# Indigenous bacterial spores as indicators of *Cryptosporidium* inactivation using chlorine dioxide

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## ABSTRACT

This objective of this study was to explore the practicality of monitoring naturally occurring organisms to predict drinking water treatment plant performance, in this case for the reduction of *Cryptosporidium*. Surface and ground water from seven drinking water treatment plants across North America that use chlorine dioxide were surveyed for aerobic and anaerobic bacterial spore concentrations. The concentrations of total spores were usually high enough in both raw and treated water to allow 4- to 5-log reductions to be observed across the treatment train by filtering up to 2 l of sample. These results suggested that naturally occurring treatment-resistant spores could be candidates as indicators of treatment performance. However, to be useful as indicators for *Cryptosporidium* reduction, the organisms would have to exhibit similar resistances to disinfection (chlorine dioxide in this case) in order to be useful. The inactivation kinetics of seven of the most common species were determined, and all were observed to be considerably more susceptible to chlorine dioxide inactivation than *Cryptosporidium* as reported in the literature. This study therefore did not identify an appropriate ambient microbial indicator for *Cryptosporidium* control.

**Key words** | *Bacillus*, chlorine dioxide, *Cryptosporidium*, disinfection, drinking water, spores

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## INTRODUCTION

It has been common in the past to use indicator organisms such as coliforms to assess the microbiological quality of treated drinking water. A weakness of this approach is that it is possible for a poorly operating treatment system to produce water that meets regulatory standards when the source water is pristine. Such a system, however, is vulnerable to deteriorations in raw water quality. A more proactive approach is to ensure that the plant is operating to its potential at all times, regardless of treated water quality.

In the United States, such an approach exists for drinking water treatment plants that use water from surface sources or ground waters influenced by surface waters. These plants generally have to provide a level of treatment consistent with at least 2-log *Cryptosporidium parvum*, 3-log *Giardia lamblia*, and 4-log virus reduction on a continuous basis, regardless of the actual measured treated water quality. Unfortunately, these target organisms are very difficult to measure, making routine pathogen monitoring impractical. Instead, surrogate methods are used to assign log reduction credits based on specific criteria. For example, filtration is assigned a log removal credit based on meeting effluent turbidity limits. Disinfection processes are assigned inactivation credits based on the calculated *Ct* value (the mathematical product of disinfectant concentration $C$ and contact time $t$).

Such prescribed credits unfortunately do not reflect site-specific factors that may influence treatment performance. For example, two filters with the same effluent turbidity may receive the same pathogen removal credit, while in reality providing very different levels of removal. The prescribed credits must therefore be very conservative and do not provide a true assessment of the level of microbiological control being obtained. It would be useful to identify methods that could provide a more accurate

prediction of treatment performance. One method is to use a naturally occurring microbial indicator to infer the actual amount of pathogen control through the treatment process.

Several criteria have to be met to use a microbial indicator to predict pathogen control. The response of both the indicator and pathogenic organisms to physical treatment and disinfection should be similar, or else the indicator should conservatively be more difficult to treat. The indicator organism should be easy to measure, and should always be present in both the ambient and treated waters at concentrations high enough to allow log reduction calculations to be performed. If such an organism could be identified, then treatment plants could continually monitor its reduction across the treatment process, ensuring that the monitored reduction exceeds that required for the pathogens of interest.

Some investigators have begun the search for such an indicator. In particular, indicators for *Cryptosporidium* have been targeted since *Cryptosporidium* is very resistant to chemical disinfectants. *Bacillus subtilis* spores and *Clostridium perfringens* spores have been suggested as potential indicators for *Cryptosporidium*, due in part to their observed resistance to disinfectants and adverse environmental conditions (Payment & Franco 1993; Rice *et al.* 1996; Nieminski *et al.* 2000; Chauret *et al.* 2001; Radziminski *et al.* 2002). There is also evidence that the ambient concentrations of such bacterial spores may be high enough in many waters to allow log reductions to be tracked across the treatment process. In particular, Nieminski *et al.* (2000) surveyed 24 utilities for indigenous physical and biological surrogates for *Cryptosporidium* and *Giardia*, and reported that bacterial spore concentrations were high enough in raw waters to commonly allow 3–4 $\log_{10}$ removal rates to be monitored.

