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Induction of *Escherichia coli* and *Salmonella typhimurium* into the viable but nonculturable state following chlorination of wastewater

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ABSTRACT

We examined the effects of chlorine disinfection on *Escherichia coli* and *Salmonella typhimurium* in secondary-treated wastewater to determine whether such treatment might induce these bacteria into the viable but nonculturable (VBNC) state. In this state, cells lose culturability but retain viability and the potential to revert to the metabolically active and infectious state. To examine the effects of chlorination on cells in different physiological states, cells from the logarithmic and stationary phases, or nutrient starved, or grown in natural wastewater, were studied. Isogenic cells with and without plasmids were also examined. Whereas a mixture of free and combined chlorine, as occurs under typical wastewater disinfection, was found to be rapidly lethal to most cells, regardless of their physiological state or plasmid content, *c*. 10^4 of the original 10^6 cells ml^{-1} did survive in the VBNC state. While we were not successful in resuscitating these cells to the culturable state, the presence of such nonculturable cells in treated wastewater offers a potential public health hazard.

Key words | chloramines, chlorination, coliforms, culturability, VBNC, wastewater

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INTRODUCTION

Wastewater is the largest reservoir of human enteric bacteria (Rockabrand *et al.* 1999) and because enteric bacteria, such as *Escherichia coli* and *Salmonella typhimurium*, are potential sources of disease when ingested, the disinfection of wastewater is essential for eliminating these and other pathogenic microorganisms to remove them from the water before they reach intakes for water treatment plants (McFeters & LeChevallier 2000). Oxidizing agents, such as chlorine and monochloramine, are recommended by the US Environmental Protection Agency to eliminate bacterial pathogens from water (Baker *et al.* 2002). Uncombined (free) chlorine in the form of hypochlorous acid is an extremely potent bactericidal agent (Belkin *et al.* 1999) which has been used as a disinfectant for more than 100 years (Baker *et al.* 2002). The mechanism by which free chlorine acts has not been fully explained experimentally, although some of the free chlorine seems to target the bacterial membrane and proteins (Belkin *et al.* 1999). When free chlorine is dosed into a wastewater effluent, it converts to mono- and dichloramines upon exposure to the nitrogenous compounds present in the wastewater (Baker *et al.* 2002). This 'combined' chlorine, while not as powerful a disinfectant, is less reactive with wastewater constituents and thus tends to remain as the principal species in the residual chlorine concentration.

At sublethal levels, the addition of chlorine would be expected to pose a stress on microorganisms in wastewater, often causing reversible cell injury. Studies indicate that as many as 90% of coliforms may be injured in this manner, and not culturable prior to injury repair (Singh & McFeters 1990;

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McFeters & LeChevallier 2000). Such environmental stresses have also been shown to induce several bacteria, including *E. coli* and *S. typhimurium*, into a viable but nonculturable (VBNC) state (Oliver 1993, 2000*a*,*b*). Cells in the VBNC state are viable, as can be demonstrated by several direct microscopic assays (Oliver 1993, 2000*b*; Breeuwer & Abee 2000; Créach *et al.* 2003) but lose the ability to be cultured on routine microbiological media. This state is characterized by significant modifications in cell morphology, metabolism, physiology and possibly genomic structure (Oliver 2000*a*,*b*).

Existence of cells in the VBNC state has a variety of significant implications. For example, *E. coli* cannot serve as an indicator of fecal pollution when the cells are present in the VBNC state, as occurs when this organism is introduced into seawater. If genetically modified cells enter the VBNC state following release to the environment in bioremediation studies, it becomes extremely difficult to track their dispersal (Oliver 2000*b*). More importantly, such cells can also represent a public health hazard. A variety of human pathogens which enter the VBNC state, including *E. coli* and *Salmonella* spp., have been shown to be capable of returning to the actively metabolizing state, with the potential to initiate disease (Oliver & Bockian 1995; Colwell *et al.* 1996; Pommepuy *et al.* 1996; Oliver 2000*a*). Thus, chlorination of wastewater might be assumed to be disinfecting when in fact some of the cells that were supposedly inactivated may remain viable, resuscitate down the line, possibly increase in number, and cause infection. Indeed, it is well documented that municipal water systems often harbour coliforms, whether chlorination or chloramination is employed (LeChevallier *et al.* 1999).

