Inactivation of environmental and reference strains of heterotrophic bacteria and *Escherichia coli* O157:H7 by free chlorine and monochloramine

Linda Wojcicka, Ron Hofmann, Carole Baxter, Robert C. Andrews, Isabelle Auvray, Julie Lière, Trent Miller, Christian Chauret and Hélène Baribeau

ABSTRACT

Reference cultures from culture collections are often used in disinfection experiments instead of indigenous environmental bacteria, but they may not accurately represent the organisms found in drinking water distribution systems due to physiological differences. This may explain why disinfectant concentrations and contact times, determined under typical laboratory conditions, are not always sufficient to control microbial growth or survival in distribution systems. The objective of this study was to investigate the effect of chlorine or monochloramine disinfection on *Escherichia coli* O157:H7 and other heterotrophic bacteria obtained from a culture collection and strains isolated from various environments, including disinfected water. It was hypothesized that previous exposure of the environmental strains to sublethal concentrations of disinfectant may allow them to develop greater resistance. Accordingly, it was observed that environmental strains of *E. coli* O157:H7 were either equally or less susceptible to chlorine and monochloramine than the reference strain. In contrast however, environmental strains of *Brevundimonas vesicularis, Pseudomonas fluorescens,* and *Sphingomonas paucimobilis* were either equally or more susceptible to free chlorine or monochloramine than their corresponding reference strains. This was counterintuitive because the environmental strains were able to survive disinfection in the pipes from which they were isolated. It is hypothesized that upon culturing in the laboratory the environmental strains may have become susceptible to low levels of disinfectant. Other researchers have suggested that changes in culture conditions may impact disinfectant sensitivity by affecting cell permeability, cell composition, or growth rate. This emphasizes the importance of designing laboratory studies to mimic environmental conditions as closely as possible to accurately represent environmental inactivation kinetics, including proper handling of organisms (subculturing, media transfers, etc.) before their use in inactivation studies.

Key words | chloramine, chlorine, disinfection, drinking water, *E. coli* O157:H7, heterotrophic bacteria

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INTRODUCTION

Reference culture strains of microorganisms (e.g. American Type Culture Collection, ATCC) are often considered in disinfection studies, however the inactivation kinetics of these reference organisms does not necessarily always match that of environmental strains. For example, despite numerous studies that have reported the general effectiveness of chlorine at inactivating waterborne bacteria and despite the presence of chlorine and chloramine disinfectant residuals in distribution systems, microorganisms are still able to survive and some can proliferate under adverse conditions. In fact, several studies have reported microbial survival and/or chlorine resistance in chlorinated water supplies (Ridgeway

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& Olson 1982; Haas *et al.* 1983; Wolfe & Olson 1984; Cargill *et al.* 1992; Abu-Shkara *et al.* 1998).

Lisle *et al.* (1998) showed that *E. coli* O157:H7 can adapt to starvation in conditions such as those potentially present in a water distribution system, and to increase its resistance to up to 0.5 mg/L of chlorine. Kuchta *et al.* (1983) tested a number of *Legionella* strains and reported that the legionellae were much more resistant to chlorine than coliform bacteria. With a chlorine residual of 0.1 mg/L, a 2-log kill of *L. pneumophila* was achieved in 40 minutes, compared to 1 minute for the same inactivation of *E. coli*. In a subsequent study, Kuchta *et al.* (1985) suggested that the higher resistance of *L. pneumophila* to chlorine may be attributed to its hydrophobic cell surface and the ability of the outer membrane composition to change under different growth conditions. Similarly, Falkinham (2003) also reported that chlorine-resistant strains of *Mycobacterium avium, M. intracellulare,* and *M. scrofulaceum* were more hydrophobic than the chlorine-sensitive strains. It was suggested that the resulting increased fluidity and permeability of the membrane might explain the increased susceptibility of the organisms to chlorine. Mir *et al.* (1997) also suggested that bacterial membrane and wall structures could be responsible for the increased chlorine resistance of gram positive organisms compared to gram negative organisms.

Studies have shown that previous exposure to disinfectants may also affect microbial resistance to chlorine disinfectants. Carson *et al.* (1978) compared inactivation of mycobacterial strains isolated from a hospital peritoneal dialysis unit to reference strains purchased from the American Type Culture Collection (ATCC). Disinfectants commonly used in hospitals were applied, such as formaldehyde and glutaraldehyde. It was found that the reference ATCC strain of *Mycobacterium chelonei* and *M. fortuitum* were rapidly inactivated; ATCC strains showed no survivors after 2 minutes of contact time with glutaraldehyde compared to the environmental isolates, which survived for 60 minutes. Similarly, the ATCC reference strains were more rapidly inactivated when formaldehyde was used as the disinfectant. These findings raise the question of whether or not reference strains should be used for inactivation studies, including those involving common water treatment disinfectants such as chlorine.

Research objectives

The first objective of this study was to determine whether microbial exposure to sub-lethal doses of disinfectants renders heterotrophic bacteria more resistant to further disinfection, to test the hypothesis that microbial survival in a distribution system environment is not accurately predicted by inactivation experiments conducted using culture collection reference strains in the laboratory. Heterotrophic plate count (HPC) bacteria were chosen for this study since they are commonly measured in public drinking water systems to assess general microbial water quality (Payment *et al.* 2003*a*). While the presence of HPCs may not be related to faecal contamination, they can be monitored to assess treatment efficiency, overall cleanliness, and the integrity of distribution systems (Payment *et al.* 2003*b*). Inactivation kinetics of environmental strains of heterotrophic bacteria isolated from disinfected distribution systems were compared to American Type Culture Collection (ATCC) reference strains. Chlorine (pH 7) and monochloramine (pH 8) were used as the disinfectants in buffered, demand-free water at 25 ± 1°C.