The purpose of our study was to continue the investigation into an indicator for *Cryptosporidium* control across a drinking water treatment process. A decision was made to focus on bacterial spores (aerobic and anaerobic) based on the promising earlier work by Nieminski *et al.* (2000). We also specifically targeted treatment approaches using chlorine dioxide ($ClO_2$) disinfection. It is reported that free chlorine and chloramines are essentially ineffective against *Cryptosporidium*, while $ClO_2$ may be a viable method to supplement other processes to meet overall *Cryptosporidium* reduction criteria.

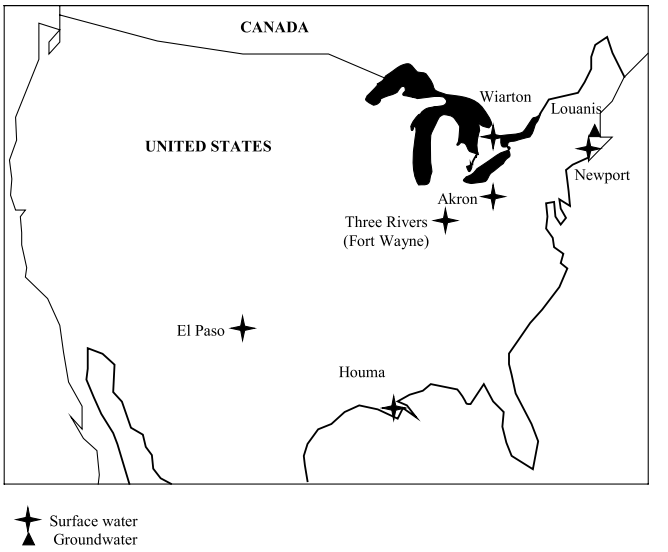


**Figure 1** | Location of the seven water utilities participating in the study.

The specific research objectives were:

1. To identify bacterial species that are commonly present in raw water and that can survive treatment at high enough concentrations to allow log reductions to be calculated across the treatment process.
2. To identify bacterial species that meet the criteria above, and that are at least as resistant to $ClO_2$ disinfection as *Cryptosporidium*.

## MATERIALS AND METHODS

### Sampling sites and sample types

Seven water utilities located in Canada and the United States participated in this study: El Paso (TX), Houma (LA), Wiarton (ON), Newport (RI), Louanis (MA), Three Rivers (IN) and Akron (OH) (Figure 1). The treatment process at each location is summarized in Table 1.

**Table 1** | Water quality and treatment processes at participating facilities

| Location | Process | Source | Raw water turbidity (NTU) | Raw water temp. (°C) | Raw water pH |
|---|---|---|---|---|---|
| Newport (1) (10-30-00) | Raw→Clar→Filt→Finished<br>↑ ↑<br>$ClO_2$ $Cl_2$ | Surface | 23 | 9 | 7.3 |
| Newport (2) (12-11-00) | Raw→Clar→Filt→Finished<br>↑ ↑<br>$Cl_2$ $Cl_2$ | Surface | 3 | 4 | 7.5 |
| Newport (3) (11-20-01) | Raw→Clar→Filt→Finished<br>↑ ↑<br>$ClO_2$ $Cl_2$ | Surface | 11 | 11 | 7.0 |
| Louanis | Raw→Flocc→Clar→Filt→Finished<br>↑ ↑<br>$ClO_2$ $Cl_2$ | Ground | NA | NA | NA |
| Three Rivers | Raw→Clar→Filt→Finished<br>↑<br>$ClO_2/Cl_2$ | Surface | 40 | NA | 7.9 |
| Wiarton (1) (28-08-00) | Raw→Clar→Filt→Finished<br>↑ ↑<br>$ClO_2$ $ClO_2$ | Surface | 0.3 | 20 | 8.4 |
| Wiarton (2) (06-09-00) | Raw→Clar→Filt→Finished<br>↑ ↑<br>$Cl_2$ $Cl_2$ | Surface | 0.3 | 20 | 8.4 |
| El Paso | Raw→Flocc→Clar→Filt→Finished<br>↑ ↑ ↑<br>$ClO_2/Cl_2$ $Cl_2$ $Cl_2$ | Surface | 52 | 24 | 8.3 |
| Houma | Raw→Clar→Filt→Finished<br>↑ ↑<br>$ClO_2$ $Cl_2$ | Surface | NA | NA | NA |
| Akron | Raw→Flocc→Clar→Filt→finished<br>↑ ↑<br>$ClO_2$ $ClO_2$ | Surface | 10 | NA | 8.2 |

Clar—clarification (sedimentation); Filt—filtration; Flocc—flocculation.

