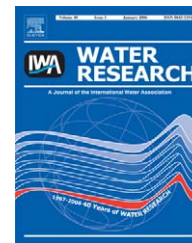


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# The impact of selected water quality parameters on the inactivation of *Bacillus subtilis* spores by monochloramine and ozone

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## ABSTRACT

Selected water quality parameters—pH, dissolved organic carbon, turbidity (NTU), and temperature—were tested for their potential effects on ozone and monochloramine inactivation of *Bacillus subtilis* spores. In oxidant demand-free phosphate-buffer, temperature had the strongest influence on inactivation kinetics when using ozone, pH had a smaller but significant impact on *B. subtilis* spore inactivation with both monochloramine and ozone. Where monochloramine was applied, modeling and experimental measurements confirmed that dichloramine levels were too low to produce significant inactivation effects under these experimental conditions. It was demonstrated that oxidant demand-free phosphate buffer may not be an adequate environmental analogue for inactivation responses in natural waters.

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## 1. Introduction

After repeated outbreaks of cryptosporidiosis caused by *Cryptosporidium parvum* oocysts, in the United States and in parts of Europe there has been an increased focus on understanding the disinfection responses of pathogenic microorganisms. Many different cyst-forming protozoa have been implicated as relatively resistant to conventional oxidative disinfection practices. While chemical disinfection has been studied for more than 100 years, the efficacy of this process under different water quality conditions has not been systematically investigated.

For chemical disinfection, the USEPA regulates inactivation using CT tables which provide oxidant exposure values (CT in min mgL<sup>-1</sup>) for different oxidants and target microorganisms (Water Quality and Treatment: A Handbook of Community Water Supply, 1999). Much of the data used to develop these tables was obtained from research conducted in oxidant demand-free phosphate-buffered waters (Finch et al., 1993; Haas et al., 1996). Further, many regulations are based on evaluating inactivation data using the Chick-Watson disinfection model which may not adequately describe disinfection behavior of many microorganisms through a broad range of environmental conditions (Finch et al., 1993). The CT tables include temperature ranges typically encountered in water

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treatment, but they do not account for changes in inactivation response that may result from changes in pH (except for free chlorine), turbidity levels, or the presence of different sources of dissolved organic matter (Water quality and treatment: A handbook of community water supply, 1999). A previous study by Haas et al. (1996), compared inactivation of *Escherichia coli*, *Giardia muris* cysts, and bacteriophage MS2 in three natural waters to oxidant demand-free phosphate-buffered water using free chlorine, ozone and monochloramine. Different water quality conditions induced a different inactivation behavior and it was not possible to predict the inactivation response for these microorganisms based on common water quality indices. Haas et al. (1996) suggested that oxidant demand-free phosphate-buffered waters were poor experimental analogues to study microorganism inactivation in natural waters, and that disinfection tests should be conducted in the actual water to be treated. Labatiuk et al. (1992) studied the effect of two natural waters in comparison to phosphate-buffered water on *G. muris* cyst inactivation using ozone, and determined that CT values obtained from experiments conducted in phosphate buffer may be too small when extrapolated to natural waters. Except for the studies done by Haas et al. (1996), Labatiuk et al. (1992), and Cho et al. (2003) there has been little work carried out to assess the effect of water quality variation on the inactivation of microorganisms.

Oocysts such as *C. parvum*, require much higher CT values for equivalent inactivation levels than most vegetative bacteria and viruses. This can be problematic in light of increased potential for disinfection by-product (DBP) formation with higher CT values. Therefore, there is a growing need to optimize disinfection processes, which requires improved understanding of disinfection under varying water quality conditions commonly encountered in water treatment. Unfortunately, reliable methods for enumerating pathogenic protozoa and their oocysts are time consuming and costly, impairing comprehensive studies on the impact of water quality parameters on their inactivation. Therefore, spore forming microorganisms such as *B. subtilis* have been investigated for their potential use as surrogates to help predict the inactivation behavior of *C. parvum* oocysts (Rice et al., 1996; Barbeau et al., 1999; Facile et al., 2000; Driedger et al., 2001b; Larson and Mariñas, 2003). For temperatures  $>15^{\circ}\text{C}$  *B. subtilis* spores appear to be a conservative indicator for *C. parvum* oocysts inactivation using ozone, and display similar behavior, but have lower CT values at temperatures  $<10^{\circ}\text{C}$ . This is because *B. subtilis* spores inactivate faster than *C. parvum* oocysts at lower temperatures, ( $1\text{--}10^{\circ}\text{C}$ ), due to lower activation energy (Larson and Mariñas, 2003). Nevertheless, *B. subtilis* spores are easy to culture and enumerate, which allows for disinfection experiments to be economically and rapidly replicated, and makes them a useful tool for studying the dynamics of microorganism inactivation (such as disinfection response to changing water quality parameters). Like *C. parvum* oocysts, *B. subtilis* spore disinfection begins with a lag phase (period before the onset of inactivation) followed by first-order inactivation using both ozone and monochloramine (Rennecker et al., 1999; Driedger et al., 2000; Larson and Mariñas, 2003). Due to their similar inactivation behavior, it is

likely that inactivation responses observed for *B. subtilis* spores due to changing water quality conditions will be representative of *C. parvum* oocyst responses.