A second objective of the study was to quantify differences in the inactivation kinetics of different isolates of *E. coli* O157:H7 (a primary pathogen) obtained from different environments and to compare these inactivation kinetics to those of a reference strain of *E. coli* O157:H7 obtained from the ATCC. *Escherichia coli* was chosen for this study because it is a commonly used indicator of faecal contamination, as it is specifically of intestinal origin and therefore only present in water polluted by faecal matter (Payment *et al.* 2003*b*). *E. coli* O157:H7 is an enterohemorrhagic strain that is found in cattle and is also a human pathogen. Because waterborne transmission of this bacterium has been documented, it is a concern for drinking water suppliers. Inactivation experiments with *E. coli* O157:H7 were conducted at 21 ± 1°C.

MATERIALS AND METHODS

Heterotrophic bacteria

Environmental strains of heterotrophic plate count (HPC) bacteria were isolated from pipe loops (pilot-scale distribution systems) at four participating utilities in Canada and the

United States. The water treatment utilities involved in this study included the Regional Municipality of Waterloo (Ontario, Canada), Jordan Valley Water Conservancy (Utah, USA), Halifax Regional Water Commission (Nova Scotia, Canada), and East Bay Municipal Utility District (California, USA). The utilities in Waterloo and East Bay used monochloramine to provide a residual, while Halifax and Jordan Valley used free chlorine. Information on the finished water quality and distribution system characteristics of each utility is provided in Table 1.

The organisms sampled from the pipe loops were then isolated in R2A medium (BD, Franklin Lake, NJ) at 20°C for 7 days. Gram staining, oxidase tests, and API biochemical profiling (Biomérieux, Hazelwood, MO) were performed on all isolates. Of the 14 strains isolated, those identified with 90% and higher confidence, and those able to grow within 2 to 3 days were selected for further study (Table 2). The strains used for inactivation experiments were identified to be *Brevundimonas vesicularis*, *Sphingomonas paucimobilis*, and *Pseudomonas fluorescens*. The corresponding American Type Culture Collection (ATCC) strains (ATCC 11426, 10829, and 17387, respectively) were purchased and also used for inactivation experiments. These reference ATCC strains served as controls, in that they were isolated from environments that do not contain chlorine-based disinfectants, unlike the environmental isolates from the present study.

All environmental and reference strains were cultured in R2A broth prior to their use in experiments. R2A broth was made by combining 0.5 g yeast extract (BD, Franklin Lake, NJ), 0.5 g proteose peptone No. 3 (BD, Franklin Lake, NJ), 0.5 g casamino acids (BD, Franklin Lake, NJ), 0.5 g dextrose (BD, Franklin Lake, NJ), 0.5 g soluble starch (BD, Franklin Lake, NJ), 0.3 g sodium pyruvate (Fisher Scientific, Fair Lawn, NJ), 0.3 g di-potassium phosphate (EM Science, Darmstadt, Germany), and 0.05 g magnesium sulfate (Fisher Scientific, Fair Lawn, NJ). Cultures were started by transferring a loop of each strain grown on R2A slants (or plates for free chlorine experiments) into 20 ml of sterile R2A broth. The cultures were incubated at 21°C with shaking at 100 rpm for 2-3 days (Standard Method 9215, APHA 1998).

Escherichia coli O157:H7

The various strains of *E. coli* O157:H7 (Table 2) were maintained in 10% glycerol at −50°C. In the short-term, strains were cultured on nutrient agar slants and kept at 4°C. To prepare a culture for an inactivation experiment, a loopful

Table 1 | Finished water quality and pipe material at each site from which heterotrophic environmental strains were isolated

Parameter	Halifax	Waterloo	Jordan Valley	East Bay
Alkalinity (mg/L as $CaCO_3$)	32.4	177	142.5	22.5
Apparent colour (PtCo)	<2.5	0	4	ND
Hardness (mg/L as $CaCO_3$)	40.9	144	201.0	18–30
Total iron (mg/L)	0.03	0.04	ND	ND
pH	8.7	7.4	7.6	9.1–9.5
TDS (mg/L)	85.0	ND	272.3	37
Turbidity (NTU)	0.32	0.26	0.05	0.06
TOC (mg/L)	1.4	2.4	1.8	0.8–1.5
Pipe Material	Ductile iron with concrete lining	Cast iron from 1930 s	Unlined ductile iron with very little wear	Unlined cast iron with tuberculation

ND = No data available.

Table 2 | Characteristics of the heterotrophic environmental strains tested

Isolate ID	Pipe Loop Source	Colony Morphology	Identification	% Confidence (API test)
1	Waterloo	yellow small convex	*B.vesicularis*	91.6
2	Waterloo	light yellow large flat	*B.vesicularis*	92.4
3 *	Jordan Valley	red small flat	*B.vesicularis*	93.8
4	Halifax	white large convex	*P.fluorescens*	98.2
5	Jordan Valley	light yellow large flat	*S.paucimobilis*	92.9
6	EBMUD	yellow large convex	*S.paucimobilis*	99.5

*Isolate 3 only used in chlorine experiments. EBMUD = East Bay Municipal Utility District.

of a slant culture was inoculated into 10 ml of tryptic soy broth (TSB) (BD) and incubated at 37°C for 18 hours. 1 ml from this culture was used to inoculate another 10 ml of TSB. After incubation at 37°C for 18 hours, this culture was washed three times by centrifugation (10,000(g, 10 minutes, 4°C) and re-suspended in 5 ml of sterile de-ionized distilled water adjusted to pH 7.0. This washed cell suspension was immediately used in inactivation experiments.