Samples for microbial analysis were obtained from various locations in the treatment train to examine the effect of treatment on the concentration of spore-forming bacteria. The sampling sites included the raw water intake, immediately before and after the addition of a primary disinfectant or a secondary disinfectant, and at various intermediate locations. Water samples were collected in sterile 1-l plastic bottles according to method 9060A (APHA *et al.* 1998). Each bottle contained 3% (w/v) sterile sodium thiosulphate solution to quench any residual disinfectant. The samples were shipped on ice to the laboratory by overnight carrier. Samples were stored at 4°C and analysed within 24 h.

## Spore isolation and enumeration

Naturally occurring spores produced by aerobic and anaerobic bacteria were examined. The protocol for enumeration was based on a method adopted by Rice *et al.* (1996) from the dairy industry (Richardson 1986). Samples were heated to 70–75°C for at least 15 min to kill all vegetative cells. A control bottle was used to ensure the water inside each bottle reached 70–75°C. After cooling, samples were filtered using the membrane filtration method. Appropriate volumes (from 1 ml to 2.0 l) were filtered in duplicate onto 0.45-μm filters (Millipore Corporation, Bedford, MA) and plated on a non-selective

nutrient agar (BD Diagnostic Systems, Sparks, MD) supplemented with 0.015 g/l of trypan blue. One set of plates was incubated at 37°C under aerobic condition for 48 h. The other set was incubated at 37°C for 48 h in a GasPak 150™ jar, which contained three anaerobic chambers (BD Diagnostic Systems). Gaspak™ disposable anaerobic indicators were used in each chamber to ensure anaerobic conditions were produced (BD Diagnostic Systems). After incubation, colonies were counted and the results were reported as colony-forming units per ml (CFU/ml). Colonies were grouped in different morphotypes according to colony characteristics (size, form, elevation, margin, colour, surface, density and consistency), Gram reaction and catalase reaction. Isolates belonging to different morphotypes were further characterized by Biolog® (Biolog, Montreal, Canada) and API® (bioMerieux, Montreal, Canada) systems.

### Maintenance of selected isolates

All isolates shown to be significantly resistant to disinfection (chlorine dioxide and/or chlorine) or to one of the treatment phases were maintained in a culture collection. Selected isolates were stored on nutrient agar slants in the dark at 4°C and were also frozen in duplicate at −80°C in sterile microtubes containing nutrient broth with 10% glycerol and 5% DMSO.

### Identification of isolates

Pure cultures of selected isolates were identified before inactivation experiments using Biolog GP and AN MicroPlate test panels containing 96 different carbon sources each. Cultures grown on solid media for 24 h were swabbed from the agar surface and resuspended in the appropriate inoculating fluid provided by the manufacturer. Biolog AN or GP Microplate wells were inoculated with, respectively, 100 µl or 150 µl of these cell suspensions that had been adjusted to the appropriate density ($\sim 3 \times 10^8$ cells per ml) by comparison with the turbidity standard supplied by the manufacturer. Microplates were incubated for 4 to 48 h at 37°C under aerobic or anaerobic conditions according to the isolate requirements. Growth was recorded and identified using Gram positive and anaerobe database software. Aerobic spore-forming bacteria identified by the Biolog system were confirmed using API 50 CH and API 20 E strips manufactured by bioMerieux. Pure cultures of these isolates were grown on nutrient agar and cells ($\sim 6 \times 10^8$ cells per ml) and resuspended in 0.85% sodium chloride using the McFarland standard provided by the manufacturer. Strips were inoculated and grown at 37°C for 24 to 48 h. Results were recorded and sent to bioMerieux for identification.

