterial inactivation and bromate formation.

Keywords: UV absorbance; Humic-like fluorescence; Ozonation; Bacterial inactivation; Flow cytometry; Bromate

1 Introduction

Ozonation is widely used for the advanced treatment of drinking water and wastewater effluent. Besides removal of odor, color and various organic micropollutants (Hollender et al., 2009; Huber et al., 2005; Ikehata et al.,

2006; von Gunten, 2003a), ozone is also an efficient disinfectant to inactivate microorganisms, especially those pathogens resistant to chlorine (Gamage et al., 2013; von Gunten, 2003b; Xu et al., 2002). Previous ozone disinfection studies which were performed in clean water matrices have determined the inactivation kinetic parameters for those laboratory-cultured indicator microorganisms, such as *E. coli*, *Bacillus subtilis* spores, and *Cryptosporidium parvum* oocysts, suggesting that ozone has inherently high reactivity with most microorganisms (Driedger et al., 2001; Finch et al., 1993; von Gunten, 2003b). As to secondary wastewater effluents containing high concentrations of particles, and dissolved organic carbon (DOC), a few recent studies have shown that ozonation can also achieve significant inactivation of indicator bacteria and various autochthonous bacterial communities at economically affordable O₃ doses, e.g., O₃/DOC mass ratios of 0.5–1.0 (Czekalski et al., 2016; Gamage et al., 2013; Lee et al., 2016). In recent years, antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) were increasingly detected in various environmental sources (Martinez, 2008). During the conventional water and wastewater treatment, chlorination is not reliable for the removal of ARB and ARGs (Pak et al., 2016; Shi et al., 2013). Recent studies have suggested that although the selection phenomena of some ARB also exist (Alexander et al., 2016), ozonation is effective for the antibiotic resistance control by reducing the amount of ARB and lowering horizontal gene transfer (Czekalski et al., 2016; Lueddeke et al., 2015; Pak et al., 2016).

For the assessment of bacterial viability and disinfection efficiency, cultivation-based plating methods have been extensively applied for quantifying the viability of laboratory-culturable indicator bacteria during various disinfection experiments (Forsyth et al., 2013; Gamage et al., 2013; Gerrity et al., 2012; Van Nevel et al., 2017). However, conventional plating methods cannot well detect the majority of autochthonous bacteria in water and wastewater, as many bacteria are not cultivable under specific conditions (Hammes et al., 2008; Van Nevel et al., 2017). In recent years, a series of cultivation-independent methods have been developed and applied for microbial detection, such as imaging, qPCR, DNA sequencing and flow cytometry (FCM). The FCM analysis combined with fluorescence staining pretreatment can be used for a range of qualitative and quantitative analyses of the microbial community including total cell count, size estimation, cell function, community structure and specific detection (Wang et al., 2010). For the assessment of bacterial viability during water and wastewater disinfection, the FCM provides a rapid and comprehensive alternative to cultivation-based methods (Berney et al., 2008; Hammes et al., 2008; Lee et al., 2016; Phe et al., 2005; Van Nevel et al., 2017).

Although the excess dose of ozone can ensure sufficient removal of micropollutants and inactivation of microorganisms, it might lead to the formation of undesirable organic and inorganic oxidation/disinfection byproducts, especially the formation of bromate in bromide-containing source water (Driedger et al., 2001; von Gunten, 2003b). Bromate is classified as a probable or likely human carcinogen, and many countries have established the maximum allowable level of 10 µg/L for drinking water (Butler et al., 2005). Besides economic costs, strategies of accurate ozone dosing are required for attaining adequate degradation of micropollutants and inactivation of bacteria with relatively low bromate formation. However, frequent monitoring of micropollutants and bacteria for online process control is a costly and time-consuming proposition, and thus many agencies (e.g., California Department of Public Health, U.S.) are embracing indicator frameworks and highlighting the need for surrogate monitoring (Chon et al., 2015; Gerrity et al., 2012).

A range of studies have described indicator frameworks for ozonation process control, which are based on dissolved O₃ concentration integrated over time (CT), O₃/DOC mass ratio, differential UV absorbance (e.g., ΔUVA254) and differential total/component fluorescence (e.g., ΔTF) (Chon et al., 2015; Gamage et al., 2013; Gerrity et al., 2012; Li et al. 2016b, 2017; Xu et al., 2002). As to wastewater, the conventional CT framework does not work well, as O₃ reacts rapidly with the effluent organic matter in the initial O₃ demand stage and the corresponding CT values are not typically measurable (Lee et al., 2013). The O₃/DOC mass ratio is an effective indicator for predicting the levels of oxidation of various micropollutants, formation of bromate and biodegradable DOC, and inactivation of microorganisms (Chon et al., 2015; Gerrity et al., 2012; Lee et al. 2013, 2016), which facilitates the comparison of ozonation studies across different water and wastewater. Lee et al. (2016) applied FCM analysis with fluorescence staining to analyze the membrane damage or DNA damage of bacteria caused under different O₃/DOC mass ratios, but the effect of ·OH on DNA damage at higher O₃/DOC mass ratios was not studied yet. It should be noted that the online measurements of O₃ and DOC concentrations require expensive instruments, and the consumed O₃ calculated from the differential O₃ concentration between inlet and outlet gas might have a higher value than the true consumed O₃ (Carvajal et al., 2017).

