



# Disinfection of *Bacillus subtilis* spores with chlorine dioxide: a bench-scale and pilot-scale study

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## Abstract

Chlorine dioxide (ClO<sub>2</sub>) inactivation of *Bacillus subtilis* ATCC 19659 spores was examined at pilot-scale during periods representative of winter and summer temperature extremes at the Britannia Water Treatment Plant in Ottawa, Canada. In addition, bench-scale experiments using the same source water (Ottawa River, Ontario, Canada), as well as buffered and unbuffered laboratory waters were conducted using *B. subtilis* spores. Bench-scale inactivation of *B. subtilis* spores by ClO<sub>2</sub> was similar to reported values for *Cryptosporidium parvum* (both organisms being more resistant to ClO<sub>2</sub> than *Giardia lamblia*), suggesting the possibility that these spores may be used as potential indicators for protozoan parasites. Additionally, spore inactivation was observed to be influenced by pH in laboratory (distilled deionised water) water but not in Ottawa River water. At pilot-scale, spore inactivation was influenced by water temperature: a ClO<sub>2</sub> dose of 2.5 mg/L resulted in a spore inactivation of approximately 2.0 log<sub>10</sub> and 0.5 log<sub>10</sub> at water temperatures of 23.2°C and 5.2°C, respectively. Chlorite concentrations remained below both the US EPA maximum contaminant level of 1.0 mg/L and the maximum contaminant level goal of 0.8 mg/L for up to 2.0 log<sub>10</sub> *B. subtilis* inactivation. © 2002 Elsevier Science Ltd. All rights reserved.

**Keywords:** Bacterial spores; ClO<sub>2</sub>; Disinfection

## 1. Introduction

Pathogens of concern to the water industry, such as *Cryptosporidium parvum*, are known to be resistant to chlorine at concentrations normally used for drinking water treatment [1–3]. Consequently, alternative disinfectants such as chlorine dioxide (ClO<sub>2</sub>) may be considered for application in water treatment. When compared to chlorine, ClO<sub>2</sub> has been shown to provide more effective pathogen inactivation while reducing the formation of certain disinfection by-products (DBPs), including trihalomethanes and haloacetic acids [4–10]. Information regarding ClO<sub>2</sub> microbial inactivation is

however lacking in the literature, and pilot studies are needed to supplement results observed at bench-scale.

Current enumeration techniques to measure *Giardia lamblia* and *C. parvum* concentrations are cumbersome, expensive, and time consuming, and are therefore not appropriate for routine monitoring [11,12]. Microbial indicators (or surrogates) of these pathogens could potentially be used to evaluate the efficacy of disinfection during water treatment. Aerobic spores of the bacterial genus *Bacillus* have been proposed as candidates for such evaluation [13,12]. Comparative studies are needed in order to relate the ClO<sub>2</sub> inactivation of microbial indicators, such as *Bacillus* spores, to that of *G. lamblia* and *C. parvum*.

Experiments were conducted at bench and pilot-scale to evaluate the inactivation of *B. subtilis* spores by ClO<sub>2</sub>. Aerobic spores of *B. subtilis* were evaluated as possible

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indicators for protozoan parasites such as *C. parvum* because of potentially similar resistance to  $\text{ClO}_2$  inactivation. Inactivation was first examined in a series of bench-scale experiments, and the results of these were used to design pilot-scale trials. Advantages of *B. subtilis* spores for studies of this type include their lack of pathogenicity in humans (unlike other potential indicators such as *Clostridium perfringens* spores or even the pathogens themselves, *B. subtilis* does not infect humans), and their ease of detection and enumeration. These properties make them suitable indicators for pilot-scale studies which require large numbers of organisms to be used, and where potential health hazards must be minimized.

A specific objective of this study was to determine the usefulness of *B. subtilis* spores as microbial indicators for the inactivation of *G. lamblia* and/or *C. parvum*. Other objectives included examining the impact of the water matrix,  $\text{ClO}_2$  dose, and temperature on aerobic spore inactivation.

## 2. Materials and methods

### 2.1. Pilot study conditions

Pilot-scale experiments were conducted at the Britannia pilot plant in November–December 1998 (Fall 1998 experiments) and July–August 1999 (Summer 1999 experiments). The pilot plant (Fig. 1) is located at the Britannia Water Treatment Plant (WTP), where both receive source water from the Ottawa River, Ottawa, ON, Canada. Chemical and physical characteristics of the Ottawa River water at various stages of treatment are summarized in Table 1.

All components of the pilot plant were constructed of inert materials, including glass, Teflon<sup>®</sup> and stainless steel to be chemically inert, and to mimic the design of the Britannia WTP at a scale-down factor of 1:6025 [14]. The pilot plant source water is obtained from the Britannia WTP intake pipe at approximately 21 m ahead of the chlorine and alum addition point, at a rate of 60 L/min [14]. The raw water enters a constant head tank at a fixed elevation to ensure that gravity flow conditions are maintained throughout the pilot train. The water is then split into two identical treatment trains (only one train was used in this study) at a flow rate of about 10 L/min. As the raw water leaves the constant head tank, metering pumps inject aluminum sulfate and sodium silicate. Mixing is provided by static mixers prior to entering a three-cell flocculation tank (downflow–upflow–downflow). As the water leaves the flocculators, it flows into a sedimentation tank equipped with inclined-plates, then passes to an anthracite/sand dual media filter in which the flow rate was held to approximately 2.5 L/min. The filter (diameter of 15 cm)

had a hydraulic loading rate of 8.22 m/h. The filter effluent is then directed to a clearwell, with a mean detention time of 50 min at a 2.0 L/min flowrate, the remainder being directed to a sampling port. When applied,  $\text{ClO}_2$  was added at the clearwell influent.