### *In vitro* production of *Bacillus* and *Clostridium* spores

Spores were produced as previously described (Chauret *et al.* 2001) at 37°C in 1/10 strength Columbia broth (Becton Dickinson Microbiology Systems, USA) supplemented with 0.1 mM $MnSO_4 \cdot 4H_2O$ and in Duncan-Strong Sporulation Medium (Atlas 1997) for 7 to 15 days for aerobic and anaerobic bacteria, respectively. The spores were further isolated from vegetative cells by heating at 80°C for 12 min. The spores were washed in sterile deionised distilled water and harvested by centrifugation at 10,000 g for 11 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 under aerobic or anaerobic conditions. The spores were stored in sterile deionised distilled water supplemented with 7% glycerol and frozen at −80°C. This procedure yielded $10^6$–$10^8$ spores/ml.

### Chlorine dioxide generation

At bench scale, chlorine dioxide was generated using a method reported by Hofmann *et al.* (1998). A 25% (w/v) solution of $NaClO_2$ was slowly introduced into a gas-wash bottle containing 12 N $H_2SO_4$. This bottle was connected to a chlorine scrubber gas-wash bottle containing a 10% (w/v) solution of $NaClO_2$. The scrubber was connected to a $ClO_2$ collection gas-wash bottle filled with deionised distilled water maintained on ice. At the end of the series, an additional $ClO_2$ trap-bottle with 10% (w/v) KI was present to trap any remaining chlorine dioxide. Overall, stock chlorine dioxide solution purities were always

greater than 99% free from chlorite, chlorate and chlorine contamination. Working solutions of chlorine dioxide were prepared by diluting samples of the stock solution to 1 g/l and were stored in headspace-free 40 ml amber vials at 4°C and in the dark.

### Inactivation experiments

Microbial inactivation experiments were conducted in sterile 500 ml demand-free plastic Nalgene bottles (Nalge Nunc International, Rochester, NY) containing sterile deionised distilled water adjusted to pH 8.0. Demand-free bottles were prepared prior to each experiment by saturating them with a 20 mg/l chlorine dioxide solution for 1 h. The bottles were then thoroughly rinsed with distilled water. Approximately $10^8$ *Bacillus* or *Clostridium* spores were added to each bottle. Chlorine dioxide was then injected (except for the control bottle) at 2.5 mg/l. The samples were mixed on a shaker at 150 rpm. Microbial samples and samples for disinfectant residual measurement were collected every 5–10 min for 90 min and placed in sterile glass tubes containing a solution of sterile 1% (w/v) sodium thiosulphate solution to quench any residual disinfectant. All experiments were conducted at $21 \pm 1$°C in the dark. Each experiment was repeated at least twice.

### Chlorine dioxide residual measurement

Chlorine dioxide residual concentrations were measured using a lissamine green spectrophotometric method (Hofmann *et al.* 1998). Standard curves were corrected for the presence of microorganisms in the water samples. Chlorine dioxide standards were prepared and tested on a regular basis.

### Calculations for inactivation experiments

Inactivation is measured as $\log_{10}(N_t/N_0)$, where $(N_t)$ is the number of viable organisms at time $(t)$ and $(N_0)$ is the number of viable organisms at the beginning of the experiment. Integrated *Ct* values are calculated as previously described (Chauret *et al.* 2001) by multiplying the disinfectant residual concentration, $(C)$, by elapsed contact time $(t)$. For example, for sample $n$ and at sampling time $t_n$ with a $ClO_2$ concentration $C_n$, the $(Ct)_n$ is calculated as follows:

$$(Ct)_n = C_n \times (t_n - t_{n-1}) + (Ct)_{n-1} \quad (1)$$

### Statistical analyses

Statistical analyses were performed using generalized linear models to assess differences among organisms in their susceptibility to $ClO_2$. The dependant variables used were the logarithm of inactivation and *Ct* values (mg · min/l), while the organism type was independent. When significant effects were detected, pairwise comparisons were conducted using Bonferroni's *t* test on adjusted mean inactivation levels generated by generalized linear models. Statistical testing was performed at 95% significance.

