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# Recovery of Chlorine-Exposed *Escherichia coli* in Estuarine Microcosms

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Laboratory microcosm experiments were performed to determine whether chlorine-exposed *Escherichia coli* are capable of recovery (i.e., increase in numbers of culturable cells) in estuarine waters and if so what water-quality parameters are responsible for this recovery. Suspensions of *E. coli* were exposed to 0.5 mg $L^{-1}$ of chlorine for 5 min followed by dechlorination with sodium thiosulfate. The chlorine-exposed bacteria were introduced into 2-L microcosms containing estuarine water collected from the Seacoast region of New Hampshire. Culturable cells in the microcosms were enumerated at 0, 10, 24, 48, and 74 h. In all estuarine microcosms the number of culturable cells increased by factors ranging from 2.8 to 50 over the 74-h incubation period. Multiple linear regression analyses indicated that ammonium and salinity were most significantly correlated with the recovery of *E. coli* over the 74-h incubation period; however, ammonium concentrations were strongly correlated with dissolved organic carbon and total dissolved nitrogen, making it impossible to determine with any degree of certainty the unique effect nitrogen or carbon had on recovery. The extensive recovery observed in our study indicates that following exposure to concentrations of chlorine that cause cell injury rather than death, numbers of culturable *E. coli* may increase significantly when discharged into estuarine waters. Thus, depending on the effectiveness of the chlorination process, the regular monitoring of chlorinated wastewater treatment effluent may underestimate the true impact on water-quality and public health risks.

## Introduction

Despite general improvements in water quality within the United States over the past 30 years, contamination of surface waters by fecal-borne microorganisms has become an increasingly important aspect of water-quality management due to concerns ranging from human illness to the degradation of economically important shellfisheries. To reduce the number of fecal-borne microorganisms released into the environment, wastewater treatment facilities (WWTFs) are required to disinfect their effluent prior to discharging into receiving water bodies. Chlorination is one of the most widely used forms of disinfection for wastewater effluent, and while this approach drastically reduces the number of microorganisms, it may not be 100% effective. For example, some cells exposed to chlorine undergo sublethal stress or injury while maintaining membrane integrity and metabolic activity (*1*−*5*), while other cells may escape the chlorination process relatively unharmed. From a public health perspective it is important to understand what happens to these organisms once they have been discharged into the environment.

While not synonymous with the viable but nonculturable (VBNC) condition described elsewhere, the characteristics of chlorine injury are functionally similar to the VBNC state as bacteria in each of these respective conditions are not considered dead but rather undetectable with selective growth media. Because chlorine-injured cells are not capable of growth on normal selective media, an underestimation of the number of microorganisms being released into the environment may occur (*6*, *7*). Public health may be threatened because chlorine-injured bacteria are capable of regaining virulence in aquatic environments (*4*) as well as regaining culturability upon removal from chlorine stress and introduction to a more favorable environment (*8*, *9*). Indeed, nutrient and energy sources have been shown to play a role in the maintenance of bacterial viability (*10*, *11*) and the initiation of bacterial resuscitation (*8*, *9*) in aquatic environments. Although reacquisition of culturability has been demonstrated in many organisms, recent studies have shown that the perceived resuscitation of VBNC populations of enteric and waterborne pathogens may be due to the regrowth of a few surviving cells upon removal from the stress (*12*, *13*). In other words, the growth of previously undetectable levels of culturable cells gives the appearance of resuscitation.

Because coliform bacteria are not expected to survive well outside their hosts, it is assumed that the few bacteria that may escape the disinfection process will rapidly die off in the environment. Management of bacterial pollution in aquatic environments has historically emphasized the need to understand the factors affecting die-off rates. As a result, studies have focused on the factors that affect bacterial decay rates in estuarine and marine environments (*14*, *15*). A few studies, however, suggest that under appropriate conditions *Escherichia coli* concentrations can increase in aquatic systems. For example, nutrient loading to surface waters by WWTFs may result in extended bacterial survival and/or increases in culturable cells in the environment. Lim and Flint (*16*) added synthetic sewage to sterilized lake water and observed an increase in culturable cells. Watkins and Cabelli (*17*) showed that *V. parahaemolyticus* densities were indirectly associated with contaminated surface waters around WWTF outfalls due to a nutrient stimulation of the food chain. Lopez-Torres et al. (*18*) showed a direct relationship between the survival of *E. coli* and *Klebsiella pneumoniae* and the presence of wastewater discharges.