Two important questions arise: does chlorination always kill the target bacteria in wastewater or can it induce them into a VBNC state? If they are induced into a VBNC state, can they recover their culturability (resuscitate) and thus be potentially infective? Such possibilities have been indicated in several previous studies (Dukan *et al.* 1997; Lisle *et al.* 1998; Zhao & Matthews 2000). However in those studies, test cells were suspended in water or phosphate-buffered saline (PBS), which do not mimic the conditions found in wastewater. A study was reported by Rockabrand *et al.* (1999) on the survival of chlorinated coliforms in raw, secondary-treated wastewater, but those investigators employed a high level (3.5 mg l^{-1}) of free chlorine. The purpose of our study was to examine the effects of chlorination on several *E. coli* and *S. typhimurium* strains present in secondary treated wastewater to determine if the cells might be induced into a VBNC state. We used chlorination methods employed at many utilities in the United States, where free chlorine is added to wastewater for disinfection and is subsequently converted into chloramines. We studied strains both with and without plasmids, to determine if such extrachromosomal elements might affect entrance or resuscitation from the VBNC state. Such a role has been reported in several *Pseudomonas* species (McDougald *et al.* 1995). Further, we studied cells present in several physiological states in the possibility that physiological status might affect sensitivity to chlorination or their induction into the VBNC state. Finally, we wished to examine if any such cells could recover culturability.

METHODS

Bacterial strains

Paired, isogenic strains of *Escherichia coli* K12 (F^+ and F^-) and *Salmonella typhimurium* (WG49 and WG45) were used in all experiments. Strains K12 F^+ and WG49 are both male strains carrying plasmids encoding for ampicillin and kanamycin resistance, respectively. These male plasmids also encode for pili, allowing these strains to be infected by male-specific coliphages. Strains K12 F^- and WG45 lack these plasmids, and are thus genetically female, but are otherwise identical to the K12 F^+ and WG49 pairs. Strains were routinely maintained on Luria Bertani (LB) agar plates.

Wastewater

Our studies were performed as part of a larger study on disinfection in wastewater. As secondary-treated sewage is the actual step in wastewater treatment plants where sewage enters the disinfection process (whether chlorine or UV), we chose this water to mimic what occurs in an actual treatment system. Non-chlorinated, secondary treated wastewater (pH 8.0) was acquired from a wastewater treatment plant in Charlotte, North Carolina. Wastewater was filtered through Whatman #1 filter paper then through a 0.4 μm, 47 mm polycarbonate filter (Fisher Scientific; Fairlawn, New Jersey). The filtered wastewater was then autoclaved. This procedure

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provided a sterile wastewater solution in which the VBNC process could be followed.

Cell preparation

A variety of methods for preparing cells in different physiological states were employed in these studies. These are described below.

Stationary phase cells

One colony of each of the strains was inoculated into LB broth and grown overnight with aeration at room temperature (22°C). An inoculum (0.1 ml) of these cells was placed in 10 ml of fresh LB broth and shaken at 37°C until the stationary phase was reached (OD_{610} of *c.* 0.80). Aliquots (1 ml) were centrifuged (*c.* 14,000 × g for 5 min), the supernatant carefully removed, and the pellet resuspended in phosphate-buffered saline (PBS). The cells were then centrifuged and washed a second time with the pellet being resuspended in 1 ml PBS.

Log phase cells

Cells were prepared as described above except that they were grown to a final OD_{610} of 0.15–0.20.

Starved cells

Cells were grown to log phase and prepared as described above but were then inoculated (1 ml) into PBS (5 ml) and incubated for 20–24 hours at room temperature.