Previous ozone and monochloramine inactivation research has demonstrated a good fit of both *C. parvum* oocysts and *B. subtilis* spore inactivation behavior to the delayed Chick-Watson model (Rennecker et al., 1999; Driedger et al., 2000; Driedger et al., 2001a, b; Larson and Mariñas, 2003):

$$\frac{N}{N_0} = \begin{cases} 1 & \text{if } CT \leq CT_{\text{lag}} = \frac{1}{k} \ln\left(\frac{N_1}{N_0}\right), \\ \frac{N_1}{N_0} \exp(-kCT) = \exp(-k\{CT - CT_{\text{lag}}\}) & \\ \frac{N_1}{N_0} & \text{if } CT > CT_{\text{lag}} = \frac{1}{k} \ln\left(\frac{N_1}{N_0}\right). \end{cases} \quad (1)$$

$CT_{\text{lag}}$  represents the lag phase, time prior to the onset of inactivation;  $k$  is the post lag phase first-order inactivation rate constant ( $\text{Lmg}^{-1}\text{min}^{-1}$ ).  $N$  represents concentration of microorganisms,  $N_0$  is equal to the initial concentration,  $N_1$  is concentration of microorganisms in the lag phase prior to inactivation. The CT in this equation can be obtained by using a constant oxidant concentration or by integrating the oxidant residual over time to obtain an integrated CT (Rennecker et al., 1999).

The objective of this study was to conduct an evaluation on the potential impacts of selected water quality parameters (pH, temperature, dissolved organic carbon (DOC), and turbidity) on *B. subtilis* spore inactivation using monochloramine and ozone.

## 2. Materials and methods

### 2.1. Experimental apparatus

Completely mixed batch reactors made from 500 mL amber glass Erlenmeyer flasks topped with silicon rubber stoppers and placed in a temperature-controlled water bath were used for all experiments. Flasks were fitted with two septum-covered sample ports, where samples could be withdrawn with a syringe. Three inch, 20 gauge syringe needles remained in the reactors for the duration of the experiment. Flasks were placed on stir plates and continuously stirred using a Teflon coated stir bar to ensure complete mixing of the reactor. Reactors and other materials were made ozone demand-free, or monochloramine demand-free, prior to use by soaking in 2 mg/L ozone or 10 mg/L monochloramine bath for 18 h. Any oxidant remaining on glassware decayed during autoclaving of the equipment, which followed the soaking in the pre-oxidation bath.

### 2.2. Reagents and procedures

All experimental waters were made using autoclaved ozone demand-free water, including 3 mM sodium phosphate buffer and 3 mM carbonate alkalinity. Specified volumes of stock solutions of 40 g/L  $\text{Na}_2\text{HPO}_4$  and 10 g/L  $\text{NaH}_2\text{PO}_4$  were used to obtain the 3 mM phosphate buffer at pH 6.6 and 7.9. To obtain 3 mM carbonate alkalinity, 5 ml from a 0.7 M stock solution of sodium bicarbonate was added to reactors after autoclaving. Carbonate was added to scavenge  $\text{OH}\cdot$  radicals, which stabilized ozone residuals over relatively long time periods

(von Gunten, 2003a,b). Clean kaolinite (Phipps & Bird, Richmond, VA, Hydrite R grade) was added to produce a target turbidity of 2.5 NTU and 5 NTU, corresponding to approximately 2.5 mg/L and 5 mg/L kaolinite. This turbidity corresponded to a total particle count of roughly 15000 particles/mL (HACH WPC1900), with approximately 90% being in the 2–5  $\mu\text{m}$  range. Turbidity was measured using a HACH 2100N turbidimeter. Suwannee River natural organic matter (NOM) isolate served as the DOC source (International Humic Substances Society (IHSS), Golden-Colorado). The dried powder isolate of Suwannee River NOM was suspended in autoclaved and filter sterilized Millipore water to make a stock solution of 100 mg/L as DOC. An aliquot of the stock solution was added to the reactor water using a sterile pipette, diluted to a concentration of 2 mg/L or 5 mg/L, after the experimental waters in the reactors were autoclaved. Organic carbon concentrations were measured using a Sievers 800 automatic total organic carbon (TOC) analyzer (Ionics Sievers, Boulder, CO). A  $10^{-2}$  dilution of a  $10^9$  CFU/ml *B. subtilis* spore stock was inoculated into the reactors to reach an approximate concentration of  $10^{4.5}$  CFU per mL and left to stir 24 h prior to experiments to allow the spores to acclimate to the experimental waters.

Lake Zurich water (Switzerland) used for experiments was drawn through an intake pipe located 30 m deep, filtered through a 0.45  $\mu\text{m}$  filter, and stored at 4 °C until use. Lake Zurich water was chosen because it has been previously well characterized and used in other disinfection studies (Driedger et al., 2001b), due to its low variability in water quality throughout the year (Elovitz et al., 2000). Lake Zurich water contains a DOC concentration of about 1.2 mg/L, 2.5 mM alkalinity (as  $\text{HCO}_3^-$ ), turbidity of 1.5 NTU, and a pH of 8.1.

For each experimental condition a control system, identical in all respects except the addition of an oxidant, was measured over an 8 h period. This ensured that *B. subtilis* spore inactivation was a result of oxidant exposure (CT), and not “natural decay” in the synthetic waters.

### 2.3. Spore culturing and enumeration

A freeze dried culture of *B. subtilis* purchased from the American Type Culture Collection (ATCC 6633) was rehydrated and grown in Tryptic Soy Broth (Difco). Streak plates were made to isolate single colonies for inoculation in Tryptic Soy Broth to grow cells for stock culture. Broth cultures were placed in 37 °C incubators and continually mixed on shaker tables for 24 h, the time required for vegetative cells to reach late log growth. A  $10^{-2}$  dilution of log growth vegetative cells was made in phosphate buffer (Standard Methods, 9050C) that contained 20 mg/L  $\text{MnCl}_2$ . The  $\text{MnCl}_2$  was used to induce vegetative cells to sporulate (Setlow, 1994). This inoculum was spread with a sterile glass rod on prepared R2 Agar (Becton Dickinson) plates. The glass rod was sterilized by sequential ethanol immersion and flame steps. Spores were harvested after 7 days when approximately a 95% *B. subtilis* spore to vegetative cell ratio was achieved. This was determined by phase-contrast microscopy, where spores appear brighter and smaller than the vegetative cells which appear dark and elongated.