### 2.2. Microbial growth and enumeration

*B. subtilis* (ATCC 19659) vegetative cells were routinely cultured in tryptic soy agar (or broth) medium (Difco Laboratories). The in vitro production of *B. subtilis* spores was conducted by cultivating the bacteria at 37°C in 1/10 strength Columbia broth (Difco Laboratories) supplemented with 0.1 mM  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  for 72–96 h (personal communication; Dr. S. Sattar, University of Ottawa, Ottawa, Canada). This procedure yielded up to  $2.0 \times 10^8$  spores/mL. The spores were further isolated from vegetative cells by heating at 80°C for 12 min. The spores were then plated out for enumeration using a spread plate method on nutrient agar medium containing 0.015 g/L of trypan blue and incubated at 37°C for 24 h. The spores were stored in sterile deionized distilled water (type I reagent water). Variability in the enumeration of spores was approximately 10%. In pilot-scale studies, disinfected water samples (100–1000 mL) were heat-treated (80°C for 12 min) and analysed by the membrane filtration method through a 0.45 µm filter (Millipore Corporation, Bedford, MA, USA) using trypan nutrient agar plates as above. Replicate samples were averaged for a given contact time.

### 2.3. $\text{ClO}_2$ generation

At bench-scale,  $\text{ClO}_2$  was generated using a method described by Hofmann et al. [15]. This procedure combined methods proposed by *Standard Methods for the Examination of Water and Wastewater*—Method 2350E [16], Sterling Pulp Chemicals, Ltd., Water Technologies Division (Toronto, ON, Canada) and the Pulp and Paper Research Centre at the University of Toronto (Ontario, Canada) to yield improved safety and performance efficiency. Stock  $\text{ClO}_2$  solution purities were always greater than 99% (as measured by the titration method described below) and essentially free from chlorite, chlorate and chlorine contamination. The stock  $\text{ClO}_2$  concentration of 2.0–2.5 g/L was subsequently diluted in deionised distilled water to obtain a concentration of about 1 g/L in order to facilitate the addition of low concentrations to water samples. Diluted  $\text{ClO}_2$  stock solutions were stored in headspace-free 40 mL amber vials at 4°C in the dark.

At pilot-scale,  $\text{ClO}_2$  was generated on-site using a ProMinent<sup>®</sup>  $\text{ClO}_2$  Generating System (Bello Zon<sup>®</sup> Type CDVa, Heidelberg, Germany) as supplied by Sterling Pulp Chemicals, Ltd.  $\text{ClO}_2$  was typically generated once

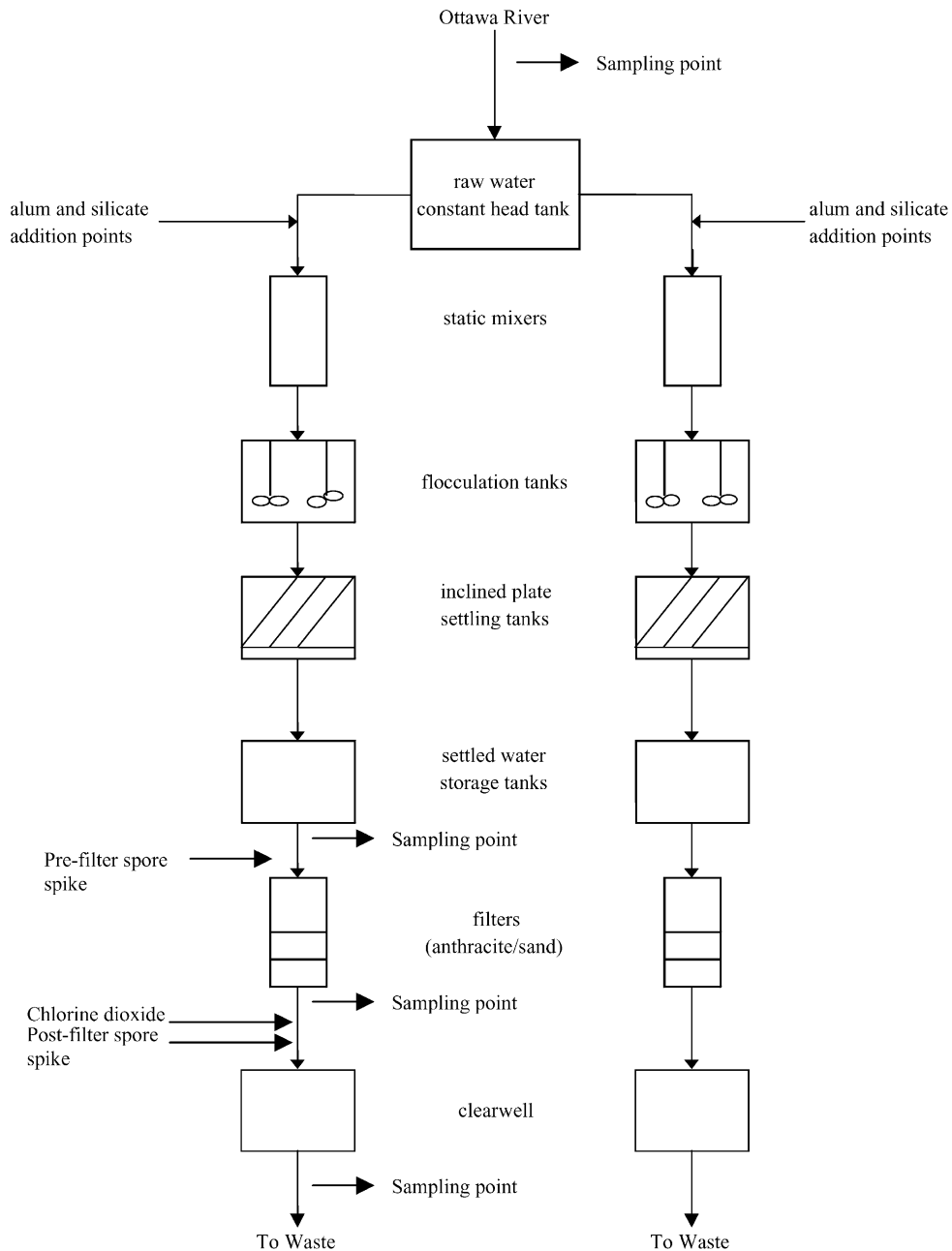


Fig. 1. Process flow diagram of the Britannia WTP pilot plant in Ottawa, Canada.