Whether due to the reacquisition of culturability of injured cells or growth of uninjured cells, increases in the number of culturable cells (defined here as recovery) of chlorine-exposed bacteria has important environmental and water-quality implications. A limited number of studies have shown that chlorine-exposed bacteria are capable of increasing in numbers when removed from the stress and introduced to a more favorable environment (*4*, *8*, *9*); however, little work has been done to quantify the extent and specific factors that control this recovery. In the present study we begin to fill this gap in our knowledge by determining to what extent conditions found within estuarine waters are sufficient to

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reverse the effect of sublethal chlorine exposure on *E. coli* culturability. Specifically, we address the following questions: (1) Does significant recovery of sublethal chlorine-exposed *E. coli* cells occur in laboratory microcosms containing estuarine water? (2) If significant recovery is observed, what are the important water-quality parameters controlling this recovery? Our findings demonstrate the potential impact that chlorinated wastewater effluent may have on the quality of our estuarine and coastal waters.

## Materials and Methods

**Cell Preparation.** Pure cultures of *E. coli* were used in all chlorination and recovery investigations described below. The strain used was isolated from the Durham, NH, WWTF. A single colony was grown to stationary phase in liquid broth, and aliquots of the resulting bacterial suspension were aseptically placed into 1 mL of saline phosphate buffer/cryoprotectant mixture and cryogenically frozen (−80 °C) until needed. Upon removal from cryogenic storage, the *E. coli* cells were grown in trypticase soy broth (TSB) and incubated at 35 °C for 18−24 h. To prevent nutrient carry over into the chlorination process, the cells were washed and centrifuged three times in $KH_2PO_4$ buffer at 3700 rpm for 10 min. (Preliminary experiments indicated that excess TSB interfered with the chlorination step) The washed cells were placed into filter-sterilized (0.2 $\mu$m pore size) autoclaved-buffered water (FSBW) to create a cell suspension of approximately $10^8$ cells $mL^{-1}$. The *E. coli*/FSBW solution was allowed to incubate at 20 °C for 18−24 h in the dark on a rotary shaker to ensure all exogenous and endogenous nutrients were utilized prior to the experiments.

**Chlorine Exposure.** To induce a sublethal chlorine-injured state in *E. coli*, chlorination procedures similar to those described elsewhere were followed (*3*, *9*, *19*). Briefly, preparation of a 500 mg $L^{-1}$ stock chlorine solution was performed on the day of each experiment from commercially available 6% sodium hypochlorite (Clorox). The chlorine stock solution was then volumetrically added to a cell suspension of $\sim 10^8$ cells $mL^{-1}$ to create a chlorine concentration of 0.5 mg $L^{-1}$. (The chlorine concentration was chosen to represent a worst-case scenario in which culturable counts were significantly reduced without significant reductions in numbers of viable cells.) Chlorine exposure was terminated after 5 min by dechlorinating with the addition of 1 mL of 1 N sodium thiosulfate (*20*).

**Bacterial Enumeration.** The enumeration of culturable *E. coli* was accomplished by using a modified drop plate method on mEndo LES agar obtained from Difco Laboratories (Detroit, MI) that included the addition of 10 or 20 $\mu$L of the sample to the agar surface (*3*, *21*). Growth medium was prepared according to manufacturer instructions and poured in 100-mm × 15-mm plastic Petri dishes. Samples were added to the mEndo LES agar surface in quadruplicate. Inoculated plates were incubated at 35 °C for 18−24 h in the dark. Green colonies with a metallic sheen on mEndo LES agar were enumerated as *E. coli*.

Total and viable cell concentrations were determined using the LIVE/DEAD *Bac*Light bacterial viability kit number L-7012 obtained from Molecular Probes, Inc. (Eugene, OR) as described elsewhere (*1*, *22*). This kit is composed of a mixture of two nucleic-acid-binding stains referred to as SYTO 9 and propidium iodide. These stains differ both in their spectral characteristics and in their ability to penetrate viable bacterial cells (*1*). SYTO 9 stains all cells green, whereas propidium iodide penetrates only those cells whose membranes have been damaged. This microscopic enumeration method, therefore, is based on the premise that membrane integrity equates with cellular viability. Only those cells with compromised cell membranes are stained red, and viable cells are stained green. Total cell counts were calculated as the sum of both red and green cells, while viable cell counts were calculated as the sum of all green fluorescing cells.