Cells grown in wastewater

In the possibility that cells which were adapted to wastewater might be more resistant to chlorination, a single bacterial colony was inoculated into filtered, autoclaved wastewater (see above) and shaken overnight at room temperature. These cells (0.05 ml) were then inoculated into 5 ml of filtered, autoclaved wastewater and shaken for 5 days at room temperature.

Cell growth/survival in wastewater

Log phase cells were inoculated (1% final concentration) into sterile wastewater. These microcosms were then placed at 5, 15 or 21°C and monitored over time for culturability.

Chlorination of cells

A 1% dilution of commercial bleach (6% sodium hypochlorite) was made in filtered and autoclaved deionized water. This was filter sterilized using a 0.2 μm Acrodisc® filter (Pall Corporation, Ann Arbor, Michigan). Two 50 ml wastewater microcosms were prepared in sterile, disposable centrifuge tubes. One tube served as the experimental sample while the other served as a control. Cells (0.5 ml except for 2.0 ml of wastewater-grown cells) were added to each wastewater microcosm and shaken to ensure even distribution. Sodium hypochlorite (free chlorine) solution was then added to provide a final concentration of $1\,mg\,l^{-1}$ of free chlorine in the wastewater. Samples were taken at times 0 (*c.* 20 s), 1, 5, 10 and 60 minutes and placed into 20% sodium thiosulfate (1:1 v/v in autoclave buffered saline) to inactivate any residual free chlorine. Chlorine levels in the microcosms at the initiation of the study and after the 10-minute sample time were determined using the DPD method (Hach Co., Loveland, Colorado).

Cell enumeration

Cells from both control (untreated) and chlorine-treated microcosms were removed, serially diluted, and plated onto LB agar to determine the numbers of culturable cells present at various treatment times. The method of Kogure *et al.* (1979) was used to enumerate total and viable (substrate responsive) cells. Briefly, samples at 0, 10 and 60 minutes were incubated with 0.025% yeast extract (Difco; Sparks, Maryland) and 0.002% nalidixic acid (Sigma; St. Louis, Missouri) to inhibit the septation of cells growing in response to the nutrient addition. After 12 hours, samples were fixed with 50 μl 37% formalin and stained with filter sterilized 0.1% acridine orange. Samples were then filtered through 0.2 μm black polycarbonate filters (Fisher Scientific). A control sample lacking Kogure reagents was employed to determine spontaneous elongation, and these values were subtracted from experimental data points. Epifluorescent microscopy (Olympus BX51) using appropriate filters was used to enumerate total and viable cells. This method has a limit of detection of *c.* 5×10^3 cells ml^{-1}.

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Resuscitation studies

Stationary phase cells were chlorine treated as described above. To attempt to recover culturable cells, reactive oxygen species (ROS) scavengers, a temperature upshift, and the use of media solidified with agarose instead of agar were examined as described below.

ROS scavengers

As there is evidence that ROS may be involved in the VBNC state of some bacteria (Oliver 2005; Kong *et al.* 2004), 200 units of bovine liver catalase, 600 units of superoxide dismutase, or a 1% solution of sodium pyruvate was spread onto LB agar plates which were then dried prior to use. All stock solutions (Sigma; St Louis, Missouri) were made with filtered, autoclaved, deionized water and kept in the dark at 5°C. At each time point, 100 μl of the chlorine-exposed cells were spread onto the ROS scavenger-treated LB plates.

Temperature upshift

A study by Gupta & Joseph (2001) provided evidence for the resuscitation of *Salmonella enterica* cells from the VBNC state following heat shock treatment. Employing their protocol, cells (250 μl) taken at each of the sample points were exposed to 80°C for 15 seconds. These heat-shocked samples (100 μl) were plated onto LB agar.

LB agarose plates

As we have observed that *Vibrio vulnificus* cells in the VBNC state show significant culturability when agarose is substituted for agar as a solidifying agent (Kong *et al.* 2004), LB medium was prepared with 15 g of agarose instead of 15 g of agar. At each time point, 100 μl of the chlorine-exposed cells were plated onto this medium.

Statistical analysis

All experiments were conducted at least twice with each of the bacterial strains. Results were graphed and the standard error of the mean (SEM) calculated and shown in the figures.