The development of miniaturized online UV absorbance/fluorescence monitor facilitates the application of spectroscopic indicator frameworks for the real-time optimization of ozonation dosage. Correlations between ΔUVA254, ΔTF or differential humic-like fluorescence, have been well developed in correlation with the oxidation of micropollutants and formation of bromate and biodegradable DOC (Gerrity et al., 2012; Li et al. 2016b, 2017). In comparison with extensive studies focusing on the oxidation performance, few studies have applied the spectroscopic indicator frameworks for estimation of disinfection efficiency. Gerrity et al. (2012), Gamage et al. (2013) and Carvajal et al. (2017) evaluated the ΔUVA254 or ΔTF as indicators for predicting inactivation of seeded or autochthonous *E. coli*, MS2 Bacteriophage and *Bacillus subtilis* Spores using cultivation-based methods, respectively. However, the information on the correlations between spectroscopic indicators and inactivation of autochthonous bacterial communities or damage of DNA is not available yet. Also, the bromate formation along with the bacterial inactivation has not been studied together in the framework of spectroscopic indicators, much less the effect of bacterial concentrations on the robustness of the indicator framework.

The objective of this study was to apply UV absorbance and fluorescence indices as surrogate indicators for predicting the inactivation of bacteria and formation of bromate during ozonation of water and wastewater effluent. The inactivation efficiencies of lab-cultured *E. coli* and autochthonous bacteria were measured by plating (for *E. coli* only) and FCM with fluorescence staining. The formation of bromate was analyzed with LC-MS/MS. Effects of water matrices and bacteria concentrations on the robustness of the spectroscopic indicator framework were evaluated. Moreover, the roles of ·OH in the damage of bacterial membrane or DNA was also studied. Ultimately, this study provides essential information for the application of UV absorbance and fluorescence indicators as feedback signals for the smart control of ozonation process.

2 Material and methods

2.1 Material and reagents

Unless otherwise noted, all chemicals and growth media in this study were purchased from commercial suppliers with at least analytical reagent grade (purity $\geq 99.0\%$) or biological reagent grade. The SYBR[®] Green I (10,000 $\times$ in dimethyl sulfoxide, DMSO) and propidium iodide ($\geq 94\%$, HPLC) for nucleic acid staining were purchased from Sigma-Aldrich. The *E. coli* strain (ATCC 8099) was purchased and cultivated in the lab to prepare the *E. coli* suspension, according to the procedure described in supporting information Text S1.

Ozone stock solutions (1.3–1.4 mM) were prepared according to the method described by Hoigne and Bader (1994), in which the ozone-containing oxygen gas is diffused into ice-cooled pure water. Notice that the ozone generator should have a capacity of 20 g O₃/hour to generate an adequate concentration of the O₃ stock solution.

2.2 Characteristics of water and wastewater effluents

Samples of secondary wastewater effluents were taken from two municipal WWTPs in China. The WWTP-WLK (DOC 9.1 mg/L) uses the oxidation ditch process, and the WWTP-DC (DOC 3.3 mg/L, 3.0 mg/L and 4.4 mg/L, sampled in different seasons) uses the conventional A²/O process. The surface water sample was collected from the Yangtze River at Nanjing (DOC 1.6 mg/L). Initial bromide concentrations in water were determined using a DIONEX ICS-2000 ion chromatograph with IonPac AS19 column. Water quality parameters are summarized in [Table S1](#).

Absorbance spectra from 200 nm to 600 nm were scanned with an Agilent 8453 UV-Visible spectrophotometer, and fluorescence excitation-emission matrixes (EEMs) were obtained with a Hitachi F-7000 fluorescence spectrophotometer (Hitachi Inc., Japan). Additionally, the previously developed LED UV/fluorescence sensor was used and the 1st order inner-filter effect of the humic-like fluorescence data was corrected according to its related absorbance spectra ([Li et al. 2016a, 2016b](#)).