Disinfectant solutions

Free chlorine experiments were conducted using a minimum 4% sodium hypochlorite solution (Sigma-Aldrich, Milwaukee, WI). Monochloramine was prepared in two separate stock solutions, each in pH 9.4 buffer. The buffer was prepared by dissolving 0.39 g Na_2CO_3 and 1.372 g $NaHCO_3$ in 200 ml of de-ionized, distilled water (ddH_2O). Stock 1 was prepared by dissolving 0.0375 g ammonium chloride (NH_4Cl) (Caledon Laboratories, Georgetown, Canada) in 75 ml of pH 9.4 buffer. In stock 2, 0.627 ml sodium hypochlorite (~4.8%) was added to 75 ml of pH 9.4 buffer. Both solutions were stored in 125 ml amber bottles at 4°C. For inactivation experiments, the two solutions were added separately to the water samples in equal quantities, with the ammonium chloride first, immediately followed by the chlorine solution. The resulting reaction between ammonia and chlorine gave a chlorine-to-ammonia-nitrogen ratio of 4:1 by mass.

The DPD Ferrous Titrimetric Method (Standard Method 4500-Cl F, APHA 1998) was used to determine the chlorine concentration of the original stock. Based on this concentration, a 100 mg/L stock was made and used for calibration and experiments. Disinfectant residuals were measured spectrophotometrically at 515 nm using the 4500-Cl G DPD Colorimetric Method (APHA 1998).

Assay reactors

Inactivation experiments were conducted using glass bottles containing buffered water to minimize fluctuations in pH. Free chlorine inactivation experiments were conducted at pH 7.0. Phosphate buffer was used, due to its buffering capacity at pH 7.0. A concentration of 5 mM was selected to represent low solute concentrations of drinking water. The 5 mM phosphate buffered demand-free (BDF) water was prepared by dissolving 0.27 mg anhydrous sodium phosphate dibasic (Na_2HPO_4) (EM Science, Darmstadt, Germany), 0.44 g anhydrous potassium phosphate monobasic (KH_2PO_4) (Sigma-Aldrich, St. Louis, MO), into 1 L chlorine demand-free water (Millipore Milli-Q® system, Billerica, MA), and adjusting to pH 7.0 using NaOH (Standard Method 5910B, APHA 1998).

All monochloramine inactivation experiments were conducted in 5 mM sterile borate buffered demand-free (BDF) water, due to the buffering capacity of borate buffer between pH 8 and 10. The 5 mM borate BDF water was prepared by dissolving 0.309 g boric acid (H_3BO_3) (EM Science, Darmstadt, Germany) and 0.04 g sodium hydroxide (NaOH) into 1 L chlorine demand-free water (Millipore Milli-Q® system), and adjusting to pH 8.0 with hydrochloric

acid (HCl). All 250 ml Pyrex® bottle reactors were filled with 245 ml of sterile BDF water, autoclaved, and cooled before use in experiments. Each assay was conducted in a laminar flow hood at 25 ± 1°C.

Inactivation protocol for HPC

On the day of experiments, 15 ml of cell cultures were transferred into sterile centrifuge tubes and centrifuged at 14,481 *g* (Sorvall RC 5C Plus, Mandel Scientific, Guelph, Canada) for 15 minutes at 4°C (Verhille *et al.* 2003). This process was repeated twice. Each time the supernatant was removed and the pellet was resuspended in 10 ml of sterile borate buffer by vortexing. After the final centrifugation step, the cultures were resuspended in 2.5 ml of sterile BDF water, and immediately used for experiments. Organisms were added to the reactors at a concentration of 10^7 to 10^8 CFU/ml for chlorine experiments, or 10^5 to 10^6 CFU/ml for monochloramine experiments, and stirred on a magnetic stir plate. Increased cell concentration for chlorine experiments was needed due to the greater inactivation of organisms. Inactivation experiments were carried out in a laminar flow biosafety hood (25 ± 1°C), in duplicate.

A control sample was taken prior to dosing with chlorine (0.1–0.3 mg/L) or monochloramine (~1.6 mg/L). Once the disinfectant was added to the reactor, a timer was started and chlorine residual samples (3 ml) were collected at 15, 30, 45, and 60 seconds. In consideration for slower inactivation kinetics, monochloramine samples (3 ml) were collected at 2, 6, 10, 14, and 18 minutes. The samples were collected into a 1-cm glass cuvette containing 150 µl phosphate buffer and 150 µl DPD. Microbial samples (1 ml) were collected 2 (±2) seconds later, placed in sterile 1.5-ml microtubes containing 10% (w/v) sterile sodium thiosulphate ($Na_2S_2O_3$; Sigma-Aldrich, St. Louis, MO) to quench any residual disinfectant. Controls were also run without chlorine or chloramine addition, to confirm that there was negligible loss of cells due to factors other than the chemical disinfection (e.g. sorption to the vessel walls).

Microbial samples were serially diluted in sterile microtubes containing sterile Milli-Q® water. At the end of each assay, 100 µl of the proper dilution was spread-plated on 100 mm × 15 mm Petri dishes containing R2A agar. The plates were incubated at 21°C for 5 to 7 days before being enumerated. Duplicates of R2A plates were conducted for each sample and the counts averaged. Only counts between 30 and 300 were considered.

Inactivation protocol for *E. coli* O157:H7

Microbial inactivation experiments were conducted in sterile 250-ml demand-free glass bottles, containing sterile buffered de-ionized distilled water. Chlorine experiments were conducted at pH 7.0 in demand free water, whereas monochloramine experiments were conducted at pH 8.0 in demand free water.

Approximately 10^7 to 10^8 bacterial cells (from a washed cell suspension) were added to each bottle. Free chlorine or monochloramine was then added to all vials (except to control vials) at a desired disinfectant dose (0.4–0.5 mg/L for free chlorine, and 1.25–1.50 mg/L for monochloramine). The samples were mixed on a magnetic stir plate. Microbial samples (1 ml) were collected at selected time intervals (every 15–30 seconds for chlorine, and every 15 minutes for monochloramine, respectively) and placed in sterile microtubes containing sterile $Na_2S_2O_3$ to quench any residual disinfectant. Samples (2 ml) for chlorine or monochloramine residual measurements were collected at each sampling time and immediately analyzed. All experiments were conducted in duplicate (duplicate samples of duplicate experiments) at 21 ± 1°C. Controls were also run without chlorine or chloramine addition, to confirm that there was negligible loss of cells due to factors other than the chemical disinfection (e.g. sorption to the vessel walls).