Spores were harvested by flooding agar plates with sterile phosphate buffer, gently scraping the agar surfaces with sterile glass rods, and washing the harvested spores three times by sequential centrifugation (3000g for 10 min each) in phosphate buffer to rid solution of vegetative cells and cellular debris. Between centrifugation steps, the supernatant was pipetted off, leaving a pellet at the bottom. The pellet contained primarily spores, while the supernatant contained vegetative cells and debris. Comparing pre- and post-washed *B. subtilis* spore solutions, using phase-contrast microscopy, confirmed that washing removed large amounts of cellular debris from lysed vegetative cells produced during sporulation. Sterile phosphate buffer was then added to the centrifuge tube and vigorously vortexed to disperse spores back in suspension. There was no observable difference in ozone decay curves in control experiments using phosphate buffered water with and without *B. subtilis* spores, indicating that addition of *B. subtilis* spore solution did not contribute a significant amount of ozone reactive material. Spores were kept for up to a year in phosphate buffer at 4 °C and the same stock was used for all experiments. A replicate of the initial experiment was executed after 12 months, to demonstrate that the sensitivity of the spore stock to ozone had not changed during the observation period.

During all experiments 3-mL samples were withdrawn through pre-placed sterile stainless steel needles with 5-mL syringes. Samples were ejected into dilution tubes containing 1-mL, 11% (w/w) sodium thiosulfate which immediately quenched the remaining oxidant. Enumeration of viable spores was done using a previously described plate filtration method (Barbeau et al., 1999). Serial dilutions of the samples were made in standard phosphate buffer to obtain the optimum concentration of colonies on the plates, 20–100 colony forming units (CFU). One milliliter of the dilution was filtered through a 0.45  $\mu\text{m}$ , 47 mm diameter, grided filter (Millipore HAWF047S1). The filters were placed on 47 mm Petri dishes with pads (Millipore AP10047S1) inoculated with 1.75-ml Tryptic Soy Broth containing 1% tetrazolium trichloride. The tetrazolium trichloride dyed the spores red as they germinated and ultimately formed red pin-head (slightly larger) colonies, aiding in the counting of microorganisms. The plates were then wrapped in water/air tight plastic bags and placed in a 75 °C water bath for 15 min, after which they were placed in a 37 °C incubator for 24 h and then counted. All samples were plated within 16 h of the experiments. Results were recorded as CFU per mL.

### 2.4. Ozone production and analysis

A 0.7 mM ozone stock solution was produced using an OREC electrostatic ozone generator which bubbled oxygen containing  $\text{O}_3$  into Millipore water placed in an ice bath. The concentration was measured using a HACH DR/4000U spectrophotometer. A molar absorptivity of  $3000 \text{ M}^{-1} \text{ cm}^{-1}$  at a wavelength of 258 nm was used to calculate the ozone concentration in the 1.1 mM stock solution (Elovitz and von Gunten, 1999). Ozone from the stock solution was transferred to batch reactors using a syringe attached with Nalgene tubing so that the ozone could be injected into the bottom of

the reactor. Ozone residual in the batch reactors was measured by the indigo method (Bader and Hoigné, 1981). Two or four millilitre samples were withdrawn from the sample ports using 5 ml, ozone demand-free syringes and then placed in 2.25 ml of 0.0125 mM or 0.0625 mM indigo solutions. Indigo solutions were made from a 1 mM stock solution of potassium indigo trisulfonate, made according to the method (Bader and Hoigné, 1981), and kept 3–4 months. The effective CT, was obtained by integrating the ozone residuals (mg O<sub>3</sub>/L) over the reaction time (minutes) using commercial software (KaleidaGraph<sup>®</sup>).

## 2.5. Monochloramine production and analysis

Fresh monochloramine stock solutions were prepared daily for experiments at a concentration no greater than 218 mg/L NH<sub>2</sub>Cl (2.1 mM Cl<sub>2</sub>), using 11% aqueous sodium hypochlorite solution and ammonium sulfate to achieve a chlorine (Cl<sub>2</sub>) to ammonia weight ratio of 4:1. Borate buffer was used to maintain the pH in the range between 9 and 10. The solution was left to stir for an hour prior to testing. Under these conditions, the dominant species was monochloramine. All monochloramine stocks were tested for the presence of free chlorine and dichloramine prior to addition to experimental waters.

Chlorine measurements were done using the DPD (N,N-diethyl-*p*-phenylenediamine) colorimetric method (Standard Methods, 4500 Cl) which measures both free chlorine and monochloramine. No free chlorine was observed in the reactors or the stock solutions.

Monochloramine decrease in experimental waters was also evaluated using a previously described kinetic model (Vikesland et al., 2001), which included the most relevant NH<sub>2</sub>Cl degradation reactions in this system. Chemical reactions and rate constants adjusted for temperature and pH were processed by shareware (ACUCHEM, National Bureau of Standards) (Braun et al., 1988) to estimate the final concentrations of NH<sub>2</sub>Cl, NHCl<sub>2</sub>, HOCl, and OCl<sup>-</sup>. The initial concentrations of 0.17 mM NH<sub>2</sub>Cl and 0.25 mM NH<sub>2</sub>Cl that

were placed in the batch reactors were modeled to estimate their final concentration over an 8 h time period.

## 2.6. Analysis of inactivation data

Log-linear regressions were done on inactivation data starting with points at which greater than a 5% difference from the initial culturable *B. subtilis* spore concentration was observed. A CT value for the end of the lag phase was determined from the log linear regression as the point at which no inactivation had occurred. Experimental data was fit to the delayed Chick–Watson model proposed by Rennecker et al. (1999) and used to describe inactivation behavior for both *C. parvum* oocysts and *B. subtilis* spores in other peer-reviewed research (Driedger et al., 2000, 2001a, b; Rennecker et al., 2000; Larson and Mariñas, 2003). This expression allows for the estimation of the CT for the lag phase, followed by a linear first-order decrease/decay of culturable microorganisms based on integrated oxidant exposure (CT). Integrated oxidant exposures provide an “effective CT,” and are reported here according to common convention as CT (L mg<sup>-1</sup> min<sup>-1</sup>).