## RESULTS AND DISCUSSION

### Raw and treated water spore concentrations

The concentrations of total aerobic and anaerobic bacterial spores in both the raw and treated waters from all facilities are shown in Figures 2a and 2b, respectively. For both aerobes and anaerobes, the concentrations in raw waters were always reasonably high (usually 10 to 100 CFU/ml). Once the water had been treated, the total counts in only 6 of the 33 samples were below detection limits (<0.0005 CFU/ml). The remaining 27 samples all had total counts that were measurable, normally in the 0.001 to 0.01 CFU/ml range. This indicates that for almost all cases studied, a 4- to 5-log reduction could be measured across the treatment process by collecting and filtering up to 2 l of sample. In the United States, regulations commonly require between 3- and 4-log reduction of target pathogens, so the ability to measure an even greater reduction in the ambient indicator organisms tracked in this study is promising.

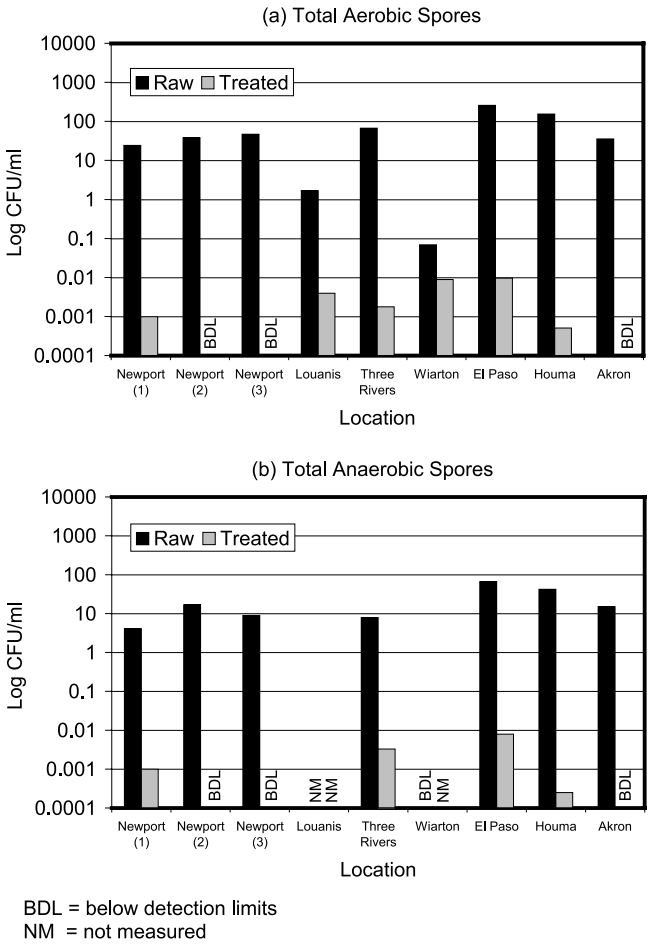


**Figure 2** | Total raw and treated aerobic and anaerobic spore concentrations.

The concentrations of total spore formers shown in Figure 2 are similar to those reported in previous studies. Nieminski *et al.* (2000), in a survey of 24 utilities, observed total aerobic spore concentrations of greater than 2 CFU/ml in 75% of the waters. Rice *et al.* (1996) reported similar findings in a study of four utilities treating surface waters. These results, together with the results of the seven waters in this study, are evidence that bacterial spore formers may be useful candidates as microbial indicators on the basis of their prevalence in nature and resistance to treatment.