The microbial samples for *E. coli* enumeration were plated out on nutrient agar plates using serial dilutions and the spread plate method. The *E. coli* plates were incubated at 37°C for 24 hours. After incubation, only plates with colony counts between 25 and 250 were considered.

Data analyses

CT values (i.e. the product of disinfectant residual concentration, C, and contact time, T) were calculated using Equation 1 below. The disinfectant residual was measured at regular time intervals. For each residual measurement, the disinfectant concentration was multiplied by the time period since the previous residual measurement. This value

was then added to the previously calculated CT value to obtain the overall CT value at each sampling point (n).

$$(CT)_n = C_n \times (T_n - T_{n-1}) + (CT)_{n-1} \quad (1)$$

Log inactivation was calculated by comparing survival at each sampling point to the original control microbial concentration. Plots of log inactivation versus CT were prepared for each organism.

For each microbial data point, two replicate measurements per sampling time were performed. The experiments were independently repeated at least twice on separate days. Comparisons were made between the slopes of two linear regressions of inactivation data using a t-test at a 95% confidence level, according to the following equation:

$$t = \frac{b_1 - b_2}{SE_{b_1 - b_2}} \quad (2)$$

where b_1 and b_2 are the slopes of the two regressions and,

$$SE_{b_1-b_2} = \sqrt{\frac{MS_{pooledерror}}{\sum(x - \bar{x})_1^2} + \frac{MS_{pooledерror}}{\sum(x - \bar{x})_2^2}} \quad (3)$$

$$MS_{pooledерror} = \frac{SS_{error1} + SS_{error2}}{DF_1 + DF_2} \quad (4)$$

$$SS_{error} = \sum(y - \bar{y})^2 \quad (5)$$

DF is the number of degrees of freedom (*n*-2 for each data set), *x* is CT, and *y* is log inactivation.

RESULTS AND DISCUSSION

For each organism, the inactivation kinetics of the environmental strains were compared to those obtained for the corresponding ATCC strain. A linear regression was applied to the linear portion of data curves. A comparison of slopes between the regression lines was performed by comparing the t-values at a 95% confidence level. If the tabulated *t*-value was greater than the calculated *t*-value, then it was concluded that the slopes were the same, and therefore the inactivation kinetics for the two strains were the same. Results for the statistical analyses are shown in Tables 4 and 5, comparing each environmental strain to its corresponding reference ATCC strain, for chlorine and monochloramine experiments.

Chlorine assays – heterotrophic bacteria

Brevundimonas vesicularis

This organism was isolated from all four utilities. Its presence in distribution systems may be undesirable as it has been occasionally implicated in cases of human infection (Gilad *et al.* 2000). Against expectation, the environmental strains isolated in this study were more susceptible to free chlorine than their corresponding reference strain (ATCC 11826) (Figure 1). CT values of more than 0.15 mg·min/L resulted in more than 4-log inactivation of the environmental strains, while less than 3-log inactivation of the reference ATCC strain was observed. The rapid inactivation of the environmental isolates was perplexing because these organisms were isolated from environments with disinfectant residuals and low nutrient concentrations, which should have selected for more resistant organisms.

One of the environmental strains, isolated from the Jordan Valley pipe loop, was more resistant than either of the two isolates from the Waterloo pipe loop. The pipe loop in Waterloo uses chloramine as the secondary disinfectant. At the time of the isolation, there was a total disinfectant residual of 0.04 mg/L. A statistical comparison of slopes (95% confidence level) for the two isolates suggests that there was no difference in susceptibility to free chlorine between the two Waterloo environmental strains. The third environmental isolate, from the Jordan Valley distribution system, was previously exposed to free chlorine. This strain was more resistant to free chlorine than the Waterloo environmental strains (Figure 1).

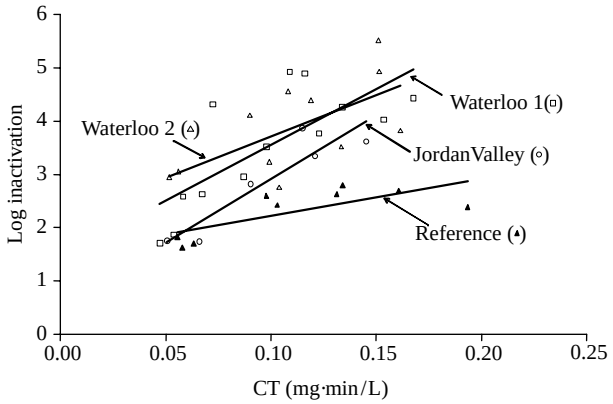

Figure 1 | Chlorine inactivation (pH 7.0, 25°C) of *B. vesicularis*.

Pseudomonas fluorescens

The environmental strain isolated from a Halifax Regional Water Commission pipe loop which used free chlorine as a secondary disinfectant, was identified as *Pseudomonas fluorescens*. In comparing the environmental isolate to the corresponding reference strain (ATCC 17387), the environmental isolate was more susceptible to disinfection with free chlorine (Figure 2). Greater than 4-log inactivation of the environmental isolate was achieved with a CT of just 0.05 mg·min/L compared to just over 3-log inactivation of the reference strain for the same CT. Statistical regression analysis comparing the two slopes concluded however, that this apparent difference was not statistically significant at a 95% confidence level (Table 3). However, the slopes shown in Figure 2 were either flat or negative, which is counter-intuitive since increasing CT should increase inactivation. This suggests that a statistical comparison of the slopes in this particular case (R^2 values ranging between 0.5–0.9 for these data sets) is perhaps less successful and further experiments would be needed to confirm the statistical conclusion. The flat slope for the environmental isolate may have been due to the data being in the "tailing" portion of the inactivation curve at high log inactivation levels (i.e. >5-log). Tailing (i.e. non-linear inactivation) can occur at high inactivation levels due to particularly resistant strains or due to aggregation or particle-association of organisms. The reason for the negative slope for the reference strain is unknown.