2.3 Ozonation experiments with spiked *E. coli* or autochthonous bacteria

For the *E. coli* inactivation experiments, water and wastewater effluents were filtered by 0.45 μm glass-fiber filters for removal of particles and native bacteria. The filtered samples were first dosed w/or w/o 100 $\mu\text{g/L}$ Br[−]. The *E. coli* suspensions of OD₆₀₀ $\sim$ 0.5 cm^{−1} (i.e., $\sim 1.2 \times 10^9$ cell/mL) were prepared for bacterial spiking (**Text S1**). For the general experiments (**Group I in Table S2**), $\sim 10^6$ cell/mL *E. coli* working solutions were used. To explore the effects of bacteria concentrations on the inactivation efficiency and bromate formation, the *E. coli* working solutions ranged from 10⁶ to 10⁸ cell/mL (Group III & IV in [Table S2](#)). Additionally, the $\sim 10^6$ cell/mL *E. coli* working solutions w/or w/o 50 mM tert-butanol (tert-BuOH) were ozonated, to study the roles of $\cdot\text{OH}$ radical during ozone disinfection (Group V in [Table S2](#)).

For the inactivation of autochthonous bacteria (Group II in [Table S2](#)), the water and wastewater samples were filtered by 8 μm glass-fiber filters. Determined by FCM, the concentrations of native bacteria in the 8 μm -filtered samples were in the range of 0.7–1.3 $\times 10^6$ cell/mL.

Each 50 mL of working solution was transferred into a 100 mL clean and sterilized glass bottle. Ozone stock solution was dosed into each bottle at a range of specific O₃ doses (O₃/DOC in the range of 0–1.5 g/g) at room temperature. The residual ozone was immediately determined by the indigo method ([Bader and Hoigne, 1981](#); [Li et al., 2017](#)). After one hour, 20 mL of samples were filtered by 0.45 μm filter for chemical and spectroscopic analyses and the residual samples were taken for plating (*E. coli* experiments only) and FCM analyses immediately. Colony forming units (CFU) were measured by means of a spot-titer plating assay ([Beck et al., 2009](#); [Forsyth et al., 2013](#)), which is characterized by dispensing 10- μL droplets of samples on the LB-agar plate ([Text S2 and Fig. S2](#)).

Compared with the recent study by [Lee et al. \(2016\)](#), a wider range of DOC levels and O₃/DOC mass ratios as well as experiments w/or w/o bromide addition were conducted, which helps establish a robust threshold or indicator for ozone dosing.

2.4 FCM analyses with fluorescence staining

FCM analyses with two fluorescence staining dyes SYBR[®] Green I (SGI) and propidium iodide (PI) were conducted according to the method described previously ([Berney et al., 2008](#); [Lee et al., 2016](#)). For SGI-only staining, the working solution was prepared by diluting SGI reagent (10,000 $\times$ in DMSO) 100-fold into DMSO. For SGI/PI double staining, the working solution consisted of 100-fold SGI and 0.6 mM PI in DMSO. These working solutions (SGI-only and SGI/PI mixture) were stored at -20°C before use. For each analysis, the 10 μL SGI or SGI/PI working solution was added to 990 μL sample. The stained samples were incubated in the dark for 20 min. No dilution of the water or wastewater sample was conducted in this study. FCM analyses were performed using a BD LSRFortessa Cell Analyzer, equipped with a 488 nm solid-state laser. Green fluorescence of SGI was collected at 520 nm, and red fluorescence of PI was collected at 630 nm. For each sample, 5000 or 10000 events were recorded. The obtained data were presented in density plots with axes of red fluorescence intensity vs. green fluorescence intensity, in which each dot

represents the fluorescence signals of each detected particle. Due to the variation of water matrices and bacterial community, the detector voltage and threshold were adjusted accordingly in order to optimize the distribution of density plots. The detailed FCM parameters for each batch experiment are listed in Table S3.

2.5 Bromate analysis

Referring to our previous literature (Li et al., 2017), bromate was determined using ion chromatography with MS/MS detection, using an Agilent 1290 series HPLC system coupled with an AB SCIEX QTRAP® 5500 LC-MS/MS operating with negative mode electrospray ionization. Separation was performed using an ion exchange column Dionex IonPac AS-16 under isocratic conditions with a mobile phase comprising 25% of a 1 M aqueous methylamine solution and 75% of acetonitrile, at a flow rate of 0.30 mL/min and an injection volume of 10 µL. The mass parameters used in multiple reaction monitoring mode for BrO_3^- identification and quantification were as follows: $^{81}\text{BrO}_3^-$, 129.0–112.9 with –50 V DP, –10 V EP, –30 V CE and –13 V CXP; $^{79}\text{BrO}_3^-$, 127.0–110.9 with –50 V DP, –10 V EP, –30 V CE and –6 V CXP. The method detection (S/N = 3) and quantification (S/N = 10) limits for BrO_3^- were ~0.03 and ~0.1 µg/L, respectively.