a week in order to obtain a fresh solution.  $\text{ClO}_2$  was produced in the generator's reaction chamber by combining a 9% hydrochloric acid solution with a 7.5% sodium chlorite solution ( $5\text{NaClO}_2 + 4\text{HCl} \rightarrow 4\text{ClO}_2 + 5\text{NaCl} + 2\text{H}_2\text{O}$ ), as supplied by Sterling Pulp Chemicals. The  $\text{ClO}_2$  produced at the effluent of the generator's reactor (20 g/L) was then diluted at 16–

18 mL/min. with plant effluent water in a bypass water line (100–150 L/h). The diluted  $\text{ClO}_2$  solution was stored in the dark (at 4°C) in a 400-L high density polyethylene (HDPE) container topped with HDPE round plastic balls to minimize headspace and  $\text{ClO}_2$  volatilization. The final  $\text{ClO}_2$  concentration ranged from 250 to 465 mg/L (depending on by-pass water line flow rate).

Table 1

Physical and chemical characteristics of Ottawa River water after different stages of treatment in the pilot plant. Each value for “Raw water”, “Pre-filter/settled water”, and “Post-filter effluent” is the average of 24 or 23 independent samples in November–December 1998 and July–August 1999, respectively

| Parameter   | Raw water | Pre-filter/<br>settled<br>water | Post-filter<br>effluent |
|---|-----------|---------------------------------|-------------------------|
| November–December 1998  |           |                                 |                         |
| Temperature (°C)  | 4.7       | 5.2                             | 5.2                     |
| pH  | 7.2       | 5.9                             | 6.0                     |
| Alkalinity (mg CaCO <sub>3</sub> /L)                          | 33.6      | 10.0                            | 10.0                    |
| TOC (mg/L)  | 5.92      | 3.21                            | 2.37                    |
| Color (TCU)   | 29.0      | 4.2                             | 2.8                     |
| UV <sub>254</sub> (A <sub>254</sub> units, cm <sup>-1</sup> ) | 0.18      | 0.04                            | 0.04                    |
| July–August 1999  |           |                                 |                         |
| Temperature (°C)  | 23.1      | 23.3                            | 23.2                    |
| pH  | 7.3       | 5.9                             | 6.0                     |
| Alkalinity (mg CaCO <sub>3</sub> /L)                          | 17.3      | 4.8                             | 4.8                     |
| TOC (mg/L)  | 5.95      | 3.16                            | 2.72                    |
| Color (TCU)   | 27.6      | 4.3                             | 3.8                     |
| UV <sub>254</sub> (A <sub>254</sub> units, cm <sup>-1</sup> ) | 0.24      | 0.07                            | 0.06                    |

During the experiments, ClO<sub>2</sub> was taken directly from the 400-L container and added to the pilot-scale treatment train using a metering pump. During pilot trials, the ClO<sub>2</sub> concentration was measured at the clearwell effluent. The ClO<sub>2</sub> stock concentration ranged from 250 to 300 mg/L (purity 93–99%), as measured by the titration method described below. The major impurities were chlorite and chlorate and the stock was free of chlorine.

#### 2.4. ClO<sub>2</sub> residual measurement

ClO<sub>2</sub> residual concentrations were measured using either the ACVK (Acid Chrome Violet K dye) spectrophotometric method developed by Masschelein et al. [17] and further reported by Hofmann et al. [15] or the Lissamine green spectrophotometric method [15]. Both authors reported these methods to be free from interferences by chlorine, chlorite, and chlorate. ClO<sub>2</sub> standards were prepared and tested on a regular basis.

#### 2.5. ClO<sub>2</sub> stock titration

A titration based on Standard Method 4500-ClO<sub>2</sub> B. was used to measure the stock concentration of ClO<sub>2</sub> [16]. To 25 mL of 10% (w/v) KI solution were added 15 mL of pH 7.0 buffer (25.4 g/L anhydrous KH<sub>2</sub>PO<sub>4</sub>,

64.4 g/L Na<sub>2</sub>HPO<sub>4</sub>). A 20.0 mL sample of the ClO<sub>2</sub> stock was then added to this solution and titrated with 0.1000 N Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> until the iodine color disappeared. The volume of titrant used was recorded as N (neutral titration). Next 20 mL of 2 N H<sub>2</sub>SO<sub>4</sub> was added; the solution was again titrated with 0.1000 N Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> until it became colorless. The volume of titrant used was recorded as A (acid titration). The concentration of ClO<sub>2</sub> (g/L) was calculated as

$$[\text{ClO}_2] = 0.27 \times T / 4,$$

where  $T$  (total volume of titration) =  $A + N$ . The purity of the stock solution was determined as

$$\text{Purity of stock solution (\%)} = (5A/4T) \times 100\%.$$

#### 2.6. Experimental setup (bench scale)

The reaction vessels used in experiments were low-density polyethylene (LDPE) bottles (Fisher Scientific, Pittsburgh, PA) of approximately 565 mL capacity. The bottles were placed in a dark, temperature-controlled incubator (Pyscrotherm™, New Brunswick Scientific, Edison, NJ) and shaken at 150 rpm. The average temperature of the incubator over the course of the experiments was 21.4°C (range 20.5–22.5°C). The water matrix under study was first brought to the desired temperature by placing in the incubator overnight.