## 2.7. Experimental design

For inactivation studies, four water quality parameters—turbidity, temperature, DOC, and pH—were selected as input (process) variables. An experimental matrix was developed using the Box and Hunter statistical method, with the aid of commercially available software (Statistica, Statsoft, 2004; Box et al., 1978). This approach was based on a partial factorial design, isolating four (three for NH<sub>2</sub>Cl) water quality variables (input variables) at two levels (minimum and maximum) with  $2^{(4-1)} = 2^3 = 8$  assays for ozone (Table 1) or  $2^{(3-1)} = 2^2 = 4$  assays for NH<sub>2</sub>Cl (Table 2). Due to extremely long experimental time frames (days), the temperature dependence for *B. subtilis* spore inactivation using monochloramine at low temperatures (7 °C) was not investigated. Tables 1 and 2 provide the range of each parameter defining the experimental conditions. This design is a resolution IV for ozone, and III for NH<sub>2</sub>Cl, which allows for

**Table 1 – Water quality conditions for *B. subtilis* spore inactivation using ozone<sup>a</sup>**

Condition	pH	Temperature (°C)	Kaolinite concentration (NTU)	DOC <sup>b</sup> (mg C/L)
1	6.6±.01	7.0±0.1	< 0.05	< 0.3
2	7.9±0.1	7.4±0.3	< 0.05	2.3±0.2
3	6.6±.01	21.4±0.4	< 0.05	2.5±0.2
4	8.1±0.1	21.5±0.2	< 0.05	< 0.3
5	6.6±0.1	7.2±0.4	4.7±0.3	2.5±0.4
6	8.0±0.6	7.4±0.5	5.6±1.2	< 0.3
7	6.6±0.1	21.8±0.7	5.5±0.3	< 0.3
8	8.1±0.1	21.0±0.2	5.6±0.2	2.6±0.1
9 (Centered)	7.4±0.1	14.1±0.7	3.1±1.1	1.3±0.3

<sup>a</sup> Values for pH, temperature, kaolinite and DOC, represent average and standard deviation of measured values for experimental waters used.

<sup>b</sup> IHSS isolate, Suwannee River NOM.

**Table 2 – Water quality conditions for *B. subtilis* spore inactivation using monochloramine<sup>a</sup>**

Condition	pH	Temperature (°C)	Kaolinite concentration (NTU)	DOC <sup>b</sup> (mg C/L)
3	6.4±0.2	22.0±2.0	< 0.05	4.9±0.7
4	8.2±0.1	21.8±0.3	< 0.05	< 0.3
7	6.4±0.2	23.1±1.0	5.5±0.5	< 0.3
8	8.2±0.1	22.0±0.5	5.2±0.6	5.1±0.4

Condition numbers correspond to the same water condition for the ozone inactivation experiments (Table 1).

<sup>a</sup> Values for pH, temperature, kaolinite, and DOC, represent average and standard deviation of measured values for experimental waters used.

<sup>b</sup> IHSS isolate, Suwannee River NOM.

the evaluation of the main effects, without requiring all the experiments necessary to individually isolate each process variable.

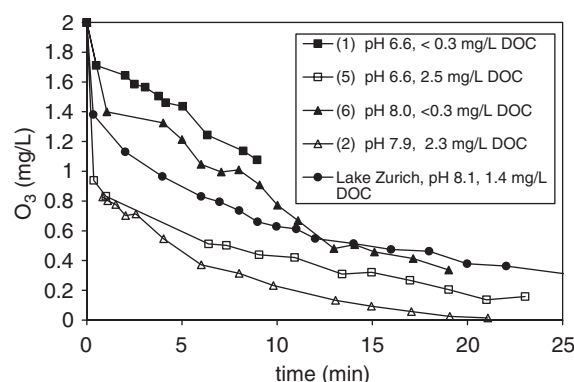
The statistical analysis was conducted in two steps; first, a quality control analysis was conducted, followed by an analysis of variance (ANOVA) for standardized effects. For quality control analysis, quality control charts (Johnson and Leone, 1964) were constructed to confirm that the effects of water quality on the dependent variables were higher than that associated with experimental error. A coefficient of variation ( $CV = (\text{standard deviation/average}) \times 100$ ) was calculated for each output variable to identify a specific disinfection condition or output variable with low reproducibility.

Once the data was determined to have statistically significant variation, and acceptable experimental error, an ANOVA was conducted to determine the influence of input variables (water quality parameters) on output variables ( $CT_{lag}$ ,  $CT_{2log}$ , and inactivation rate). This approach identified the main parameters affecting the output variables under investigation using a thorough sensitivity analysis. Standardized effects were calculated for each water quality parameter. Effects were defined here as the variation in an output variable (e.g.  $CT$ ) caused by modification of a control variable (e.g., pH). With the statistical power provided by the experimental matrix, the output values provided were used to demonstrate the magnitude of effects when isolating changes in a single water quality variable, which were normalized by their respective experimental error. For the standardized effects,  $p$  values were calculated at a 95% confidence level. Standardized effect values greater and less than the  $p$  value demonstrated that a water quality parameter (input variable), had a significant impact on the output variable, inactivation. A negative standardized effect value indicates that when the input variable value was increased from a low to a higher “set point” the output variable value was reduced, while a positive value indicates that increasing the input variable value results in an increase in the output value.