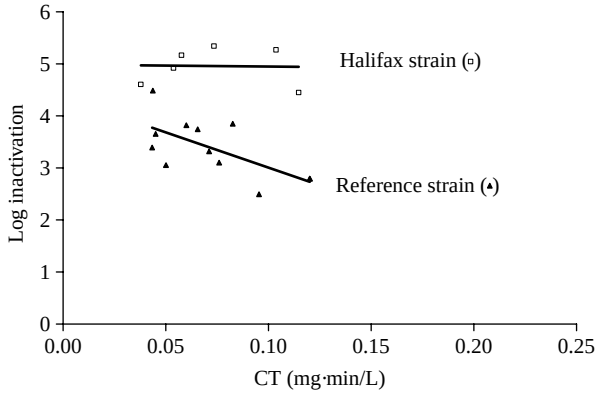

Figure 2 | Chlorine inactivation (pH 7.0, 25°C) of *P. fluorescens*.

Sphingomonas paucimobilis

The genus *Sphingomonas* includes species that are opportunistic pathogens, particularly by waterborne routes (Laskin & White 1999). *S. paucimobilis* has been reported to cause bacteremia, meningitis, urinary tract infection, and wound infections (Reina *et al.* 1991). *Sphingomonas* species are commonly found in biofilms of distribution systems and because some may be opportunistic pathogens, their presence may be undesirable (Koskinen *et al.* 2000).

The *S. paucimobilis* strains used in this study were isolated from the bulk water of the Jordan Valley pipe loops (previously exposed to 0.08 mg/L free chlorine) and the East Bay Municipal pipe loop (previously exposed to 1.69 mg/L monochloramine). Figure 3 shows that the Jordan Valley environmental isolate is slightly more susceptible to free chlorine, with a CT value less than 0.1 mg·min/L being sufficient for a 5-log reduction of this strain, whereas the reference strain was inactivated by less than 4-log at a similar CT. Statistically, this apparent difference was not significant with a 95% confidence (Table 3). In contrast, statistical analysis revealed that the inactivation kinetics, as indicated by the linear regression of slopes, for the East Bay strain were different from those of the reference strain (95% confidence level) (Table 3).

Monochloramine assays – heterotrophic bacteria

A summary of the statistical analyses for monochloramine inactivation results are shown in Table 3. As with chlorine inactivation data analysis, a comparison of slopes between the regression lines was performed by constructing 95% confidence intervals around the slope parameter. Monochloramine experiments with all but one strain (Jordan Valley) showed no statistical difference between the environmental strains and their corresponding reference strain (Table 3).

Brevundimonas vesicularis

When monochloramine was used as the disinfectant in this study, the patterns of inactivation were similar to those found in the chlorine experiments. When the two Waterloo environmental strains of *B. vesicularis* were subjected to monochloramine, it was found that there was no statistically

Table 3 | Characteristics of the *E. coli* O157:H7 strains tested

Strain	Isolated from	Obtained from	Identification analyses
ATCC 35150	Human feces	ATCC	Serology
FRIK 2516	Farm water	Food Research Inst.[a]	Biochemical, serological & genomic subtyping
FRIK 2531	Farm water	Food Research Inst.	Biochemical, serological & genomic subtyping
FRIK 2536	Farm water	Food Research Inst.	Biochemical, serological & genomic subtyping
FRIK 2542	Steer feces	Food Research Inst.	Biochemical, serological & genomic subtyping
FRIK 2552	Cow feces	Food Research Inst.	Biochemical, serological & genomic subtyping
FRIK 2568	Cow feces	Food Research Inst.	Biochemical, serological & genomic subtyping
WSLH	Lake water	WSLH[b]	Serology

[a]Food Research Institute, University of Wisconsin, Madison, WI.
[b]Wisconsin State Laboratory of Hygiene, Madison, WI.

significant difference (95% confidence level) in the inactivation kinetics between the environmental and reference strains (Figure 4). It was also observed that for a monochloramine residual concentration of 1 mg/L throughout the experiment, the highest log inactivation of organisms was approximately 1.5-log, for CT values in the order of 30 mg·min/L.

Pseudomonas fluorescens

The inactivation kinetics of the *P. fluorescens* environmental strain was statistically the same as that for the reference strain, at a 95% confidence level (Figure 5). A CT of 25 mg·min/L achieved greater than 3-log inactivation of this organism from either source. Of the three HPC organisms tested, *P. fluorescens* was the most easily inactivated by monochloramine. Based on the environment from which it was isolated, it is not surprising that this organism was most susceptible to both chlorine and monochloramine disinfection. The residual concentration of 0.02 mg/L free chlorine was the lowest of all the pipe loop samples collected for microbial isolation.

Sphingomonas paucimobilis

Inactivation kinetics of the *S. paucimobilis* reference strain were statistically different (95% confidence level) from for the Jordan Valley environmental strain, but statistically the

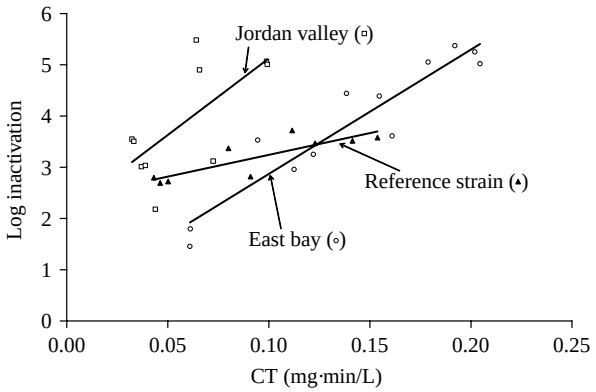

Figure 3 | Chlorine inactivation (pH 7.0, 25°C) of *S. paucimobilis*.