A 1:1 SYTO 9 and propidium iodide stock solution was prepared and stored at −20 °C in the dark and thawed at room temperature when needed. Two milliliter samples were removed from each experimental microcosm, treated with 6 $\mu$L of the *Bac*Light stock solution, and incubated in the dark at room temperature for 15−20 min. After incubation 1 mL of the subsamples was filtered through 0.2-$\mu$m Nucleopore Track-Etch Polycarbonate Membrane filters (Whatman) in duplicate and mounted on 25 × 75 × 1-mm microscope slides with *Bac*light mounting oil as described by the manufacturer. Completed slides were stored at 4 °C in the dark for no more than 5 days and viewed at 1000× magnification under a fluorescent microscope. A minimum of 20 fields were counted for each mounted filter. For all direct counts a 470−490-nm excitation filter and a 520-nm barrier filter were used. The concentration of bacteria (cells $mL^{-1}$) was calculated as described by Boulos et al. (*1*).

**Estuarine Microcosm Experiments.** To obtain a wide range of nutrient concentrations and salinities, estuarine water for use in the microcosms was collected at low tide during the spring of 2003 from nine locations in the Seacoast region of New Hampshire (Figure 1). A 5-L amount of estuarine water was collected in sterile HDPE bottles from each location. The estuarine water samples were filter sterilized using 47-mm 0.2-$\mu$m pore size filters. Water samples were stored in sterilized 2-L flasks at 4 °C in the dark until used in the microcosm experiments. (Maximum storage time was 5 days.) Upon inoculation with the chlorine-treated *E. coli*, replicate estuarine microcosms (EMs) were placed on an orbital shaker and incubated at 20 °C for 74 h in the dark. Samples were collected two times the first day of the experiment (0 and 10 h) and once a day for the remaining 4 days of the experiment (24, 48, and 74 h). Total, viable, and culturable cell concentrations were enumerated for each sample as previously described. Control microcosms consisted of inoculating FSBW with chlorine-treated *E. coli*.

Concentrations of ammonium ($NH_4^+$−N), nitrate ($NO_3^-$−N), total dissolved nitrogen (TDN), phosphorus ($PO_4^{-3}$), and dissolved organic carbon (DOC) were determined for each microcosm prior to inoculation. Samples were analyzed in duplicate for $NH_4^+$−N, $NO_3^-$−N, and $PO_4^{-3}$ using a Lachat "QuikChem" method by the Water Quality Analysis Lab at the University of New Hampshire. Specifically, $NH_4^+$−N was analyzed with the phenol hypochlorite method and sodium nitroprusside enhancement (Lachat QuickChem Method 10-107-06-1F), and $NO_3^-$−N was analyzed by cadmium reduction (Lachat QuickChem Method 10-107-04-1B). Phosphorus concentrations were determined using automated ascorbic acid reduction (Lachat QuickChem Method 10-115-01-1B). DOC and TDN were quantified with a Shimadzu TOC 5000 (platinum-catalyzed high-temperature combustion) and an ANTEK Nitrogen detector (*23*, *24*). DOC was determined by calculating the difference between total dissolved carbon and dissolved inorganic carbon.

**Data Analysis.** In viability studies the increase in number of culturable cells following exposure to environmental or chemical stress is typically attributed to bacterial regrowth (i.e., the growth of uninjured cells) and/or resuscitation (i.e., the reacquisition of culturability by injured cells following removal from stress) (*8*). In this study we define recovery as any increase in the number of culturable cells, whether from regrowth or resuscitation. Recovery is calculated as

$$R_i = \log\left(\frac{PC_i}{PC_0}\right) \quad (1)$$

where $R_i$ is recovery, $PC_i$ is the cell concentration according to plate counts, $PC_0$ is the cell concentration at the beginning