3 Results and discussion

3.1 Assessment of ozone inactivation by spot-titer and FCM analyses

3.1.1 FCM analyses with SGI-only or SGI/PI fluorescence staining

The 0.45 µm membrane-filtered water and wastewater effluent samples w/and w/o 100 µg Br^- addition were spiked with $\sim 10^6$ cell/mL lab-cultured *E. coli* and were treated by ozone with the specific ozone doses up to 1.5 g/g O_3/DOC mass ratio. The results of FCM analyses with SGI/PI or SGI-only staining for Group I experiments were provided in Fig. S7-S12, and Fig. 1 shows the representative FCM density plots of WLK wastewater effluents. The green-fluorescent dye SGI can pass through intact cell membrane and intercalate and bind with double-stranded DNA, i.e., each dot represents a cell irrespective of its viability or cultivability; while the red-fluorescent dye PI can also stain DNA, but it is normally excluded outside of the intact bacterial cell until the membrane is damaged (Berney et al., 2008; Ramseier et al., 2011; Van Nevel et al., 2017). With increasing O_3 doses (Fig. 1e–h), the dots of FCM with SGI-only staining shifted gradually and to the left out of the fixed gate with lowering green-fluorescence intensity, which indicates considerable damage to DNA, e.g., double-strand breaks or separation into single-stranded DNA (Lee et al., 2016). The dots of FCM with SGI/PI staining shifted counterclockwise quickly out of the fixed gate with lowering green-fluorescence intensity but increasing red-fluorescence intensity (Fig. 1a–c), suggesting the *E. coli* membrane is fragile to ozone attacks (Ramseier et al., 2011). At high O_3 doses (Fig. 1d), the red-fluorescence intensity also decreased as well as the green-fluorescence intensity, suggesting the severe damage occurred to cells. Thus, the cell counts in the fixed gate of FCM analyses with SGI/PI or SGI-only staining can be used for quantitatively assessing bacterial membrane damage or DNA damage, respectively (Lee et al., 2016; Ramseier et al., 2011).

Fig. 1 The representative FCM density plots (X-axis 520 nm, Y-axis 630 nm) of the 0.45 µm filtered WLK wastewater effluent spiked with the lab-cultured *E. coli* ($\sim 10^6$ cell/mL) and treated with increasing O_3 doses. In the first row, samples were stained with SGI/PI for membrane damage analysis; in the second row, samples were stained with SGI-only for DNA damage analysis.

alt-text: Fig. 1

3.1.2 Comparison of CFU, membrane damage and DNA damage

Fig. 2 shows the variations of the live *E. coli* concentrations regarding CFU, membrane damage and DNA damage during ozonation of WWTP-WLK and WWTP-DC effluents. For both wastewater samples, the live *E. coli* concentrations measured by these viability assessment methods decreased in the order of CFU > membrane damage > DNA damage for the O₃/DOC mass ratio in the range of 0–0.60 g/g. The sensitivities of these viability parameters were consistent with the previous inactivation of *E. coli* by ozonation (Lee et al., 2016). For WWTP-WLK experiments, the *E. coli* viability quantified by CFU and membrane damage decreased steadily with increasing specific O₃ doses at lower range (O₃/DOC < 0.40 g/g) and then decreased relatively slowly at higher O₃ doses. For WWTP-DC experiments, there were obvious lags in the changes of cultivability and membrane damage at the lower specific O₃ doses (O₃/DOC < 0.25 g/g for CFU and O₃/DOC < 0.35 g/g for membrane damage), and then these viability parameters suddenly dropped down to $-3 \log(N/N_0)$. The relative concentration of intact cell seem to increase slightly with O₃/DOC mass ratios greater than 0.6 g/g for WWTP-DC-SGI and WWTP-DC-SGI/PI plots, because some dots of severely damaged cell also fell into the gate of intact cell in FCM analysis. Lee et al. (2016) attributed this lag phase to the high concentration of nitrite. However, it seems this could not explain the results of WLK batch experiments well, where there was no significant lag phase. Due to its high DOC concentration, the absolute O₃ concentrations dosed into WWTP-WLK effluent were also relatively high at the initial O₃/DOC mass ratios, while the *E. coli* was spiked with the same cell concentration for both samples. Given that the cell membrane is also fragile to O₃ attack, the high absolute O₃ doses led to no lag phase in WWTP-WLK experiments. The DNA damage for both wastewater matrices showed significant lag phases until O₃/DOC reached to ~0.4 g/g or membrane integrity reached to $-1 \log(N/N_0)$, consistent with previous observations (Czekalski et al., 2016; Lee et al., 2016). In prior work addressing inactivation of ARB cells and degradation of ARGs, in which wastewaters spiked with *E. coli* J53 containing ARG *sul1* were ozonated, 2-log of DNA damage and ARG *sul1* abatement were achieved at O₃/DOC = 0.38–0.40 g/g, i.e., the FCM with SGI-only staining and qPCR measurements showed congruent trends (Czekalski et al., 2016).