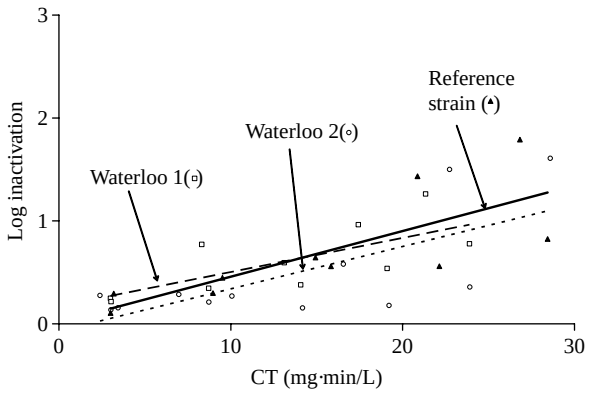

Figure 4 | Monochloramine inactivation (pH 8.0, 25°C) of *B. vesicularis*.

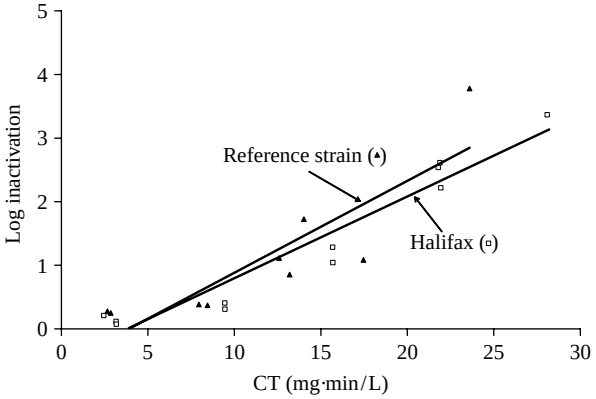

Figure 5 | Monochloramine inactivation (pH 8.0, 25°C) of *P. fluorescens*.

same as the East Bay environmental isolate (Figure 6). The Jordan Valley isolates were more susceptible to disinfection than the reference strain, which was again counterintuitive to the research hypothesis.

The higher susceptibility of the Jordan Valley isolate compared to the East Bay strain could be a result of the latter being isolated and exposed to higher disinfectant residual concentrations (1.69 mg/L monochloramine) than the Jordan Valley strain (0.08 mg/L free chlorine). However, the reference strain also had not been previously exposed to disinfectant, and was more resistant to monochloramine than the Jordan Valley isolate. The differences may be attributed to strain-to-strain variability. If this is the case, then it implies that one strain is not representative of the species.

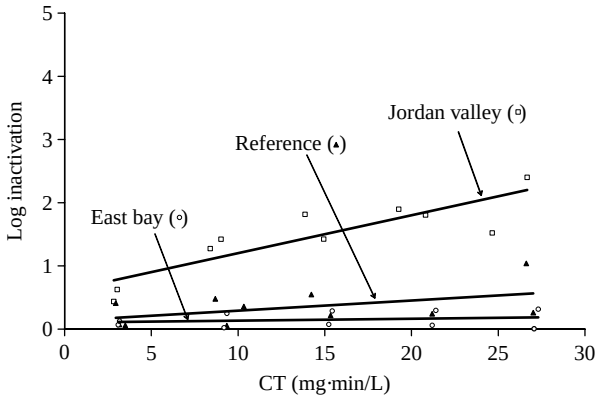

Figure 6 | Monochloramine inactivation (pH 8.0, 25°C) of *S. paucimobilis*.

Inactivation of *E. coli* O157:H7

Bench-scale inactivation studies were carried out with a reference strain of *E. coli* O157:H7 (ATCC 35150) and seven environmental strains of *E. coli* O157:H7 isolated from dairy farms (Shere *et al.* 1998) and a lake in Wisconsin. Table 3 describes these various strains.

In bench-scale experiments, the inactivation of pure cultures of various *E. coli* strains by free chlorine and monochloramine was tested (Table 4). The inactivation experiments for all strains of *E. coli* were replicated at least twice (three and four times for selected strains). Figure 7 is a representative example of a comparison between the chlorine inactivation kinetics of *E. coli* O157:H7 ATCC reference strain to the inactivation kinetics of the one environmental strain. The *E. coli* strains were very sensitive to free chlorine; in all cases, CT values < 0.30 mg· or ·min/L were sufficient to inactivate at least 2- to 3-log of these organisms (data not shown), with the exception of the FRIK 2536 strain, which was markedly more resistant. These data are in agreement with the data from Rice *et al.* (1999) who found that the average chlorine inactivation rates at 5°C (pH 7.0, 1.1 mg/L of free chlorine) for seven *E. coli* O157:H7 isolates (from cattle) and four wild type *E. coli* isolates (from cattle manure) were identical (2.93-log/sec.). Therefore, chlorination appears to adequately control this pathogen, at least in clean water. The statistical analysis for chlorine experiments is shown in Table 5. The chlorine inactivation of three of the FRIK strains (2536, 2542 and 2568) was shown to be statistically different from that of the ATCC strain. Although significant statistical variations were observed between the inactivation kinetics of certain strains, these differences are not important from a water treatment perspective in terms of the amount of disinfectant used or contact time necessary.