### 3. Results and discussion

#### 3.1. Ozone stability

Due to the radical type chain reactions involved in ozone decomposition as the result of NOM (von Gunten, 2003a),



**Fig. 1 – Ozone decrease profiles for different experimental conditions and Lake Zurich water, at 7 °C and an initial ozone concentration of 2 mg/L; open and closed squares represent pH 6.6, and open and closed triangles represent pH 8.0 and pH 7.9. Open symbols represent water containing Suwannee River NOM. Legend numbers in parenthesis refer to experimental condition listed in Table 1. The initial fast decrease varies for the individual waters and is followed by a first-order decay further described in Table 3.**

stable residuals could not be maintained in the presence of Suwannee River NOM ( $t_{1/2} < 1$  min.), if no additional  $OH\cdot$  radical scavenger was added. To overcome this effect, it was necessary to include 3 mM of carbonate alkalinity to demand-free-buffered waters. This maintained ozone residuals long enough to obtain at least 2-log inactivation for all experimental conditions. Bicarbonate provided a more realistic defined system for ozonation experiments which was similar to natural waters that contain some natural alkalinity (0–5 mM carbonate alkalinity) or other  $OH\cdot$  radical scavengers. Bicarbonate scavenges  $OH\cdot$  radicals because it interrupts the chain reactions initiated by  $OH\cdot$  radicals, thereby stabilizing ozone residuals.

Fig. 1 shows ozone residual concentrations obtained for four experimental conditions at 7 °C with an initial ozone concentration of 2 mg/L (with 3 mM of carbonate alkalinity) and that from a natural water source, Lake Zurich. Table 3 summarizes the first-order rate constants for ozone decrease under four experimental conditions. Depending on the water

**Table 3 – Ozone decay rate constants at 7 °C for different experimental conditions presented in Table 1 and Lake Zurich water<sup>a</sup>**

Water	pH	DOC (mg/L)	Alkalinity (mM)	Rate constants— $k$ (L mg <sup>-1</sup> min <sup>-1</sup> )	R <sup>2</sup>
Lake Zurich water	8.1	1.2	2.5	0.24	0.98
1	6.6	< 0.3	3	0.03	0.95
2	7.9	2.3	3	0.078	0.97
5	6.6	2.5	3	0.30	0.96
6	8.0	< 0.3	3	0.036	0.95

<sup>a</sup> The corresponding ozone decrease profiles are presented in Fig. 1, the R<sup>2</sup> of the regressions are provided. Refer to Table 1 for standard deviation of pH and DOC.

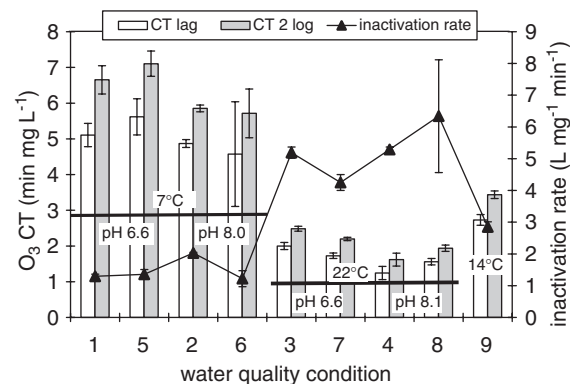
quality conditions, the ozone decrease profiles significantly changed. Similar to that observed by Driedger et al. (2001b), at 22 °C ozone residual concentrations decreased an order of magnitude faster than at 7 °C, and displayed similar effects as a result of water quality conditions. Experiments varying the kaolinite concentration, under otherwise identical conditions, between 0, 2.5 and 5 NTU, were conducted at 7 °C and pH 6.6 without the addition of DOC or microorganisms (data not shown). There was no observable difference in ozone decrease due to kaolinite addition. These data suggest that the changes in the ozone chemistry in these experiments were due to the addition of DOC, and/or changes in pH and temperature. However, changes in the ozone residual profile preclude direct comparisons of inactivation responses due to water quality variations. It cannot be stated whether the observed differences in inactivation resulted from changes in the ozone chemistry/water chemistry or if they resulted from water quality effects exerted on the microorganism themselves.

### 3.2. Ozone inactivation studies

Fig. 2 summarizes the inactivation response from each experimental condition for *B. subtilis* spore inactivation using ozone. Regressions were used to obtain the dependent variables— $k$ , L mg<sup>-1</sup> min<sup>-1</sup>, the CT lag (mg min L<sup>-1</sup>), CT 2log (mg min L<sup>-1</sup>). The water quality conditions in Fig. 2 are ordered to aid in reading the graph; 22 and 7 °C are grouped together within that by pH. This data grouping suggests that temperature has the greatest impact on *B. subtilis* spore inactivation rate, CT<sub>lag</sub>, and CT 2log. While pH also demonstrates an impact, it was necessary to conduct an ANOVA to determine if pH effects were significant.

Output data were checked to ensure acceptable experimental error and reproducibility. The quality control charts demonstrated that the variation of the output variables for each experimental condition (rate of inactivation, CT<sub>lag</sub> and CT 2log inactivation) among the experimental conditions was higher than the variation due to experimental error. The coefficient of variation (CV) obtained for the output variables of most experimental conditions was less than 10% and all were below 20% (five output variables were between 10% and 20%).

Fig. 3 summarizes the standardized effects on the output variables in response to changes in water quality parameters. These values represent the effect caused by changing a water



**Fig. 2 – Output values obtained for the inactivation of *B. subtilis* spores using ozone. Output variables were obtained from regressions of triplicate experiments and average and standard deviation were calculated. Numbers on the x-axis refer to experimental conditions in Table 1. Experiments were re-ordered in this graph for comparison purposes. Temperature and pH values are marked on the graph for ease in comparing water quality conditions.**

quality parameter from its lowest to its highest “set point,” for example increasing DOC from 0 to 2 mg/L, or increasing the pH from 6.6 to 8.1. The values represent the magnitude of the effect and a positive value indicates that a control variable increased the output variable, while a negative value means that the output variable was significantly decreased. For 95% confidence level, the  $p$ -value corresponded to 2.07. Therefore, effects higher than 2.07 (absolute values) were considered significant.