#### 2.7. Water matrices

The laboratory-reagent waters studied included type I deionised distilled water (ddH<sub>2</sub>O)-phosphate buffered to pH 6.0, and ddH<sub>2</sub>O adjusted to pH 8.0 with 0.5 M NaOH (no buffer). The former was prepared by adding a phosphate buffer (88.9 mL/L of monopotassium phosphate solution (63.5 g/L KH<sub>2</sub>PO<sub>4</sub>) and 11.1 mL/L of disodium phosphate solution (249.2 g/L Na<sub>2</sub>HPO<sub>4</sub> · 7H<sub>2</sub>O) drop-wise to ddH<sub>2</sub>O to obtain pH 6.0. Pilot plant post-filtration Ottawa River water (Ontario, Canada) which had not been exposed to disinfectant was collected September 21, 1999, and used by January 18, 2000. Depending on the experiment, the Ottawa River water was either unmodified (pH 6.0) or adjusted to pH 8.0 using 0.5 M NaOH. The ClO<sub>2</sub> demand in laboratory water and Ottawa River water was 0.42 mg/L ( $n = 4$ ) and 0.65 mg/L ( $n = 4$ ), respectively. Each bottle received 565 mL of the water matrix under study. Spores were added to obtain a concentration of approximately 10<sup>6</sup> CFU/mL and ClO<sub>2</sub> was added to a target dosage of approximately 2.5 mg/L for most trials. At various time intervals, 12 or 22 mL samples were removed with 10 mL of sample being used for ClO<sub>2</sub> residual, with the remainder being quenched by 1.5% (w/v) Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> for spore enumeration.

## 2.8. Experimental setup (pilot scale)

The filter was backwashed with air scour using disinfectant-free filter effluent water and 30 min were allotted for filter ripening before  $t = 0$  samples were collected. For experiments involving  $\text{ClO}_2$  addition, the stock  $\text{ClO}_2$  solution was titrated twice and the flow rate was adjusted to achieve the desired  $\text{ClO}_2$  concentration in the clearwell effluent by delivery at the clearwell influent. Fifteen-mL stock suspensions of *B. subtilis* spores were cultured in the laboratory (approximately  $1 \times 10^{10}$  spores in 15 mL aliquots) and shipped on ice to the pilot plant by overnight courier. The contents of one 15-mL aliquot was diluted in 3.5 L of pH 6 buffered Milli-Q<sup>®</sup> water in a 4-L flask with a continuously running magnetic stir bar. Depending on the trial, bacterial spores were spiked into the pilot plant at either the filter influent (pre-filter) or clearwell influent (post-filter) using a peristaltic pump (Cole–Parmer Instrument Co., peristaltic pump model 7520–25, Chicago, IL, USA). The actual flow rate (15–19 mL/min) of the microbial pump used to deliver the microbial spike was measured at each of  $t = 0$  and  $t = \text{equilibrium}$  ( $n \geq 3$  at each time), resulting in two separate calculated values for the microbial concentration injected at the clearwell influent. The mean of these two calculations was used in the computation of spore inactivation for the experiment using the following equation:

$$\begin{aligned} &(\text{microbial concentration injected at clearwell influent}) \\ &= (\text{microbial stock concentration}) \\ &\quad \times (\text{microbial pump rate}) / (\text{clearwell flow rate}) \end{aligned}$$

After commencing the addition of  $\text{ClO}_2$  and/or microbial spores (depending on the experiment), the time elapsed before “equilibrium” samples were collected was 140 min for post-filter spike runs and 180 min for pre-filter spike runs. Equilibrium conditions at 140 min for post-filter spike experiments were confirmed in an experiment where  $\text{ClO}_2$  residuals were monitored over time at the clearwell effluent (data not shown). Therefore, in inactivation experiments, duplicate samples were collected at time “0” and once an equilibrium had been reached (140 min) from the filter effluent and clearwell effluent (post-filter spiking experiments) to allow the determination of microbial inactivation resulting from the addition of  $\text{ClO}_2$  to the clearwell. Duplicate samples for the pre-filter spiking experiments were taken at time “0” and at equilibrium (180 min) at the filter influent and effluent, as well as the clearwell effluent. The mean detention time from the clearwell influent to the clearwell effluent at the Britannia Plant was shown to be 50 min by using a tracer study [18]. Using the data obtained from these sampling points, microbial removal through the filter as well as  $\text{ClO}_2$  inactivation of the spores through the clearwell were

calculated. All samples were collected in duplicate in 125-mL or 1-L high-density polyethylene bottles which contained 1.5% (w/v)  $\text{Na}_2\text{S}_2\text{O}_3$  to quench any residual disinfectant.

## 2.9. Inactivation, removal, and $Ct$ calculations

At bench-scale, inactivation was measured as the  $\log_{10}(N_t/N_0)$ , where  $N_t$  is the number of viable organisms at a time “ $t$ ” and  $N_0$  is the number of viable organisms at the beginning of the experiment. At pilot-scale, spore inactivation was calculated as  $\log_{10}(\text{spore concentration at clearwell effluent}/\text{spore concentration at clearwell influent})$ . Spore removal through the filter was calculated as  $\log_{10}(\text{spore concentration at filter effluent}/\text{spore concentration at filter influent})$ .  $Ct$  values were calculated at bench-scale by multiplying the disinfectant residual concentration, “ $C$ ”, measured at a given contact time, “ $t$ ”. At pilot-scale,  $Ct$  values were calculated by multiplying the measured  $\text{ClO}_2$  residual by the contact time of the clearwell (50 min).