Figure 8 is a representative example of a comparison between the monochloramine inactivation kinetics of the *E. coli* O157:H7 ATCC reference strain to the inactivation kinetics of one environmental strain. All strains tested showed approximately 2- to 3-log of inactivation with CT values ranging from 20 to 30 mg or min/L (data not shown). These data are in agreement with previous studies on chloramine disinfection of *E. coli* (Ward *et al.* 1984). Interestingly, all environmental strains of *E. coli* O157:H7 were consistently more resistant to monochloramine than

Table 4 | Statistical comparison of environmental isolates to reference strains in terms of inactivation by chlorine and monochloramine

	Chlorine				Monochloramine			
Environmental strain	**DF**	$t_{\alpha,\upsilon}$	t_{calc}	**Result**	**DF**	$t_{\alpha,\upsilon}$	t_{calc}	**Result**
Waterloo 1	18	2.10	2.15	More Sensitive	16	2.12	0.63	Same
Waterloo 2	16	2.12	1.29	Same	18	2.10	0.18	Same
Jordan Valley 3	11	2.20	3.27	More Sensitive	–	–	–	Not Tested
Halifax 4	14	2.14	1.64	Same	16	2.12	0.51	Same
Jordan Valley 5	15	2.13	2.09	Same	17	2.11	2.90	More Sensitive
East Bay 6	17	2.11	3.88	More Sensitive	17	2.11	1.21	Same

If $t_{\alpha,\upsilon} > t_{calc}$, then it can be assumed that there is no difference in kinetics between strains.
Note: $t_{\alpha,\upsilon}$ values vary according to degrees of freedom (DF).

the ATCC 35150 strain (data not shown), and these differences were statistically significant (Table 5). This agrees with the hypothesis that indigenous strains may be better adapted to survival in adverse conditions than a culture collection strain because of physiological differences. However, this is the opposite of what was observed in terms of the other heterotrophic bacteria considered in this study.

Comparison of reference versus environmental strains

In this study, it was hypothesized that microorganisms obtained from environmental isolates would be more resistant to disinfection than reference strains. While this was observed with respect to the inactivation of *E. coli* O157:H7 by monochloramine, in all other cases the inactivation of environmental isolates was either equal to *or greater than* the inactivation of reference strains, which is contrary to the hypothesis. Due to these unexpected results, the literature was again reviewed in an attempt to explain the observations of this study.

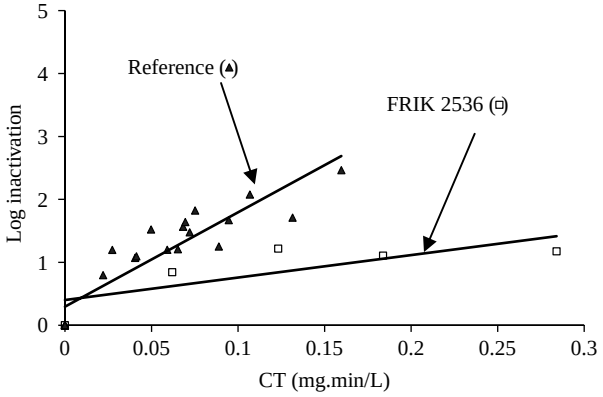

Figure 7 | Inactivation by free chlorine (pH 7.0, 21°C) of *E. coli* O157:H7 ATCC 35150 (▲) and *E. coli* O157:H7 FRIK 2536 (■) strains.

One possible explanation for the unexpected results may stem from the method in which organisms are cultured before experiments. Although the type of medium and other conditions such as pH, temperature, and shaking were kept the same in each experiment of each strain, there was variation in factors such as inoculum size, time of growing the cultures, and the age of the stock cultures which were used to initiate the experimental cultures. According to Johnston *et al.* (2000), inoculum levels may have a significant effect on microbial inactivation. The extent to how much these variables affect the results could only be determined by direct comparisons, i.e. grow a culture for five versus six days, and determine the effect on kinetics.

The differences in the inactivation among the environmental isolates from different pipe loops were likely due to the disinfectant conditions in the loops. For example, the Waterloo isolates of *Brevundimonas vesicularis*, which were

Table 5 | Statistical comparisons between the slopes of linear regressions of inactivation data (chlorine and monochloramine inactivation of the ATCC 35150 and the environmental isolates of *E. coli* O157:H7) to determine if two data sets are statistically equivalent within a 95% confidence level (t-test)

	Chlorine				Monochloramine			
Environmental Isolates	**DF**	**$t_{\alpha,\upsilon}$**	**t_{calc}**	**Result**	**DF**	**$t_{\alpha,\upsilon}$**	**t_{calc}**	**Result**
FRIK 2516	26	2.06	1.88	Same	22	2.07	2.57	More resistant
FRIK 2531	26	2.06	1.21	Same	22	2.07	2.38	More resistant
FRIK 2536	22	2.07	5.28	More resistant	23	2.07	3.53	More resistant
FRIK 2542	26	2.05	3.66	More sensitive	21	2.08	3.88	More resistant
FRIK 2552	27	2.05	0.13	Same	21	2.08	3.70	More resistant
FRIK 2568	23	2.07	5.23	More sensitive	21	2.08	3.75	More resistant
WSLH	24	2.06	1.66	Same	19	2.09	3.53	More resistant

If $t_{\alpha,\upsilon} > t_{calc}$, then it can be assumed that there is no difference in kinetics between strains.
Note: $t_{\alpha,\upsilon}$ values vary according to degrees of freedom (DF).

more susceptible to free chlorine than the Jordan Valley isolate (Figure 1), were previously exposed to chloramines, not free chlorine as was the Jordan Valley strain. Also, the Jordan Valley strain was isolated from pipe loops with higher residual concentrations (0.14 mg/L free chlorine) compared to the Waterloo isolates (0.04 mg/L total chlorine). Likewise, the East Bay environmental strain of *Sphingomonas paucimobilis*, which was less susceptible to chlorine than the Jordan Valley strain (Figure 3), was isolated from a more disinfected environment (1.69 mg/L monochloramine) than the Jordan Valley strain (0.08 mg/L of free chlorine).