RESULTS

Cell growth/survival in wastewater

Strains of *E. coli* and *S. typhimurium* all responded similarly to incubation in wastewater. As shown in Figure 1, there was sufficient nutrient present in the wastewater to support growth of cells when incubated at either 15 or 21°C. Culturability remained high at these temperatures for the duration of our study (150–200 days). In contrast, both organisms exhibited a gradual drop in culturability when incubated at 5°C, and entered into the VBNC state within 60–80 days.

Response of cells to chlorination

Following the addition of chlorine to microcosms harbouring log, stationary, starved or wastewater-grown cells, the levels of free and total (free + combined) chlorine were measured at 30 s and 10 min. Levels were similar in all studies, and averaged 0.96 mg l^{-1} free and 1.70 mg l^{-1} total chlorine at the initial determination. By 10 min, these levels had decreased to 0.13 and 1.10 mg l^{-1} for free and total chlorine, respectively. Whereas the culturability of control F^+ (Figure 2a) and F^- (Figure 2b) *E. coli* cells in the stationary phase remained at *c.* 10^6 CFU ml^{-1}, cells exposed to this chlorination protocol typically declined to <10 CFU ml^{-1} by the first (20 s) sampling. Total cell counts revealed the continued presence of $>10^6$ total cells in all cases. When assayed by the substrate responsiveness method, control cells exhibited *c.* 75–95% viability. In contrast, *E. coli* K12 F^+ (Figure 2a) showed a high level of viability following 30 seconds of chlorine exposure, but by 10 minutes, only 1.2% of the cells remained viable, and at 60 minutes of treatment the level was reduced to 0.36%. Similarly, and in contrast to the untreated cells, stationary phase cells of *S. typhimurium*, both WG45 (Figure 3a) and WG49 (Figure 3b) exhibited a total loss of culturability on chlorination by the first (20 s) sampling. The viability assay again indicated significantly reduced levels of cell survival at 10 minutes, with little or no viability at the 60-minute point.

In all remaining studies, regardless of whether the cells were in log phase, starved, or grown in wastewater instead of

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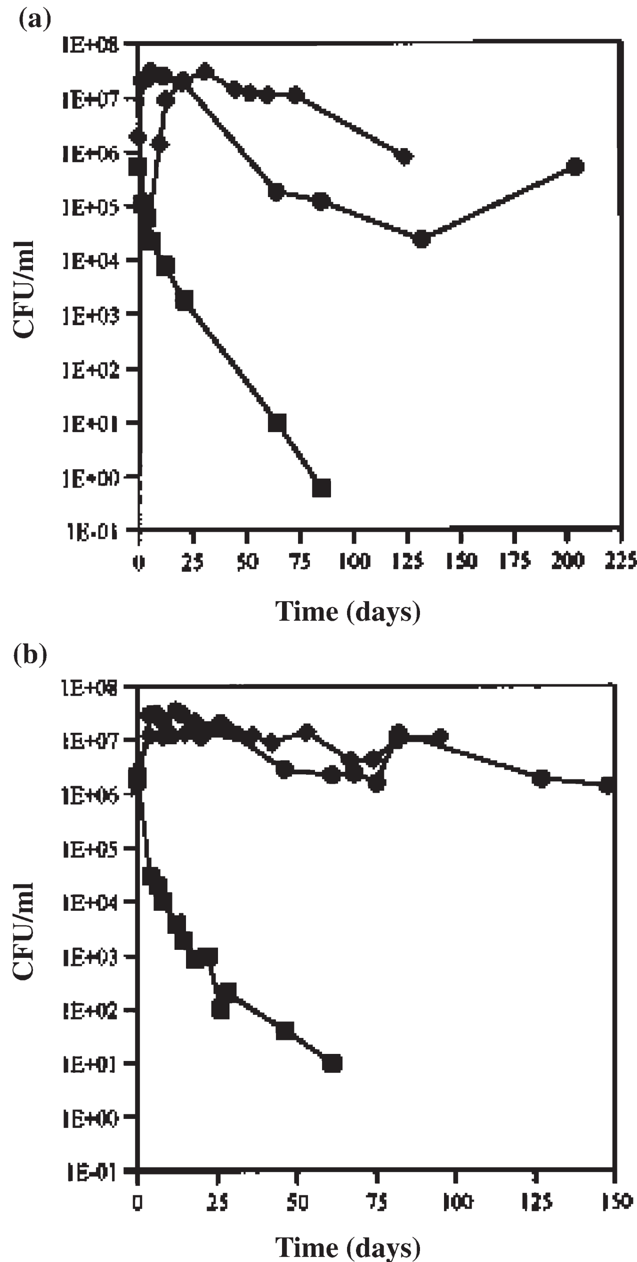