Fig. 2 Variations of cell viability parameters $\log(N/N_0)$ regarding CFU, membrane damage and DNA damage versus the O₃/DOC mass ratios during ozonation of *E. coli* spiked into (a) WWTP-WLK effluents and (b) WWTP-DC effluents.

alt-text: Fig. 2

For smart online control, it is interesting to note the inflection points of O₃ doses for sufficient inactivation. For the WLK wastewater matrices, the inactivation of *E. coli* as measured by CFU reached to $-3 \log(N/N_0)$ when the O₃/DOC was > 0.6 g/g. For the DC wastewater matrices, the inactivation of *E. coli* as measured by CFU reached to $-4 \log(N/N_0)$ and fell below the detection limit at O₃/DOC > 0.4 g/g. At these inflection points, the corresponding membrane damage and DNA damage were roughly $-2 \log(N/N_0)$ and $-1 \log(N/N_0)$ (Fig. S3). Therefore, 99% of membrane damage or 90% DNA damage is conservatively proposed as an indicative threshold for sufficient inactivation of bacteria as well as degradation of DNA.

3.2 Application of UVA/fluorescence indices as indicators for microbial inactivation

The UVA254 index has been widely applied as a surrogate indicator for online monitoring purposes because it correlates well with dissolved organic matter (DOM) aromaticity and UV light at 254 nm can be easily obtained from a low-mercury lamp (Gerrity et al., 2012; Korshin et al., 1997). UVA280 can also be used as a DOC indicator, by using commercially available 280 nm UV light emitting diodes (LED) (Li et al., 2016a). For DOM in water samples, the UVA280/UVA254 ratios are frequently ~0.74 (Li et al., 2016a), but these ratios might decrease slightly with increasing ozone doses (Li et al., 2017).

For EEM spectra, there are representative protein-like peaks and humic-like peaks (Fig. S4). During ozonation of water samples containing bacteria, the extracellular and endogenous proteins could be detached and leaked into water as dissolved components (Meng et al., 2016), which might affect the monitoring of the protein-like fluorescence. Compared to the protein-like fluorescence, the humic-like fluorescence is more sensitively detected by online sensors using silicon photodiode (Li et al., 2016b). Therefore, the UVA254, UVA280 and humic-like fluorescence indices were applied as indicators for microbial inactivation in this study.

3.2.1 Inactivation of *E. coli* versus changes of UVA/fluorescence indicators

Fig. 3 summarizes the membrane damage or DNA damage as a function of the reduction of UVA254, UVA280 and humic-like fluorescence during ozone inactivation. With the reduction of UVA254 and UVA280, the membrane damage of the *E. coli* spiked into different water matrices increased relatively linearly, and the lags at the initial points were not very significant, suggesting that the kinetics of *E. coli* membrane damage are faster than the destruction of aromatic moieties in DOM. However, there were significant lags in the plots of membrane damage versus the reduction of humic-like fluorescence, indicating that O₃ preferentially attacks the fluorophores containing rich electron-donating groups in the very initial stage, i.e., at very low ozone

doses with $O_3/DOC < 0.1$ g/g. When ~40% of UVA254, ~50% of UVA280 or ~75% of humic-like fluorescence were lost, the extents of membrane damage of all water and wastewater samples reached 99%, which is taken as an indicative threshold for sufficient inactivation. The plots also showed that the bacterial inactivation would not be sufficient when the losses of UVA254, UVA280, and humic-like fluorescence were below ~25%, ~30% and 45%, respectively. For all the plots of DNA damage versus UVA or fluorescence indices, there were significant lags in the initial O_3 demand stage, because highly reactive constituents in water and wastewater effluents consumed O_3 quickly (Fig.S5) and little O_3 passed through the *E. coli* cell membrane to attack DNA. When ~50% of UVA254, ~60% of UVA280 or ~80% of humic-like fluorescence were lost, DNA damage reached 90%. Notice that the plots for Yangtze River (DOC 1.6 mg/L) and WWTP-WLK (DOC 9.1 mg/L) showed relatively similar curves of DNA damage, despite their very different DOC levels. Thus the specific reactivity of UV-absorbing and fluorescent DOM constituents, rather than DOC concentration, is a better measure of the relationships between bacterial inactivation efficiency and DOM content during ozonation.