## 2.10. Statistical analyses

Generalized linear models were used to compare inactivation data in selected experiments. These models were constructed using a statistical application software (SAS version 8.0, SAS Institute, Cary, NC) with the logarithm of inactivation as the dependant measure and  $Ct$  values (mg min/L) as independent variables. Model adequacy checks were performed for each of these models and included residual plots, q–q plots, normality tests, and boxplots. When significant effects were detected, pairwise comparisons were performed using the Bonferroni’s  $t$ -test on adjusted mean inactivation levels generated by generalized linear models.

## 2.11. Chlorate and chlorite

Amber-glass, 125 mL bottles were half-filled with samples, and residual  $\text{ClO}_2$  was purged with nitrogen ( $\text{N}_2$ ) for at least 10 min using a gas-dispersion tube. A 20 mL colorless glass vial with a Teflon<sup>®</sup>-lined cap was filled with the purged sample, and 2–3 drops of ethylene diamine stock (EDA; 45 g/L) were added. Samples were shipped to the University of Toronto for analysis in accordance with US EPA method 300.0 [19]. Ion chromatography was performed using a Dionex DX 500 ion chromatograph equipped with an AS9-SC analytical column ( $4 \times 250 \text{ mm}^2$ ), AG9-GC guard column, and CD20 conductivity cell detector (Dionex Corp., Sunnyvale, CA). The sample loop was 50  $\mu\text{L}$ , with pump rates of 2.0 mL/min for eluent (120 mM  $\text{H}_3\text{BO}_3$  and 30 mM NaOH) and about 8 mL/min for regenerant (25 mM  $\text{H}_2\text{SO}_4$ ). The detection limit for each of chlorate and chlorite was 0.05 mg/L. Values reported

represent the arithmetic mean of duplicate samples. Travel standards containing 0.1 mg/L of each of chlorate and chlorite were prepared from stock solutions, treated with 2–3 drops of EDA, and included in each shipment. Travel blanks containing laboratory-reagent water were treated likewise. Measured mean concentrations (standard deviation) of the travel standards ( $n = 10$ ) were 0.13 (0.04) mg chlorite/L and 0.14 (0.09) mg chlorate/L. Chlorite and chlorate concentrations were less than their detection limits for all travel blanks ( $n = 10$ ).

### 2.12. Alkalinity, colour, $UV_{254}$ , and total organic carbon (TOC)

Alkalinity was measured by titration (Method 2320 B.; [16]). Color was measured by the technicians at the Britannia Water Purification Plant using a spectrophotometric method at 420 nm.  $UV_{254}$  measurements were conducted using Standard Method 5910 B. [16]. TOC was analyzed by Standard Method 5310D [16] using an OI Analytical Model 1010 TOC Analyzer (OI Corporation, College Station, TX, USA).

## 3. Results and discussion

*B. subtilis* spore bench scale experiments were conducted to evaluate the impact of pH and water matrix on  $ClO_2$  inactivation, as well as to compare spore inactivation to that of published data for *C. parvum*. The data show that there was a significant difference ( $p < 0.05$ ) in the  $ClO_2$  inactivation rate of *B. subtilis* spores at 21.5°C in deionized distilled water buffered to pH 6.0 and in deionized distilled water adjusted to pH 8.0 (Fig. 2). There was, however, no significant difference ( $p < 0.05$ ) in the sensitivity to  $ClO_2$  for *B. subtilis* spores at 21.5°C in post-filtration Ottawa River water at

pH 6.0 or 8.0 (Fig. 3).  $ClO_2$  disinfection efficiency is not expected to vary with pH. Noss and Olivieri [20] examined the  $ClO_2$  inactivation of f2 bacterial virus (ATCC 15776-B) and presented evidence that  $ClO_2$ , and not its disproportionation products such as chlorite and chlorate, was the active disinfecting species. This was confirmed by [21], who reported the inactivation of *Bacillus subtilis* vegetative cells (strain unreported) in phosphate buffered water (pH 7.0) at 23°C using a “stabilised” aqueous solution of  $ClO_2$ . Liyanage et al. [33] presented a similar conclusion for “drinking water disinfection conditions” after conducting  $ClO_2$  inactivation experiments using *C. parvum* oocysts in oxidant demand-free 0.05 M phosphate buffer (pH 8.0) at 22°C. Further, in contrast to aqueous chlorine ( $HOCl/OCl$ ), the chemical structure of aqueous  $ClO_2$  does not change with pH. As such, the reactivity of  $ClO_2$  does not change significantly from pH 2.0 to 10.5 when the speciation (i.e. the chemical charge) of the substrate does not vary (Hoigné and Bader, [34]). Coupling this with the evidence that free  $ClO_2$  is the major disinfecting species,  $ClO_2$  inactivation efficiency is not anticipated to vary significantly in the pH range of 2.0–10.5. However, the literature contains conflicting reports on the effects of pH on  $ClO_2$  efficiency. Botzenhart et al. [22] reported that  $ClO_2$  disinfection efficiency on *B. subtilis* spores increased when the pH of a continuous-flow, bench-scale reactor was increased from 6.0 to 8.0 at temperatures of 5°C or 15°C (water matrix unspecified). Likewise, Berman and Hoff [23] reported that at 5°C, simian rotavirus SA11 was more rapidly inactivated by  $ClO_2$  at pH 10.0 than at pH 6.0. [24] showed that the  $ClO_2$  inactivation rate of *Naegleria gruberi* cysts in buffered,  $ClO_2$  demand-free ddH<sub>2</sub>O water matrices increased when pH increased from 5.0 to 9.0. The authors speculated that this might have been the result of changes in the organism surface or an increased

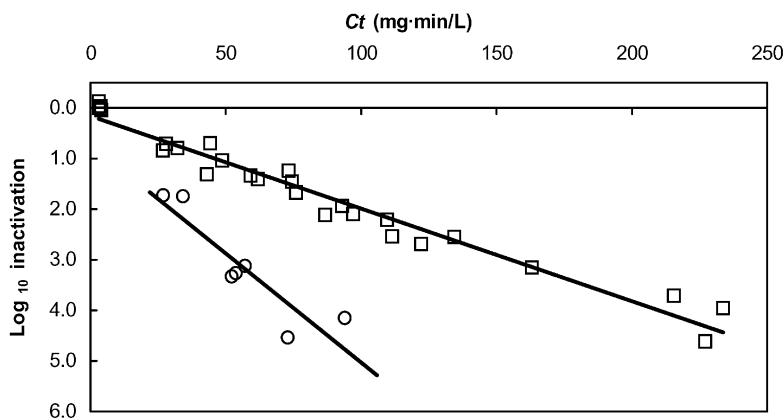


Fig. 2. Linear regression comparison of the inactivation by  $ClO_2$  at 21.4°C of *B. subtilis* spores suspended in laboratory water at pH 6 (□) and pH 8 (○).