The reduction in organism concentrations across the treatment train is a combination of physical removal and chemical inactivation (disinfection). In this study, organism and disinfectant concentrations were tracked at intermediate locations in some cases, however it was beyond the scope of the project to separate the effects of physical and chemical reduction (for example, the combined physical removal and disinfection across a sedimentation basin where pre-disinfection is practised). Nevertheless, limited data are available that distinguish physical and chemical reductions for the Louanis and Three Rivers facilities, which did not practise pre-disinfection (Table 1). In both facilities, approximately 1.3-log reduction in total aerobic spores was observed across the sedimentation process. Subsequent filtration and post-disinfection contributed a further 1.3-log and 2.3-log reduction for the two plants, respectively. This suggests that physical removal can be a major contributor to overall reduction in organism concentrations, a result that has been reported by others. For example, Payment & Franco (1993) observed a 4.7-log reduction in *Cryptosporidium* oocysts across a conventional treatment plant, primarily due to physical removal. Nieminski *et al.* (2000), in their study of aerobic spores at 24 utilities, reported that the reduction of the spores during treatment was mostly the result of physical removal, and that disinfection contributed little to their overall reduction. Further evidence that the spore reductions observed in our study were greatly influenced by physical removal is the relative similarity in log reductions for total aerobic and total anaerobic spores, always within 0.8-log of each other for every water (Figure 2). If it is assumed that the inactivation kinetics for the aerobic and anaerobic spores may be different (as qualitatively suggested later on by data shown in Figure 3), then the similarity in total log reductions suggests that physical removal processes are dominant.

## Isolate identification

Total aerobic and anaerobic bacterial spores as described in the previous section probably cannot be used directly to assess treatment performance, since the inactivation kinetics of 'total bacteria' would depend on the types of bacteria present. The intent was to first identify whether enough bacterial spores in general were present to theoretically provide a quantitative assessment of treatment performance. With the results suggesting that 4- or

**Table 2** | Identification of *Bacillus* and *Clostridium* species

| Species | Newport | Louanis | Three Rivers | Wiarton | El Paso | Houma | Akron |
|---|---|---|---|---|---|---|---|
| *B. amyloliquefaciens* | ✔ | — | ✔ | ✔ | — | — | — |
| *B. pumilus* | ✔ | ✔ | ✔ | ✔ | — | — | — |
| *B. cereus/thuringiensis* | — | — | — | ✔ | — | — | ✔ |
| *B. halodurans* | — | — | — | — | — | — | ✔ |
| *B. licheniformis* | ✔ | — | — | — | ✔ | ✔ | — |
| *B. subtilis* | ✔ | — | — | — | — | — | — |
| *B. mycoides* | ✔ | — | — | — | — | — | ✔ |
| *Clostridium* spp | — | — | ✔ | — | ✔ | — | ✔ |

✔ — species present at location.

5-log reductions could routinely be monitored, the next step was to identify individual species that were common and contributed to a large fraction of the total spores present. If any such predominant species exist, then their specific inactivation kinetics could be determined, and the concentration of that species could then be tracked through the treatment process to extrapolate the reduction of a target pathogen, such as *Cryptosporidium*.

Ideally, it would be useful to take the total spores identified in Figure 2 and determine which species are contributing to the total amounts. Unfortunately, such quantitative analyses of a water sample are difficult because of the great similarity between various species of spore-forming *Bacillus*. Several biochemical (and often molecular) tests must be performed on each isolate to accurately identify it. Even with identification kits such as the Biolog® system, it is nearly impossible to fully characterize each colony when thousands exist on hundreds of plates. Instead, the 'predominant' four or five colony types from the total spore plates (from each sample) were visually identified, isolated and subcultured to allow identification. In this way, a semi-quantitative analysis of the water samples could be conducted and common species identified.

Three hundred and fourteen spore-forming colonies were isolated from water samples collected throughout the treatment train of the seven utilities. Gram staining and catalase testing (data not shown) revealed that most of the aerobic spore-forming bacteria presented characteristics of the genus *Bacillus*. Cells were Gram-positive, catalase-positive rods arranged in pairs or chains, with rounded or squared ends. Most of the endospores were ovals and some were cylindrical. Some of the isolates were facultative anaerobic bacteria. There was a great diversity of *Bacillus* colonies. Most of the anaerobic spore-forming bacteria presented characteristics of the genus Clostridium. Cells were Gram-positive rods and formed oval or spherical endospores that distended the cells, and were arranged in pairs or short chains and were positive for catalase.