Fig. 3 The membrane damage (left column) or DNA damage (right column) of *E. coli* spiked into WWTP-WLK, WWTP-DC and Yangtze River matrices versus the changes of spectroscopic indicators UVA254 (a & d), UVA280 (b & e) and humic-like fluorescence (c & f).

alt-text: Fig. 3

3.2.2 Inactivation of autochthonous bacteria versus changes of UVA/fluorescence indicators

The concentrations of autochthonous bacteria in water and wastewater effluent samples ranged from 7.64×10^5 to 1.27×10^6 cell/mL, as counted by FCM analysis (Table S2). The FCM density plots for assessing membrane damage or DNA damage are provided in Fig. S13-20. Because of the high heterogeneity of autochthonous bacteria, the FCM operation parameters were adjusted accordingly, as shown in Table S3. Notably, the FCM density plots of the autochthonous bacteria in DC wastewater

effluents showed two main bacterial clusters, which were previously designated as high nucleic acid and low nucleic acid content bacteria (Berney et al., 2008; Ramseier et al., 2011).

Fig. 4 shows the inactivation of native bacteria in water and wastewater effluents versus changes of O₃/DOC mass ratio, UVA254, UVA280 and humic-like fluorescence. With increasing O₃ doses, the membrane damage increased steeply and then reached a plateau at O₃/DOC ~0.25 g/g for WWTP-WLK and Yangtze River samples and at O₃/DOC ~0.40 g/g for WWTP-DC samples. In previous work, it has been reported that more than 99% of autochthonous bacteria were usually inactivated (as inferred from membrane permeability) when O₃/DOC > 0.50 g/g (Lee et al., 2016). Notice that the membrane damage rates of WWTP-DC samples fluctuated slightly above 90% at high O₃/DOC mass ratios. Due to the broad fluorescence intensity spectrum of WWTP-DC samples, the intact cells of low nucleic acid content could not be distinguished from the severely membrane-damaged cells by the fixed gate (Fig. S17). Therefore, we conservatively assumed that points on the plateaus in Fig. 4 indicate sufficient inactivation of bacteria. For the plots of membrane damage versus the losses of UVA254, UVA280 and humic-like fluorescence, the inflection points indicative of sufficient bacteria inactivation were ~30%, ~40%, and ~60%, respectively. In Fig. 4d, the significant lags visible at low O₃/DOC mass ratios further suggest that DOM fluorophores enriched in electron-donating groups consumed O₃ rapidly (Li et al., 2017), and thus out-competed bacteria for O₃ at low O₃ doses. Regarding DNA damage, Fig. 4e showed that the O₃/DOC mass ratio of ~0.6 g/g is a reasonable threshold for sufficient inactivation (based on ~90% degradation of DNA). The plots of DNA damage as a function of UVA254, UVA280 and humic-like fluorescence rate exhibited as S curves. It can be seen that the inflection points between initial lags and slopes were in the range of 15–20%, 20–25% and 30–35% decreases of UVA254, UVA280 and humic-like fluorescence, respectively; while the inflection points reaching to ~90% of DNA damage were in the range of 40–48%, 48–55%, and 70–78% decreases of UVA254, UVA280 and humic-like fluorescence, respectively. These findings indicate that the O₃ doses required for sufficient inactivation of spiked *E. coli* and autochthonous bacteria (taking 99% membrane damage and/or 90% DNA damage as measures of sufficient inactivation) correspond to ~0.6 g/g O₃/DOC, ~50% loss of UVA254, ~60% loss of UVA280, and ~80% loss of humic-like fluorescence.

Fig. 4 The inactivation of autochthonous bacteria in water and wastewater effluents inferred from membrane damage (left column) or DNA damage (right column) versus changes of O_3 /DOC mass ratio (a & e), UVA254 (b & f), UVA280 (c & g) and humic-like fluorescence (d & h), respectively.

alt-text: Fig. 4

3.3 Effects of bacterial concentration and roles of oxidative species during ozone disinfection

Specific thresholds of O₃ doses, or UVA or fluorescence indices established as indicators of sufficient bacterial inactivation in water and wastewater effluents have mostly been obtained at cell concentrations of around 10⁶ cell/mL. The effects of cell concentration on the relationships between inactivation efficiency and these surrogate indicators have not been evaluated in depth, and are required to evaluate the robustness of the control strategies.