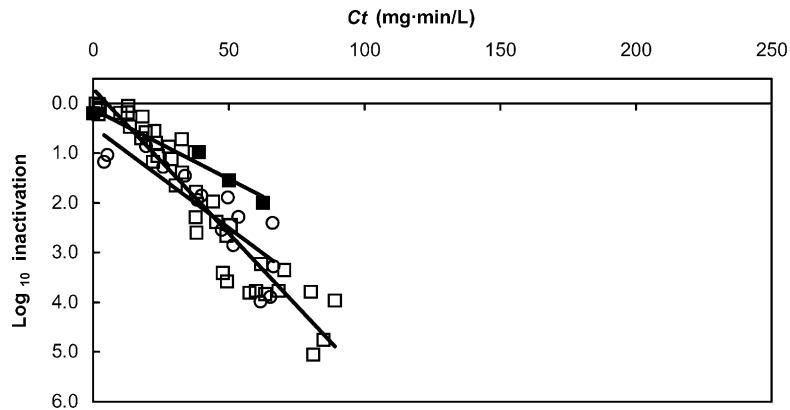


Fig. 3. Linear regression comparison of the inactivation by  $\text{ClO}_2$  at 21.4°C of *B. subtilis* spores suspended in post-filtered Ottawa River water at pH 6 (□) and pH 8 (○). Pilot plant inactivation data (■) (summer 1999 results from Table 3) for *B. subtilis* spores by  $\text{ClO}_2$  are shown for comparison.

effectiveness of the disinfectant with increased pH [24]. Noss and Olivieri [20] presented evidence that f2 bacterial virus was more easily inactivated by  $\text{ClO}_2$  as the pH increased from 5.0 to 9.0. Commenting on these results, the authors suggested that there may have been a change in reactivity between  $\text{ClO}_2$  and the virus, but dismissed the idea of a change in the species of the oxychlorine compound present [20]. More specifically, they hypothesized that the results may have been attributable to a change in the chemical structure of the virion and/or the concentration of hydroxyl ions in the solution, if the hydroxyl ions were necessary for the inactivation reaction to occur [20]. Similarly, Liyanage et al. [33], commenting on research that suggested  $\text{ClO}_2$  disinfection of *G. lamblia* cysts improved as the pH increased, postulated that the increased efficiency of  $\text{ClO}_2$  at a higher pH may be due to possible chemical or physical changes in cyst structure. These conclusions on the pH effect may also be applied to *B. subtilis* spores, based on the data presented in this study. However, since spore inactivation in the Ottawa River was not affected by pH, it may also be that the presence of the phosphate buffer (deionized distilled water at pH 6.0) may have afforded some indirect or direct protection of the spores.

Spore inactivation levels at bench-scale were compared to inactivation levels from the literature for *C. parvum* and *G. lamblia* (Table 2). *Ct* values for *G. lamblia* were obtained from the Surface Water Treatment Rule (SWTR), while those for *C. parvum* at pH 6 and 20°C were reported in the literature [2,25,32,33,35,36]. Finch and Li [36] used a mouse infectivity assay to report a 1.8 and 2.1  $\log_{10}$  inactivation of *Cryptosporidium* oocysts at a *Ct* of 120 mg·min/L in a laboratory buffer at pH 6 and at 22°C. Korich et al. [2]

reported a 1.0  $\log_{10}$  inactivation of *C. parvum* oocysts for a *Ct* of 78 mg min/L in a phosphate buffer at pH 7 and at 25°C. Finch et al. [37] recalculated the animal infectivity data of Korich et al. [2] to standardize the reporting method for animal infectivity. While Finch et al. [37] admitted that the calculations were crude because of the necessary assumptions, they reported that, based on the data of Korich et al. [2], a maximum theoretical *Ct* of 78 mg min/L resulted in 2.0  $\log_{10}$  inactivation rather than the 1.0  $\log_{10}$  kill reported originally. In the present study, a 2.0  $\log_{10}$  inactivation of *B. subtilis* spores required *Ct* values of 100 and 25 mg min/L at pH 6 and 8, respectively. Therefore, it appears that *C. parvum* oocysts and *B. subtilis* spores have similar levels of inactivation, whereas *G. lamblia* is more sensitive to  $\text{ClO}_2$  (Table 2). Due to the apparent similarities between *B. subtilis* spores and *C. parvum* inactivation, it was decided to use the spores in pilot-scale trials to evaluate the efficacy of  $\text{ClO}_2$  during treatment.