The values for the standardized effects indicate that increasing the temperature had the most impact on inactivation rate, an impact of roughly eight times higher than the other variables. Increasing temperature, also led to a corresponding decrease in the lag phase and CT 2log. The effect of temperature has been previously documented in literature (Finch et al., 2001; Barbeau et al., 2004; Rennecker et al., 1999). The only other water quality variable in which all three output variables were significantly affected was pH. Increasing the pH resulted in an increased inactivation rate and a corresponding decrease in the lag phase and CT 2log. While the standardized effects suggest changes in turbidity and

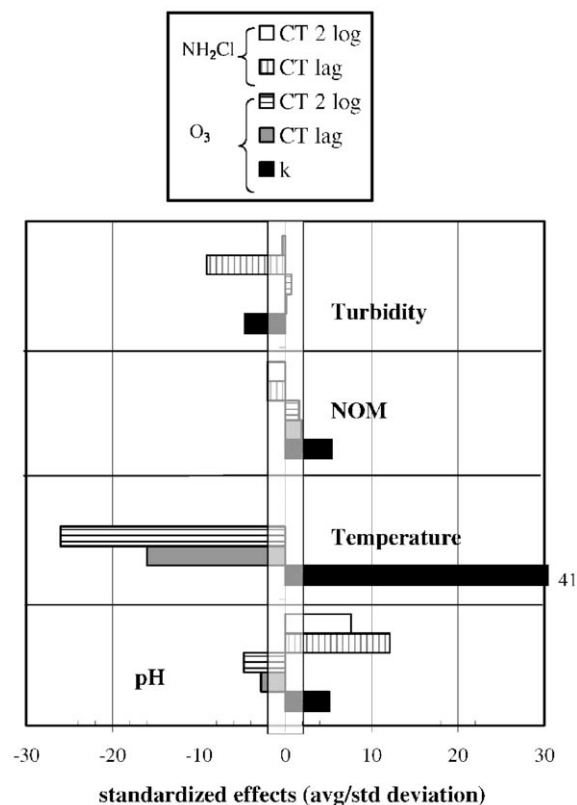


Fig. 3 – Summary of standardized effects for *B. subtilis* spore inactivation using ozone and monochloramine.

The values within the column represent the values below the  $p = 0.5$  significance level (equaling a value of 2.07, 95% confidence). An increase in standardized effects for  $CT_{lag}$  and  $CT_{2\log}$  indicate that increasing a control variable (i.e. turbidity, NOM, temperature, and pH) results in increased CT values. Increase in standardized effects for inactivation rate ( $k$ ) indicates faster inactivation.

DOC significantly affected the inactivation rate,  $CT_{lag}$  and  $CT_{2\log}$  were not significantly affected. Thus, there was no significant impact on the inactivation of *B. subtilis* as a result of increasing turbidity and DOC concentrations in these experiments.

Changes in the inactivation profiles in response to pH increases are presented in Fig. 4. Experiments at 7 °C were executed with the same initial ozone concentration of 2 mg/L for all four experimental conditions. Changes in inactivation profiles were also observed for experiments conducted at 22 °C. Compared to experiments conducted at pH 8.0, equivalent inactivation of *B. subtilis* spores required higher CT values at pH 6.6. A similar effect of pH on the inactivation of *B. subtilis* using ozone was also observed by Larson and Mariñas (2003) where equivalent inactivation at pH 10 required lower CT values than at pH 8.

As a result of the pH dependence of ozone decomposition,  $OH\cdot$  radicals are formed faster at pH 8, than at pH 6.6. However, even in waters where a high  $OH\cdot$  radical to ozone ratio is present, the rate constant necessary for a  $OH\cdot$  radical

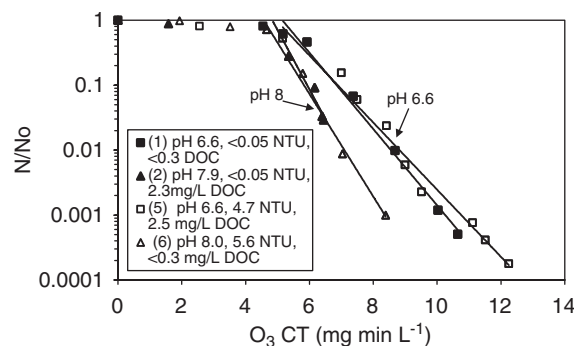


Fig. 4 – Inactivation of *B. subtilis* spores with ozone at about 7 °C and an initial ozone concentration of 2 mg/L.

The legend numbers in parenthesis refer to Table 1 experimental conditions.

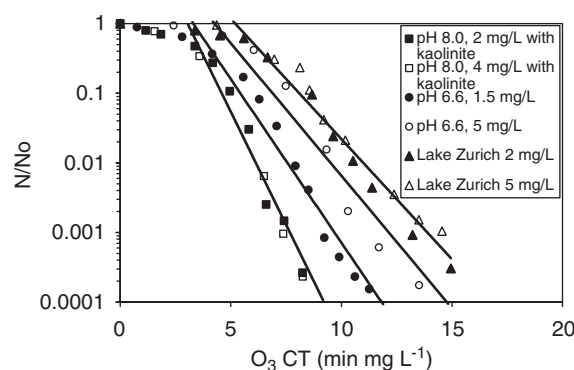


Fig. 5 – Inactivation of *B. subtilis* spores using ozone in oxidant demand-free phosphate-buffered water at pH 8.0 and pH 6.6 with 3.0 mM carbonate alkalinity, with 5 NTU and without kaolinite compared to inactivation in (unmodified) Lake Zurich water at pH 8.1, 2.5 mM carbonate alkalinity at 7 °C. Values in legend (mg/L) are initial ozone concentrations and do not refer to kaolinite concentration.