In the range of low O₃ doses (0.10 g/g < O₃/DOC < 0.25 g/g) in [Fig. 5a](#), the higher concentration of *E. coli* was spiked, the higher membrane damage rate it showed. This phenomenon may indicate that: (i) compared to the reactive chromophores and fluorophores, the cell membrane was relatively more fragile to O₃ attacks; (ii) in the ozone demand stage, the dosed O₃ was competitively consumed by *E. coli* cells rather than chromophores and fluorophores; and (iii) the higher cell concentration increased the contact opportunity of *E. coli*, and thus led to the higher membrane damage rate. Similar trends could also be observed in the plots of membrane damage versus UVA254, UVA280, and humic-like fluorescence ([Fig. 5b-d](#)). The minimal ozone dose required for sufficient bacterial inactivation as inferred from membrane damage was ~0.32 g/g O₃/DOC, corresponding to ~35% losses of UVA254, ~47% losses of UVA280, and ~70% losses of humic-like fluorescence.

Fig. 5 The effects of *E. coli* concentration (1×10^7 , 1×10^8 , 2×10^8 & 2.5×10^8 CFU/mL) on inactivation efficiency, as inferred from membrane damage (left column) or DNA damage (right column), versus changes of O_3 /DOC mass ratio (a & e), UVA254 (b & f), UVA280 (c & g) and humic-like fluorescence (d & h), respectively.

alt-text: Fig. 5

Fig. 5e depicts the plots of DNA damage versus O_3 /DOC mass ratios. It can be seen that at low O_3 doses (O_3 /DOC < 0.25 g/g), the *E. coli* concentration did not have obvious effects on the DNA damage; while at high O_3 doses (O_3 /DOC > 0.25 g/g), the high cell concentration reduced the DNA damage rate significantly. Inferred from the overlapped S curves in Fig. 5f-h, the DNA damage versus the UVA and fluorescence indicators were not influenced by the *E. coli* concentration. In summary, the minimal O_3 doses required for sufficient bacterial inactivation as inferred from DNA damage was O_3 /DOC ~ 0.60 g/g, corresponding to ~40% losses of UVA254, ~52% losses of UVA280 and ~80% losses of humic-like fluorescence. These values did not exceed the thresholds obtained from the above inactivation experiments of *E. coli* and autochthonous bacteria.

As shown in Fig. 6a, with respect to membrane damage, ozonation with *tert*-BuOH yielded slightly lower levels of inactivation when O_3/DOC was <0.15 g/g (marked by arrow), suggesting that ~40% of cell membrane damage was mainly attributed to the direct O_3 attacks at low O_3 doses. With increasing O_3 doses, the divergences in membrane damage between ozonation w/and w/o *tert*-BuOH remained less than 20% before reaching a plateau at $O_3/DOC >0.40$ g/g, suggesting that direct O_3 attack contributed over 80% to the membrane damage at high O_3 doses. No matter w/or w/o *tert*-BuOH, the damage of DNA kept in lag phase until the membrane damage reached to approximately ~90%, consistent with the results observed in Figs. 2 and 3. At higher O_3 doses, the differences of DNA damage w/and w/o *tert*-BuOH were less than 30%, and thus the DNA damage was mainly attributed to direct O_3 attacks rather than $\cdot OH$ attacks. As illustrated in Fig. 6b-d, the presence of *tert*-BuOH resulted in the left shifts of the plots of the membrane/DNA damage versus spectroscopic indicators. For the same membrane/DNA damage level, the left shifts of the losses of UVA254 and UVA280 were generally within 10%; while the left shifts of the losses of humic-like fluorescence were up to ~30%.

Fig. 6 The membrane (square) and DNA damage (circle) of the *E. coli* spiked into WWTP-DC effluents versus the changes of (a) O_3/DOC mass ratio, (b) decrease of UVA254, (c) decrease of UVA280 and (d) decrease of humic-like fluorescence for ozone inactivation w/(half-filled) and w/o (full-filled) the addition of 50 mM *tert*-BuOH.

alt-text: Fig. 6

3.4 Formation of bromate and its control strategies for ozone disinfection

Fig. 7a depicts the plots of bromate formation versus O_3/DOC mass ratios, which illustrate two phases in bromate formation. During the initial ozone demand stage ($O_3/DOC <0.4$ g/g) (Fig. S5), the dosed ozone was rapidly consumed - likely by reactions with bacterial membrane and electron-donating DOM chromophores and/or fluorophores, resulting in suppression of bromate formation below $10 \mu g/L$. At higher O_3 doses, when residual O_3 could be detected ($O_3/DOC >0.6$ g/g), bromate formed linearly with increasing O_3 doses, and the higher initial Br^- formed the higher bromate concentration. These results are in agreement with previous studies (Chon et al., 2015; Li et al., 2017), which found that bromate yields ($[BrO_3^-]/[Br^-]$, mol/mol in %) were negligible for $O_3/DOC <0.4$ or 0.6 mg O_3/mg DOC.