Predominant isolates sampled post-disinfection were identified at the species level using the Biolog® system and were found to belong to seven different species: *B. pumilus*, *B. amyloliquefaciens*, *B. cereus/thuringiensis*, *B. halodurans*, *B. licheniformis*, *B. subtilis* and *B. mycoides* (Table 2). Identifications were further confirmed by using the API system, except for the species *Bacillus halodurans* that was not

included in the bioMerieux database. *Bacillus cereus* and *Bacillus thuringiensis* are species that are closely related and therefore could not be clearly differentiated by either identification system. The seven species identified are well distributed in the environment and commonly found in soil and water. Some appeared to be common to several utilities. The most widespread ones were *Bacillus amyloliquefaciens*, *Bacillus pumilus* and *Bacillus licheniformis* and were isolated in finished water (Table 2), indicating that they are very resistant to treatment.

None of the anaerobic spore-forming isolates could be consistently identified at the species level using the Biolog® system. Results were only consistent at the genus level and isolates were found to belong to the genus *Clostridium*.

The results of these efforts to identify common species of spore-forming bacteria showed no single *Bacillus* species present at all sites, and thus no single species may be a candidate by itself for use as a treatment performance indicator (it is also possible that some isolates and/or species were not detected by the cultivation methods used in this study). The identified species that were common to the seven treatment facilities were relatively resistant to physical and chemical treatment, and may be present at high enough concentrations to measure log reductions across the treatment train. Note, however, that at present there are no fast and easy commercial methods to measure a single *Bacillus* species (let alone a cocktail of species). In the near future, it may be possible to detect various species of *Bacillus* without relying heavily on biochemical tests. For example, in a recent study, a polymerase chain reaction (PCR) test based on the 16S rRNA gene was set up that could identify *Bacillus subtilis* and four other closely related species (*B. pumilus*, *B. atrophaeus*, *B. licheniformis* and *B. amyloliquefaciens*) from wastewater samples (Wattiau *et al.* 2001). In addition, these authors reported that this PCR test allowed a rough estimation of the proportion of the target species when assayed under well-defined conditions. Even though this test is limited to a few species of *Bacillus* and quantification needs to be improved, it suggests that this type of analysis will become available in the future.

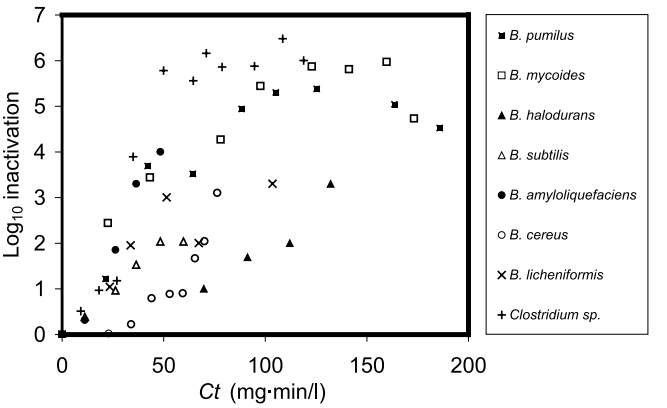


**Figure 3** | $ClO_2$ inactivation of naturally occurring environmental spores (21°C, distilled water, pH 8). Data points are the average of duplicates.

## Inactivation experiments

Spores produced by the identified environmental isolates were cultured in the laboratory and resuspended in distilled deionised water for inactivation experiments with chlorine dioxide. All experiments were conducted at 21°C. Results are shown in Figure 3.

The most resistant spores were produced by *Bacillus halodurans* and *Bacillus cereus*, and the least resistant ones by *Bacillus amyloliquefaciens* and *Clostridium*. A notable feature of the data in Figure 3 is that the most sensitive spores to $ClO_2$ showed a plateau at 5–6 $\log_{10}$ of inactivation. This may be attributed to the aggregation properties of the spores, and may have been one of the reasons why some of these spores survived the entire disinfection process at the plants at which they were sampled. Gauthier *et al.* (1999) suggest that pathogens are usually aggregated or associated with cell debris in environmental waters, and consequently some of them may not be removed entirely by treatment processes. These authors also explained that once microbes are entrapped in particles or absorbed to surfaces, they can be shielded from disinfection. Spores, therefore, would be more resistant in an actual treatment facility because of their aggregation properties, whereas bench-scale inactivation kinetics are based on laboratory studies using primarily dispersed suspensions of organisms. Nevertheless, $ClO_2$ was able to effectively provide up to approximately 5-log inactivation of all species examined