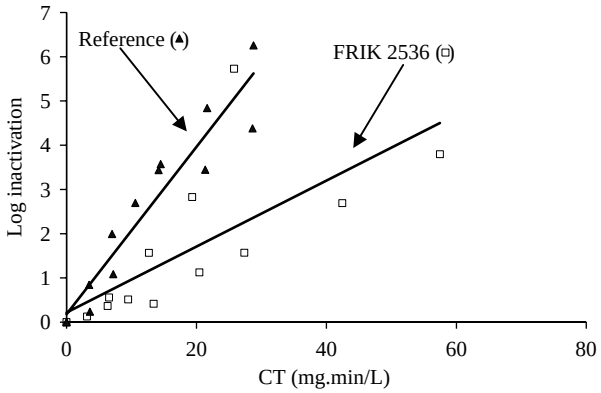

Figure 8 | Inactivation by monochloramine (pH 8.0, 21°C) of *E. coli* O157:H7 ATCC 35150 (▲) and *E. coli* O157:H7 FRIK 2536 (■) strains.

Although this observation supports the hypothesis that organisms originating from disinfected environments are able to better withstand further disinfection than those adapted to more temperate conditions, it does not explain why the inactivation kinetics were not lower for the East Bay strain when compared to the reference strain. It may be valuable, however, to compare environmental strains from disinfected environments to isolates from non-disinfected environments, where in both cases the isolates would have been exposed to similar conditions, unlike the reference strain, which has acclimated to optimum conditions.

The results from the chlorine assays do not support the hypothesis that environmental strains isolated from disinfected environments are more resistant to chlorine than reference ATCC strains. In fact, the environmental strains were equally or more susceptible to chlorine than their corresponding reference strains. The strains exposed to higher levels of disinfectant residual in the pipe loops were more resistant to further chemical insult than those strains of the same species. This is supported by previous studies by Ridgeway & Olson (1982), where they showed that bacteria from a chlorinated system were more resistant to chlorine than those from an un-chlorinated system. Mechin *et al.* (1999) also reported that pre-exposure to disinfectants renders organisms more resistant to further

exposure. The strains used in that study and similar studies (Langsrud *et al.* 2003), however, were not grown in the presence of disinfectant in the medium.

Several authors have shown that the methods used to conduct inactivation experiments have a great influence on the inactivation of microorganisms in the presence of chlorine. LeChevallier *et al.* (1988) showed that resistance to chlorine can change as much as 10-fold by changing previous growth conditions (i.e. growth medium or temperature). Taylor *et al.* (2000) reported a 10-fold increase in resistance to chlorine in cells grown in water compared to medium-grown cells. Other studies show similar results with changes in resistance to disinfectants depending on the type of medium in which the microorganisms are grown (Kuchta *et al.* 1985; Cargill *et al.* 1992; Abu-Shkara *et al.* 1998; Le Dantec *et al.* 2002). A few studies have shown that subculturing naturally occurring organisms even once decreased the resistance to disinfectants (Carson *et al.* 1972, Wolfe & Olson 1984). This may explain why the environmental strains of the heterotrophic bacteria were not more resistant to free chlorine, as initially hypothesized. Kuchta *et al.* (1985) showed that when highly chlorine-resistant *Legionella pneumophila* strains were subcultured in agar medium, their resistance fell to levels comparable to organisms which were never exposed to chlorine. If inactivation experiments fail to mimic proper environmental factors (nutrient limitation, dormancy, growth rate, etc.), the accuracy of the experimental results decreases (Gilbert & Brown 1995).

Inactivation experiments are designed to mimic disinfection systems and predict microbial responses to disinfectants in the field. The microorganisms used for such experiments originate from either reference (laboratory) cultures or environmental sources. The advantages of using reference strains in such experiments include increased reproducibility (Gilbert & Brown 1995), limiting interferences from the complex natural water environment, and to simplify the interpretation of experimental results (Shang & Blatchley 2001). Unfortunately, such experiments may not adequately represent actual field conditions, such as those found in distribution systems. It is generally known that microorganisms are able to physiologically adapt to changes in their growth environments. It makes sense then, to utilize environmental strains of microorganisms which have been exposed to low nutrient environments with disinfectant residual present, such as chlorine. However, this study has shown that it may be inappropriate to simply culture an environmental organism, expecting the inactivation kinetics of the inoculum to be the same as the organisms in the environment. It is hypothesized that given proper antecedent growth conditions (cultured in the presence of disinfectant), environmental isolates would be more resistant than those organisms never exposed to disinfectants. If in fact previous exposure of microorganisms to sublethal concentrations of disinfectant causes disinfectant-tolerant organisms to evolve, laboratory results using reference cultures for inactivation experiments may overestimate the effectiveness of a given residual concentration. Implications of this may be that residual concentrations or the type of secondary disinfectant used in the distribution system may have to be periodically changed.

CONCLUSIONS

The results from these inactivation studies indicate that *E. coli* O157:H7, *Brevundimonas vesicularis, Pseudomonas fluorescens,* and *Sphingomonas paucimobilis* are susceptible to free chlorine or monochloramine at typical concentrations used in water treatment practices, when tested under laboratory conditions. A comparison of inactivation kinetics was made between organisms obtained from different sources (ATCC reference strains vs. environmental strains). While environmental isolates of *E. coli* O157:H7 were either equally or less susceptible to inactivation than the reference strains, environmental isolates of the other heterotrophic bacteria were generally more easily inactivated than the reference ATCC strains. The increased susceptibility of some of the environmental isolates in the laboratory may be a result of antecedent growth conditions (exposure to nutrient-containing media for culturing organisms, higher temperatures, etc.). Sub-culturing may also affect the resistance of environmental isolates, and needs to be studied further to determine if resistance capabilities are lost by sub-culturing. Lastly, the strain-to-strain variability seen in these results shows that several strains of one species are needed to be representative of that species. Proper antecedent growth conditions, representative of

actual environmental conditions, should be evaluated in further studies to determine if they will more accurately represent inactivation kinetics in disinfected environments.

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