Fig. 7 The bromate formation versus changes of (a) O_3 /DOC mass ratio, (b) UVA254, (c) UVA280 and (d) humic-like fluorescence during ozone inactivation of the spiked *E. coli* and autochthonous bacteria in water and wastewater effluents.

alt-text: Fig. 7

Fig. 7b-d presents the bromate formation during ozonation as a function of the relative changes of UVA254, UVA280 and humic-like fluorescence. Similarly, changes in bromate formation versus spectroscopic indicators could be further divided into two stages by significantly different slopes. The breakthrough points related to the appreciable formation of BrO_3^- (10 $\mu g/L$) were approximately in the range of 44–58%, 54–65% and 78–90% losses of UVA254, UVA280, and humic-like fluorescence, respectively. In our previous ozonation study – conducted without the presence of bacteria, the losses of UVA254, UVA280, and humic-like fluorescence corresponding to the maximum concentration limit of BrO_3^- were in the range of 45–55%, 52–57%, and 86–90%, respectively (Li et al., 2017). As shown in Fig. S6, the addition of *E. coli* bacteria suppressed the bromate formation at the same O_3 /DOC mass ratios, and might lead to the breakthrough points shifting to the right. Rather than the presence of bacteria, it is the differences in initial Br^- concentration and water matrices that contributed to the left shifts of the indicative spectroscopic ranges for predicting bromate formation in this study.

The present work supports that it is possible to reach a compromise between bromate formation and bacterial inactivation in terms of membrane or DNA damage during ozonation of water and wastewater effluents, where the O_3 /DOC mass ratio was in the range of 0.40–0.60 g/g and the losses of spectroscopic indicators were approximately around 50%, 60% and 80% for UVA254, UVA280 and humic-like fluorescence, respectively. In the context of the oxidation roles, such ozone doses could also eliminate most trace organic contaminants that have moderate reactivity with O_3 and high reactivity with $\cdot OH$ (i.e., Group I and Group II micropollutants) (Gerrity et al., 2012; Lee et al., 2013; Li et al., 2016b). When ozone is applied for inactivation of recalcitrant microorganisms (e.g., spores or protozoan cysts) and oxidation of refractory trace organic contaminants (e.g., TCEP), higher O_3 doses or changes of spectroscopic indicators are necessary (Gamage et al., 2013; Gerrity et al., 2012); however, the initial Br^- concentration or BrO_3^- concentration should be carefully evaluated before or after ozonation.

4 Conclusions

- The FCM analyses with SGI/PI or SGI-only staining can be used for qualitative and quantitative assessment of membrane damage and DNA damage, respectively. During ozonation of *E. coli* spiked into wastewater effluents ($\sim 10^6$ cell/mL), the priority of inactivation efficiency determined by different cell viability methods was in the order of CFU > membrane damage > DNA damage. Approximately, 99% membrane damage and/or 90% DNA damage are conservatively taken as indicators for sufficient bacterial inactivation, i.e., $< -3 \cdot \log(N/N_0)$ or $< -4 \cdot \log(N/N_0)$.
- In this study, the O_3 dosing thresholds required for sufficient inactivation of spiked *E. coli* and autochthonous bacteria, as inferred from 99% membrane damage and/or 90% DNA damage, were ~ 0.6 g/g O_3 /DOC, $\sim 50\%$ loss of UVA254, $\sim 60\%$ loss of UVA280, and $\sim 80\%$ loss of humic-like fluorescence.

- Within the range of 10^6 – 10^8 cell/mL, the bacterial concentration did not have significant effects on the required thresholds of O_3 /DOC mass ratios or spectroscopic indicators required for bacterial inactivation. The addition of *tert*-BuOH increased the specific ozone doses but decreased the losses of spectroscopic indicators for sufficient bacterial inactivation, Membrane and DNA damage were found to be due primarily to direct attack by O_3 .
- The presence of spiked bacteria (10^6 cell/mL) slightly suppressed bromate formation compared to experiments undertaken in the absence of spiked bacteria at equivalent ozone doses. When O_3 was added within the range of thresholds required for sufficient bacterial inactivation, bromate formation was suppressed below 10 µg/L, suggesting that it is possible to reach a balance between bacterial inactivation and bromate formation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.watres.2018.08.030>.

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Appendix A. Supplementary data

The following is the supplementary data related to this article:

[Multimedia Component 1](#)

20180811_Supporting Information

alt-text: 20180811_Supporting Information

Graphical abstract

alt-text: Image 1

Highlights

- UVA & fluorescence were used as indicators for O₃ disinfection efficiency.
- Bacterial membrane damage and DNA damage by O₃ were analyzed by flow cytometry.
- Direct O₃ attacks mainly contributed to the bacterial membrane/DNA damage.
- Balance between bacterial inactivation and bromate formation can be reached.
- ~80% decrease of humic fluorescence indicating sufficient bacterial inactivation.

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