Figure 1 | Culturability of *E. coli* and *S. typhimurium* cells following incubation in wastewater at 5 (■), 15 (●), or 21°C (◆); (a) *E. coli* F +, (b) *S. typhimurium* WG45

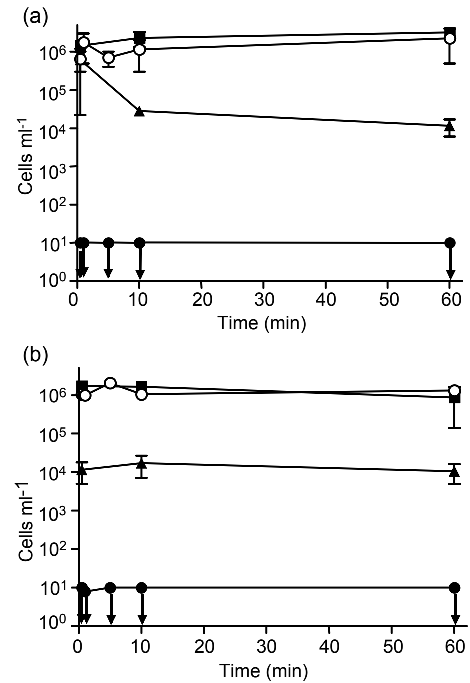

Figure 2 | Levels of *E. coli* cells in control and chlorine-treated microcosms. Stationary phase cells were placed in autoclaved and filtered wastewater (secondary treated), then exposed to *c*. 1.0 mg l^{-1} free chlorine. Cell survival was measured after 0.5, 1, 5, 10 and 60 minutes of treatment. Total cell population (■), culturable levels of control (untreated) cells (○), culturable levels of chlorine-treated cells (•), and levels of VBNC cells (▲)are shown for (a) F^+ cells and (b) F^- cells.

nutrient medium, the results were essentially the same as shown in Figures 2 and 3, with chlorinated cells exhibiting a *ca*. six-log reduction in culturability during the first 30 seconds. On average, regardless of the physiological state of the cells, 0.39% of the treated cells responded to the viability assay after 60 min of chlorination, indicating a small portion of the cells were able to resist this treatment. While such a percentage is low, it equates to c. 10^3–10^4 cells/ml.

Resuscitation studies

Neither of the strains of *E. coli* or *S. typhimurium* exhibited growth ($<10\,\mathrm{CFU\,ml^{-1}}$) under any of the resuscitation conditions tested.

DISCUSSION

Prior to disinfection, wastewater harbours numerous bacterial species, many of which are pathogenic. Figure 1 indicates that strains of *E. coli* and *S. typhimurium* are capable of long-term survival in natural wastewater, although a gradual entrance into the VBNC state was observed when cells were incubated at low (5°C) temperature. These results