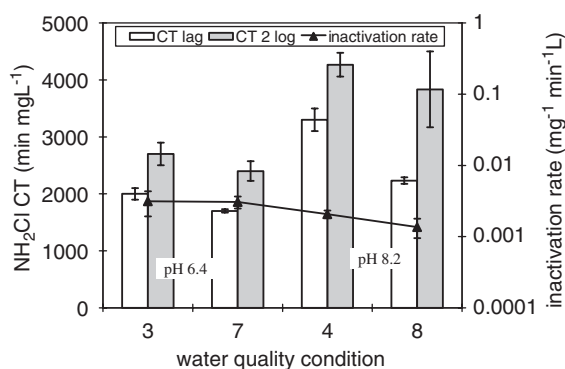
inactivation of *B. subtilis* spores would have to be significantly higher than the rate constants typically observed for  $OH\cdot$  radical oxidation (von Gunten, 2003b). In addition, the short half-life of  $OH\cdot$  radicals would make it unlikely for inactivation to be influenced by  $OH\cdot$  radicals. It is possible that radical species formed during ozone decomposition may affect the inactivation of *B. subtilis* spores. Recent research indicated that  $OH\cdot$  radicals may contribute to the inactivation of *B. subtilis* spores during the ozonation process (Cho et al., 2003). This research demonstrated that the effect of  $OH\cdot$  radical inactivation was most pronounced in the lag phase, resulting in lower CT values when more  $OH\cdot$  radicals were available for inactivation. Since  $OH\cdot$  radical concentrations were not measured in the present study, it was not possible to determine the difference in  $OH\cdot$  radicals available for inactivation at the different pH values. Another possible explanation for the observed differences is the pH-dependent speciation of the surface moieties (von Gunten, 2003a).

Further experiments were conducted to compare a natural water, Lake Zurich water, to the oxidant demand-free

buffered waters used in these experiments using ozone at 7 °C (Fig. 5). The inactivation profile for oxidant demand-free phosphate-buffered water at pH 6.6 and 8.0 resulted in lower CT values than that observed in Lake Zurich water (pH 8.1). These data suggest that water quality parameters other than pH were also affecting the inactivation of *B. subtilis* spores in Lake Zurich water, because none of the experimental waters tested resulted in similar inactivation responses (as those observed in Lake Zurich water). These experiments indicate that dissolved and/or colloidal material present in Lake Zurich water had an adverse influence on the ozone inactivation behavior of *B. subtilis* spores.

### 3.3. Monochloramine inactivation studies

Fig. 6 summarizes the inactivation response from each experimental condition for *B. subtilis* spore inactivation using

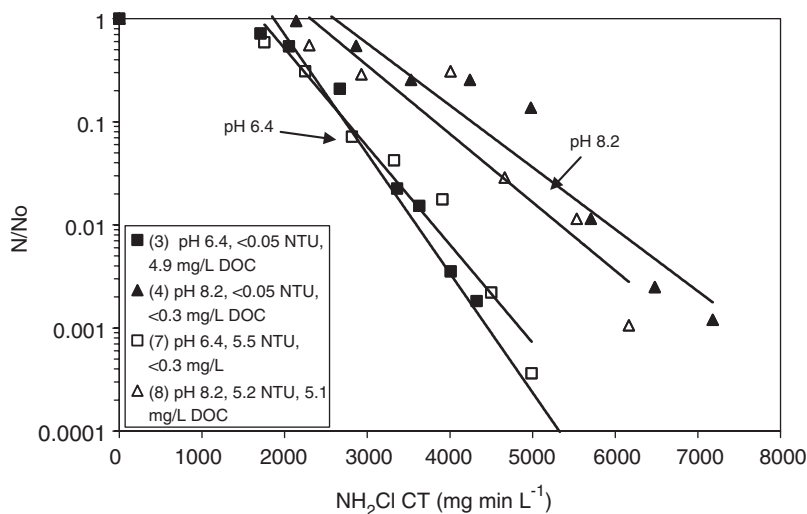


**Fig. 6 – Output values obtained for the *B. subtilis* spores inactivation using monochloramine. Output variables were obtained from regressions of triplicate experiments and averaged. Error bars provide the individual standard deviations obtained for each of the output variables.**

monochloramine. Regressions were used to estimate values of dependent variables—inactivation rate ( $k$ ,  $L\text{mg}^{-1}\text{min}^{-1}$ ), the  $CT_{lag}$  ( $\text{mgmin L}^{-1}$ ),  $CT\ 2\log$  ( $\text{mgmin L}^{-1}$ ). The experimental conditions in Fig. 6 are grouped together with the same pH to aid in reading the graph. These data suggest that pH had the greatest impact on *B. subtilis* spores using monochloramine for  $CT_{lag}$  and  $CT\ 2\log$ , but there does not appear to be a significant difference in the inactivation rate across the different water quality conditions.

Before the impact of water quality parameters on the output variables was further evaluated, the data was checked for statistical significance. The CV was calculated to be less than 10% for most output variables, except two inactivation rate constants demonstrated a CV greater than 10% but less than 20%. A quality control chart further demonstrated that the variation among the inactivation rates for each of the respective water quality conditions was not significantly greater than their associated experimental errors at  $p = 0.05$ . Therefore, a statistical analysis was only conducted on  $CT_{lag}$  and  $CT\ 2\log$  variables for which quality control charts exhibited significant variations. Of all the water quality parameters observed, pH had the most significant effect on inactivation rates,  $CT_{lag}$  and  $CT\ 2\log$  (Fig. 3). As judged by the standardized effects (Fig. 3), as the pH increased, the  $CT_{lag}$  and  $CT\ 2\log$  decreased for equivalent inactivation levels. The standardized effects also suggest that when kaolinite concentrations increased, the  $CT_{lag}$  significantly decreased. Fig. 7 shows the inactivation profiles obtained for each of the four water quality conditions tested. At pH 6.4, lower CT values were required for equivalent inactivation than at pH 8.2.