These aerobic spores have also been proposed by other investigators as potential indicators for protozoan parasites in pilot-scale and full-scale studies. For example, *B. subtilis* spores were used as surrogates for the removal and inactivation by chlorine of *C. parvum* [12]. The present study used these spores to evaluate  $\text{ClO}_2$  inactivation at pilot-scale. In addition, seasonal effects such as the influence of water temperature were studied by repeating the pilot runs at two different time periods: November–December 1998 (Fall period) and July–August 1999 (Summer period). It was not feasible to measure  $\text{ClO}_2$  concentration over time in a specific flow segment, and thus the concentration of  $\text{ClO}_2$  measured at the clearwell effluent was used as the value for *Ct*. This means that the *Ct* values reported in Table 3 may be low when compared to integrated approaches

Table 2

Summary of selected data for the ClO<sub>2</sub> inactivation of *C. parvum* oocysts, *G. lamblia* cysts, and *B. subtilis* spores and comparison to the present study

| Experimental setup   | Ct<br>(mg min/L)  | Log <sub>10</sub><br>inactivation | Reference <sup>a</sup>                |
|--|-------------------|-----------------------------------|---------------------------------------|
| (1) <i>C. parvum</i> , room temperature  | 12.9 <sup>b</sup> | 1.24 (1.0 <sup>c</sup> )          | Peeters et al. [35]                   |
| (2) <i>C. parvum</i> , 0.01 M phosphate-buffered pH 7 water, 25°C  | 78 <sup>b</sup>   | 1.0 (2.0 <sup>c</sup> )           | [2]                                   |
| (3) <i>C. parvum</i> , oxidant demand-free 0.05 M phosphate-buffered pH 8 water, 22 (±1)°C                   | 60 <sup>b</sup>   | 0.99                              | Lymanage et al. [33]                  |
| (4) <i>C. parvum</i> , 0.05 M phosphate-buffered pH 6 water, 22°C  | 120               | 1.8 and 2.1                       | Finch and Li [36]                     |
| (5) <i>C. parvum</i> 0.01 M phosphate-buffered pH 6 and 8 ddH <sub>2</sub> O, 20°C                           | 150               | 2.0 <sup>d</sup>                  | Ruffell et al. [25]                   |
| (6) <i>C. parvum</i> (Iowa strain from the University of Alberta), ddH <sub>2</sub> O adjusted to pH 8, 21°C | 75                | 2.0 <sup>d</sup>                  | [32]                                  |
| (7) <i>G. lamblia</i> , phosphate-buffered ddH <sub>2</sub> O  | 15                | 3.0                               | Surface water treatment rule (US EPA) |
| (8) <i>B. subtilis</i> spores, ddH <sub>2</sub> O-buffered to pH 8, 21°C                                     | 25                | 2.0 <sup>d</sup>                  | Present study                         |
| (9) <i>B. subtilis</i> spores, ddH <sub>2</sub> O adjusted to pH 6, 21°C                                     | 100               | 2.0 <sup>d</sup>                  | Present study                         |
| (10) <i>B. subtilis</i> spores, Ottawa River water-buffered to pH 6, 21°C                                    | 40                | 2.0 <sup>d</sup>                  | Present study                         |
| (11) <i>B. subtilis</i> spores, Ottawa River water adjusted to pH 8, 21°C                                    | 40                | 2.0 <sup>d</sup>                  | Present study                         |

<sup>a</sup> Selected data from each reference are shown.

<sup>b</sup> Ct value is the theoretical maximum Ct, calculated by multiplying the initial disinfectant dose by the exposure time. Liyanage et al. [33] provided data for the final and initial ClO<sub>2</sub> residuals in their *C. parvum* oocyst inactivation experiments, and in all cases the final residual was 60% or less of the initial value.

<sup>c</sup> Recalculation by Finch et al.

<sup>d</sup> Approximate value.

because the method does not account for a potentially higher concentration of ClO<sub>2</sub> at the clearwell influent. Data reported in Table 3 show spore inactivation levels (for ClO<sub>2</sub> doses of 2.42–2.54 mg/L) of 1.70–2.50 log<sub>10</sub> and 0.60–0.45 log<sub>10</sub> in the summer and winter, respectively. The average water temperature during the summer runs were 23.2°C, whereas the average water temperatures during the winter runs was 5.2°C (Table 1). Thus, inactivation was much lower at the lower water temperature since inactivation kinetics is slower in cold water. In fact, inactivation at 23.2°C was approximately four times greater than at 5.2°C. ClO<sub>2</sub> demand during the winter and summer pilot-scale experiments was on average 0.48 and 1.09 mg/L, respectively, and was the same, irrespective of the initial ClO<sub>2</sub> dose (data not shown). Although ClO<sub>2</sub> inactivated approximately 2.0 log<sub>10</sub> of spores in warm waters, it does not appear to be as effective at the low temperatures encountered during the Fall pilot runs (Table 3). ClO<sub>2</sub> may, however, be more efficient at low temperatures when used synergistically with another disinfectant as postulated by [26]. This hypothesis remains to be investigated at pilot-scale.

Numerous studies [22,27,28] have shown that increases in disinfectant concentration promote microbial inactivation. As expected, a similar trend was observed during the pilot-scale inactivation of *B. subtilis* spores (Fig. 4). The pilot plant clearwell mean detention time was not varied during the course of the experi-

ments. Thus, within the context of the Chick–Watson model, increased inactivation would necessitate an increase in ClO<sub>2</sub> concentration. The increased residual ClO<sub>2</sub> measured at the clearwell effluent at equilibrium indeed corresponded to increased spore inactivation (Fig. 4). The levels of ClO<sub>2</sub> in the clearwell effluent for 1.5 and 2.0 log<sub>10</sub> inactivation were 1.0 and 1.25 mg/L, respectively (Fig. 4). Chlorite concentrations were below the US EPA [29] maximum contaminant level (MCL) of 1.0 mg/L and below the maximum contaminant level goal (MCLG) of 0.8 mg/L for all levels of inactivation studied (Fig. 4). The general trend of increasing concentrations of chlorite and chlorate with increasing ClO<sub>2</sub> residual was expected as the former are the major by-products associated with ClO<sub>2</sub> disinfection.