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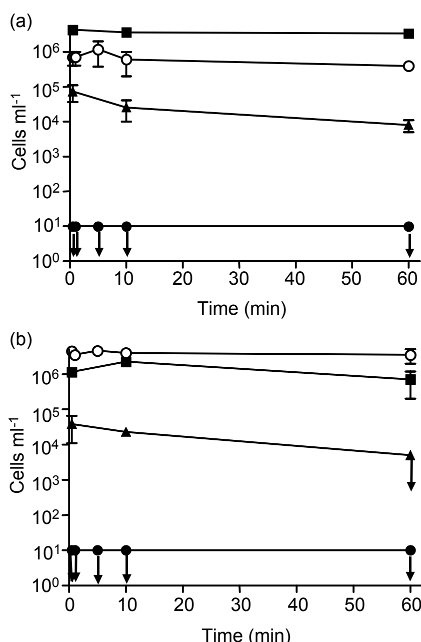

Figure 3 | Levels of *S. typhimurium cells* in control and chlorine-treated microcosms. Cells were prepared and treated as described in Figure 2; (a) WG45 cells and (b) WG49 cells. See Figure 2 for key to symbols.

confirm that temperature is the major VBNC-inducing factor in these bacteria (Nelson *et al.* 1996; Rigsbee *et al.* 1997).

Several reports have described the 'revival' of disinfection-injured cells to an actively growing state (McFeters & LeChevallier 2000), and others have indicated that cells grown in wastewater, as opposed to laboratory media, may be significantly more resistant to chlorination (Rockabrand *et al.* 1999). While bacterial cell injury and revival are well established, the possibility that chlorine treatment also leads to cells in a viable but nonculturable state has not been adequately investigated. Our study examined *E. coli* and *S. typhimurium*, as representatives of the bacterial populations that exist in such environments, and their possible induction by a mixture of chlorine and chloramines, as occurs under actual wastewater disinfection, into the VBNC state.

Initial experiments conducted with cells grown in nutrient broth prior to wastewater exposure showed very low viability once chlorine was added to the microcosms. This suggested that, even though the chlorine levels in our experiments declined rapidly as a result of combining with, and scavenging by, constituents in the wastewater, a finding consistent with other investigators (Taylor *et al.* 2000), they were sufficient to completely eliminate culturability in the strains tested. These results are consistent with several studies demonstrating the rapid inactivation by chloramines of coliforms and other bacterial pathogens found in wastewater (Blaser *et al.* 1986; Rockabrand *et al.* 1999; Taylor *et al.* 2000).

Dukan *et al.* (1997) reported that, following chlorination of *E. coli* with 0.4–1.0 mg l^{-1} HOCl in PBS, three populations developed: a majority of dead cells, a few (10^2–10^4) culturable cells, and a large population (*c.* 10^7) of cells in the VBNC state. Except that we did not observe culturable cells, our findings parallel those of Dukan *et al.* (1997) and offer further evidence that a portion of chlorine-treated populations remain alive, but in the VBNC state. Our studies also confirm those of Zhao & Matthews (2000), who also treated stationary cells of *E. coli*. Like them, we observed a complete loss of culturability within 30 seconds, although cells in the VBNC state were detected in their study for up to 5 days. We employed wastewater as the diluent, and thus a combination of free and combined chlorine (e.g. chloramines) resulted as our disinfectant. Although combined chlorine is known to be less effective than free chlorine for inactivation of bacteria (Sobsey 1989), the exposure of the microbes to the combined chlorine during our chlorination step was sufficient to result in survival levels similar to that found by Dukan *et al.* (1997) and Zhao & Matthews (2000). Finally, we note the study by Rockabrand *et al.* (1999), who also reported that, while coliforms naturally occurring in raw wastewater underwent a 100–1,000-fold decrease in culturability when chlorinated, the treatment elicited the nonculturable state. Those authors went on to suggest 'that current standard procedures for wastewater analysis which rely on detection of culturable cells likely underestimate fecal coliform content'.

Since very low viability was observed when cells were grown in nutrient broth, we decided to examine whether starvation-induced cross protection would serve to make the bacterial cells more resistant to chlorination. Such an effect has been reported by Lisle *et al.* (1998) and by Saby *et al.* (1999). Again, however, the cells in our studies showed only minimal viability. The difference between our results and those of Lisle *et al.* (1998) and of Saby *et al.*

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(1999) may be attributed to significant differences in methodology. Lisle *et al.* (1998) employed distilled water as the starvation medium, while Saby *et al.* (1999) used PBS.