Using ACUCHEM, the concentrations of  $\text{NH}_2\text{Cl}$ ,  $\text{NHCl}_2$ ,  $\text{NH}_3$ ,  $\text{NH}_4$ ,  $\text{OCl}^-$ , and  $\text{HOCl}$  were evaluated using initial  $\text{NH}_2\text{Cl}$  concentrations of 13 and 9 mg/L (Table 4). The values generated by ACUCHEM estimated the time-dependent concentration of the monochloramine within 10% of that measured during the duration of the experiments (<8 h) (Table 4). For all experimental conditions, less than 15%



**Fig. 7 – *B. subtilis* spore inactivation using monochloramine at 22 °C. Values in the legend in parenthesis correspond to water conditions in Table 2.**

**Table 4 – Output values for chloramine modeling using ACUCHEM software for pH 8.0 and 6.6 at 22 °C for initial monochloramine concentrations of 9 and 13 mg/L (0.17 and 0.25 mM)**

	Time (h)	[NH <sub>2</sub> Cl] mM	[NHCl <sub>2</sub> ] mM	[NH <sub>3</sub> ] mM	[NH <sub>4</sub> ] mM	[OCl <sup>-</sup> ] mM	[HOCl] mM
pH 8.0 9 mg/L	0	$1.7 \times 10^{-4}$	$1.0 \times 10^{-30}$	$3.5 \times 10^{-5}$	$5.1 \times 10^{-4}$	ND	ND
	8	$1.7 \times 10^{-4}$	$1.4 \times 10^{-13}$	$3.5 \times 10^{-5}$	$5.1 \times 10^{-4}$	$2.5 \times 10^{-10}$	$5.9 \times 10^{-11}$
13 mg/L	0	$2.5 \times 10^{-4}$	$1.0 \times 10^{-30}$	$2.4 \times 10^{-5}$	$3.6 \times 10^{-4}$	ND	ND
	8	$2.5 \times 10^{-4}$	$8.4 \times 10^{-14}$	$2.4 \times 10^{-5}$	$3.6 \times 10^{-4}$	$2.5 \times 10^{-10}$	$5.9 \times 10^{-11}$
pH 6.6 9 mg/L	0	$1.7 \times 10^{-4}$	$1.0 \times 10^{-30}$	$5.5 \times 10^{-7}$	$5.5 \times 10^{-4}$	ND	ND
	8	$1.1 \times 10^{-4}$	$5.1 \times 10^{-12}$	$5.5 \times 10^{-7}$	$5.5 \times 10^{-4}$	$1.9 \times 10^{-10}$	$2.9 \times 10^{-9}$
13 mg/L	0	$2.5 \times 10^{-4}$	$1.0 \times 10^{-30}$	$3.8 \times 10^{-7}$	$3.8 \times 10^{-4}$	ND	ND
	8	$2.2 \times 10^{-4}$	$3.1 \times 10^{-12}$	$3.8 \times 10^{-7}$	$3.8 \times 10^{-4}$	$1.9 \times 10^{-10}$	$2.9 \times 10^{-9}$

Initial OCl<sup>-</sup> and HOCl concentration was considered non-detectable.

difference between initial and final monochloramine concentration was observed: the results obtained from ACUCHEM agreed with the experimentally measured results. The dichloramine concentrations predicted by ACUCHEM were many orders of magnitude lower than monochloramine and 2–4 orders of magnitude less than the free chlorine concentrations (Table 4). Using an inactivation rate for these water quality conditions obtained from previously published literature (Vikesland et al., 2001), our model calculations suggest that no inactivation occurred as a result of free chlorine exposure. Currently, there are no inactivation rates available for dichloramine inactivation of *B. subtilis* spores. However, in order for dichloramine to have any significant impact on inactivation, it would have to be many orders of magnitude greater than the inactivation rates of monochloramine and free chlorine, given the relative concentrations (Table 4). Direct chemical measurements and ACUCHEM modeling suggests that the inactivation observed at pH 6.6 was not due to dichloramine.

#### 4. Conclusions

Our experiments demonstrated that changes in water quality conditions can impact the inactivation of *B. subtilis* spores using ozone and monochloramine. Although integrated oxidant exposures (CT) were used to determine inactivation, predicting the impact of water quality parameters in other waters, from these experiments, may not be possible. This is not only because water quality conditions clearly affected inactivation responses, but also because of oxidant speciation. For inactivation of *B. subtilis* spores using ozone and monochloramine, temperature had the most significant impact on inactivation, followed by pH. The more efficient inactivation at pH 6.6 observed for *B. subtilis* spores using monochloramine could not be explained by chloramine speciation (i.e., dichloramine) contributing to inactivation. Changes in inactivation as a result of pH using ozone and monochloramine could be a result of changes in the proteins

at the spore surfaces, resulting in differences in rate of oxidation of the spore surfaces.

Changes in pH alone could not explain the changes in inactivation rate, CT<sub>lag</sub> and CT 2 log when *B. subtilis* spores were exposed to ozone in Lake Zurich water, as compared to their responses in oxidant demand-free phosphate-buffered water. We hypothesize that dissolved and colloidal matter present in Lake Zurich water exerted an effect on *B. subtilis* spore inactivation. These results are in accordance with other disinfection reports which suggest that oxidant demand-free phosphate-buffered water is, in many cases, a poor experimental analogue for extrapolating inactivation behavior to actual drinking water supplies (Labatiuk et al., 1992; Haas et al., 1996). This, among other research, indicates the need to further investigate the effects of individual water quality parameters on microorganism inactivation behavior.

With increasing numbers of research reports indicating that inactivation behavior can be markedly affected by changes in water quality conditions, the current regulatory structures based on the CT concept may need to be amended to account for more than simple temperature effects, especially since these temperature effects may vary with pH changes (Barbeau et al., 2004) or other water quality parameters (Finch et al., 2001). Conservative oxidant exposures (CT), to ensure safe levels of disinfection, may result in high concentrations of DBPs, while lower applications may result in increased immediate health risks. It may be beneficial to conduct simple bench scale inactivation experiments for specific waters to optimize the overall process with regard to disinfection, DBP formation, and cost.

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