**Table 3** | Chick-Watson specific lethality coefficients (*k*, base 10) measured at 21°C

| Organism | *k* (l · min/mg) |
|---|---|
| *Bacillus amyloliquefaciens* | 0.082 |
| *Clostridium* spp | 0.080 |
| *Bacillus mycoides* | 0.064 |
| *Bacillus pumilus* | 0.060 |
| *Bacillus subtilis* | 0.038 |
| *Bacillus licheniformis* | 0.037 |
| *Bacillus cereus* | 0.025 |
| *Bacillus halodurans* | 0.020 |
| *Cryptosporidium parvum* (source 1)[a] | 0.0061 |
| *Cryptosporidium parvum* (source 2)[a] | 0.0026 |
| *Cryptosporidium parvum* (source 3)[a] | 0.00071 |

[a]From Chauret *et al.* (2001); oocysts obtained from three different sources.

prior to the plateau, with a reasonably linear relationship between log inactivation and *Ct*.

For a clearer comparison of the inactivation kinetics for each species, the data shown in Figure 3 were regressed to give the Chick-Watson coefficients of specific lethality (i.e. the slopes of the curves). The regressions were only applied to data up to 4-log inactivation, since greater inactivation levels exhibited a plateau. The linear regressions also involved forcing the intercept through zero. While these simplifications may not be strictly appropriate when determining the true inactivation kinetics of an organism, it was deemed appropriate in this case where the interest was primarily to compare resistances to chlorine dioxide among the different organisms. The coefficients of specific lethality are shown in Table 3, along with coefficients for *Cryptosporidium parvum* that have been reported in the literature.

The intent of this study was to try to identify surrogates for *Cryptosporidium parvum* inactivation in water treatment. The data in Table 3 suggest that while the bacterial spores isolated in the study were quite resistant to chlorine dioxide, they were still not as resistant as *Cryptosporidium parvum* oocysts by a factor of at least 3 under the best of conditions. The data in Table 3 also indicate that experiments examining *Cryptosporidium* show great inconsistencies in the reported inactivation kinetics depending on the source of the oocysts used for the tests, possibly due to genetic drift in stock populations or differences in preparation of the oocysts. The result is that the issue of identifying an appropriate surrogate for *Cryptosporidium* may remain unresolved until the inactivation kinetics for *Cryptosporidium* when using $ClO_2$ are better understood. In any case, even the most sensitive *Cryptosporidium* examined is reportedly much more resistant than the bacterial spores examined in this study.

## SUMMARY AND CONCLUSIONS

The rationale of this study was to first identify microorganisms that are common in the environment and can survive conventional treatment (using $ClO_2$) at levels that can be readily measured. Such organisms would then be candidates for serving as microbiological surrogates for *Cryptosporidium* control provided that it could be demonstrated that they were at least as resistant to $ClO_2$ as *Cryptosporidium*, and also assuming that their physical removal was similar to *Cryptosporidium* (an issue not addressed in this study).

A survey of seven locations, including both surface and ground waters, indicated that the predominant aerobic spore-forming bacteria included certain species of *Bacillus*, while predominant anaerobes included the genus *Clostridium*. Both *Bacillus* and *Clostridium* spores were routinely isolated in treated water, demonstrating their resistance to treatment. Furthermore, the ambient concentrations of total aerobic and anaerobic spores in both raw and treated water were high enough that a 4- to 5-log reduction across the treatment train could often be monitored by filtering up to 2 l of water. These results were considered to be promising in terms of identifying a naturally occurring and common surrogate for

*Cryptosporidium* control. Unfortunately, when the inactivation kinetics of the common species were determined, it was observed that even the most $ClO_2$-resistant spore (*Bacillus halodurans*) was about three times more susceptible to $ClO_2$ than the least resistant *Cryptosporidium* strain reported in the literature.

The results of this study therefore indicate that while certain species of aerobic and anaerobic spores are abundant in nature and survive conventional drinking water treatment (with $ClO_2$), the species identified in this study would not be appropriate surrogates for monitoring *Cryptosporidium* control.

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