In a final set of experiments, we felt that our results might reflect the use of cells which had been cultured in a very high nutrient ($15\,g\,l^{-1}$) bacterial medium and that growth in wastewater might more accurately reflect the cells' response to the natural environment. Indeed, Taylor *et al.* (2000) reported *Mycobacterium avium* strains to exhibit significantly greater survival when water-grown compared with being medium-grown. Thus, we used cells cultured in wastewater for 5 days at room temperature. Our results, however, did not differ significantly from those obtained using log, stationary or starved cells.

As shown in Figures 2 and 3, whether or not cells harboured plasmids did not appear to have any effect on the sensitivity of the cells to chlorination, to entry into the VBNC state, or to resuscitation from that state (see below). Harbouring plasmids was found in a previous study (McDougald *et al.* 1995) to have a dramatic effect on entry into the VBNC state by *Pseudomonas fluorescens* and *P. syringae*. For those species, however, the VBNC state was induced by incubation at high (35°C) temperature, and was a gradual process. It is likely that the near immediate effects of chlorine on cells preclude such a phenomenon.

Resuscitation

A number of bacterial species which enter the VBNC state have been shown to regain culturability upon elimination of the stress that induced the VBNC state, or on exposure to certain chemicals or culture conditions (for reviews, see Oliver 1993, 2000*a,b*).

Dukan *et al.* (1997) diluted chlorine-treated cells ($2\,mg\,l^{-1}$ in PBS for 20 min) of *E. coli* O157:H7 into fresh PBS, and observed increases in culturability which they attributed to both resuscitation of VBNC cells as well as to regrowth of the culturable cells remaining after treatment. Approximately 7 days was required for a return to a level of culturability comparable to the starting population. We did not employ such a protocol in our studies, but did examine for culturability after allowing the cells to remain overnight in the treated microcosms. Although the level of combined chlorine remaining in the microcosms was quite low ($<0.13\,mg\,l^{-1}$), it is possible that the VBNC cells were incapable of resuscitation under these conditions. Rockabrand *et al.* (1999) reported that the chlorinated coliforms they studied were capable of resuscitation following nutrient addition. However, under the conditions of their studies, between 100 and 1,000 culturable cells remained after chlorine treatment, making it likely that the 'resuscitation' they reported was actually due to regrowth of these residual culturable cells.

The addition of ROS scavengers to the culture medium in our study was used because of recent data suggesting that the same defences involved in hydrogen peroxide resistance are effective against free chlorine (Belkin *et al.* 1999). Calabrese & Bissonnette (1990) reported that the culturability of chlorine-stressed coliforms could be increased by the addition of catalase and pyruvate to the recovery medium. However in our studies these same ROS scavengers, along with temperature up-shifts and the use of media with agarose in place of agar, all failed to result in recovery of the stressed cells. This lack of recoverability does not mean that cells surviving in the VBNC state were not capable of resuscitation; it is well documented that resuscitation is not always easy to demonstrate, and may require conditions not yet established for specific microorganisms (Oliver 2000 *a,b*).

CONCLUSIONS

Based on the results of our study, a mixture of free and combined chlorine, as occurs under typical wastewater disinfection, was shown to be a highly effective agent against *E. coli* and *S. typhimurium*, regardless of their physiological state, with $>99\%$ loss of culturability. However, a small portion of the stressed populations survived in the VBNC state. While the percentage of cells in this state was low ($<0.4\%$ in our studies), this can equate to significant population sizes (*ca*. 10^4 in our studies). Further, considering the infectious dose of these two pathogens, as low as 20 cells for *Salmonella* and 10 for some *E. coli* strains, such residual levels can be potential health risks. While we were not able to document resuscitation of these cells to the culturable state, evidence from other studies

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indicates that cells of *E. coli* and *S. typhimurium* are capable of resuscitation (Oliver 2000*a*, 2005). The presence of VBNC cells in chlorinated wastewaters should be considered when evaluating the epidemiology of infections mediated by this environment.

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