Finally, in several runs, the spores were spiked prior to filtration in the pilot treatment train rather than post-filtration (prior to the clearwell). Nevertheless, no difference was observed in inactivation rates (Table 3), suggesting that any stress attributable to filtration did not cause the spores more sensitive to ClO<sub>2</sub> inactivation. Spore removal rates through the filter, in both cold and warm waters, were approximately 2.0 log<sub>10</sub>, and similar to the results of Rice et al. [12], who reported spore removals via filtration of 1.68–2.75 log<sub>10</sub> in four full-scale plants. Swerfeger et al. [30] also reported similar results; they found that indigenous spores were removed by filtration by 1.48–2.37 log<sub>10</sub>.

Table 3

Summary of *B. subtilis* spore inactivation and removal through the Britannia WTP pilot plant (runs 1 and 2 in Fall 1998; runs 3–10 in Summer 1999)

| Pilot run # | Study period | Spiking         | Applied ClO <sub>2</sub> dose (mg/L) | ClO <sub>2</sub> residual in clearwell (mg/L) | Chlorite in clearwell (mg/L) | Log <sub>10</sub> removal by filtration | Log <sub>10</sub> inactivation | Ct (mg min/L)     |
|-------------|--------------|-----------------|--------------------------------------|---|------------------------------|---|--------------------------------|-------------------|
| 1           | Fall 1998    | Post-filtration | 2.50                                 | 2.02  | 0.30                         | —                                       | 0.60                           | 101.0             |
| 2           | Fall 1998    | Pre-filtration  | 2.50                                 | 2.00  | 0.30                         | 1.85                                    | 0.45                           | 100.0             |
| 3           | Summer 1999  | Post-filtration | 0                                    | 0   | 0                            | —                                       | 0.16                           | 0                 |
| 4           | Summer 1999  | Pre-filtration  | 0                                    | 0   | 0                            | 2.15                                    | 0.27                           | 0                 |
| 5, 6, 7     | Summer 1999  | Post-filtration | 2.42 <sup>a</sup>                    | 1.24 <sup>a</sup>                             | 0.77                         | —                                       | 1.70 <sup>a</sup>              | 62.0 <sup>a</sup> |
| 8, 9        | Summer 1999  | Pre-filtration  | 2.54 <sup>b</sup>                    | 1.54 <sup>b</sup>                             | 0.61                         | 2.40 <sup>b</sup>                       | 2.50 <sup>b</sup>              | 77.0 <sup>b</sup> |
| 10          | Summer 1999  | Post-filtration | 1.74                                 | 0.78  | 0.51                         | —                                       | 1.00                           | 39.0              |

<sup>a</sup> Mean of three runs.

<sup>b</sup> Mean of two runs.

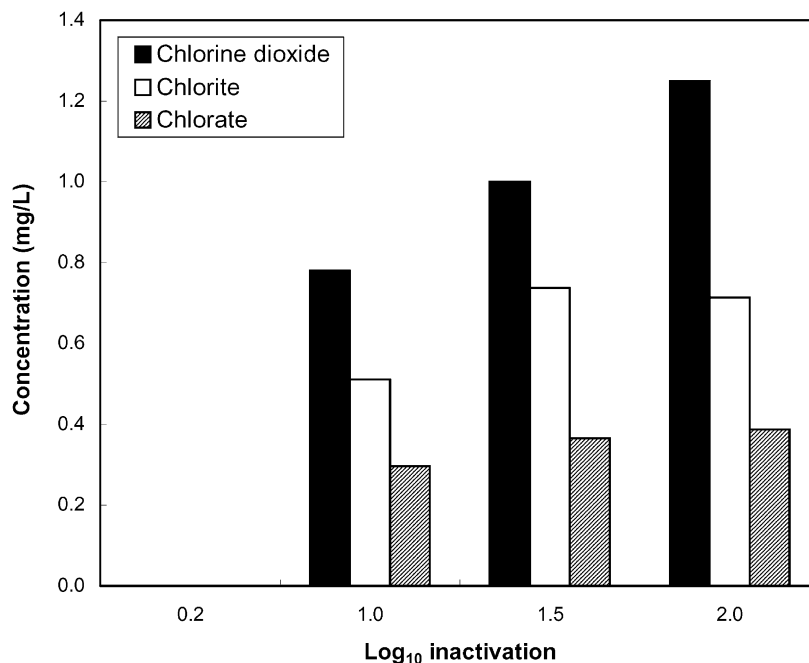


Fig. 4. Chlorite, chlorate and residual ClO<sub>2</sub> concentrations in the clearwell effluent for pilot-scale *B. subtilis* spore inactivation studies. The x-axis indicates the log<sub>10</sub> inactivation of spores by ClO<sub>2</sub> corresponding to the data. The method detection limits (MDL) were 0.017 mg ClO<sub>2</sub>/L [15] and 0.05 mg/L for each of chlorite and chlorate.

#### 4. Conclusions

In conclusion, as indicated by other researchers [12,13,31], bacterial spores may serve as potential indicators (along with other parameters) of water treatment plant efficiency for *C. parvum* inactivation. These spores may be considered adequate indicators because of their similar resistance to ClO<sub>2</sub> (albeit somewhat lower) as *C. parvum*, their ease of detection,

and the fact that they are not pathogenic to humans. Other strains of *B. subtilis*, and other spore-forming bacteria, including natural isolates from distribution system, will be examined in the future. Finally, the data show that 2.0 log<sub>10</sub> inactivation of *B. subtilis* spores can be achieved in warm waters without exceeding the US EPA MCL for chlorite. Results of this study may be used to assist researchers in selecting an appropriate microbial indicator for the measurement of disinfectant

efficacy, especially for pilot-scale studies. The impact of  $\text{ClO}_2$  dose, pH, and temperature on microbial indicator inactivation will provide additional insight with respect to the factors affecting microbial inactivation when employing  $\text{ClO}_2$ .

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