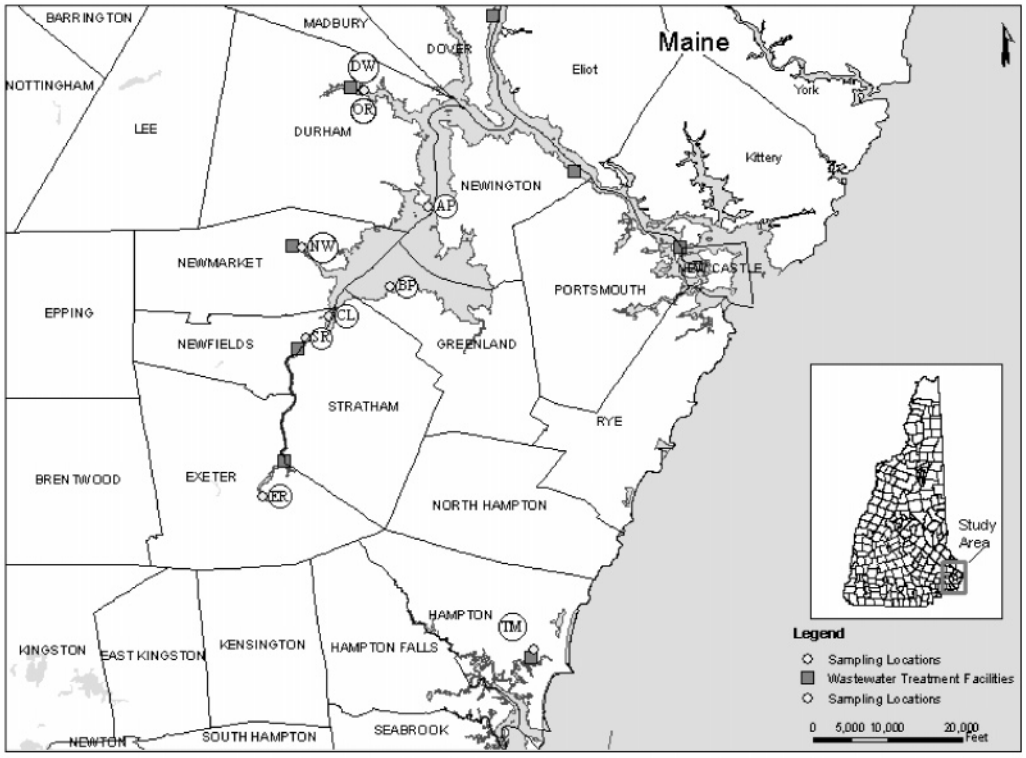


**FIGURE 1. Map of the Seacoast region of New Hampshire showing locations where estuarine water was collected for use in microcosms.**

of the experiment, and $i$ is time in hours. The recovery value after 74 h ($R_{74}$) was used in all statistical comparisons of the EMs to identify overall recovery of *E. coli* under different treatments.

**Statistical Analysis.** Single-factor analysis of variance (ANOVA) was used to determine if statistically significant increases in number of culturable cells (i.e., recovery) occurred over the 74-h incubation time. Single-factor ANOVAs were also used to compare $R_{74}$ values for each microcosm. Where necessary, Tukey's pairwise comparisons were made to determine which microcosms were significantly different from one another. Single-factor ANOVAs were performed using Systat 10.0.

Regression and correlation analyses were used to determine whether water-quality parameters such as dissolved organic carbon, salinity, or nutrient concentrations were significantly correlated with $R_{74}$. To account for the effects of multiple parameters on $R_{74}$, multiple regression analyses were performed. Multiple regression analyses were manually performed for all possible parameter combinations. The best regression model for each number of parameters used (i.e., two-, three-, four-, or five-parameter model) was determined from analysis of the following statistical measures: $R^2$, adjusted $R^2$ (accounts for number of observations and regression parameters), $F$-statistic, root-mean-square error, and $p$ value. Regressions were performed using MATLAB.

## Results

Exposure to 0.5 mg $L^{-1}$ of chlorine for 5 min resulted in significant reductions in *E. coli* culturability when compared to total and viable counts; initial concentrations within the estuarine microcosms of total and viable counts were approximately $10^7$ cells $mL^{-1}$, whereas the numbers of culturable cells were only in the range of $10^3$ and $10^4$ CFU $mL^{-1}$ (Figure 2). The significant reduction in the number of culturable cells in conjunction with the maintenance of membrane integrity indicated that very little bacterial death occurred during the chlorination treatment and that the chlorine concentrations and contact times used in our experiments resulted in sublethal injury of the *E. coli*. This represents a worst-case scenario in which culturable counts are significantly reduced without a concomitant reduction in cell viability as determined by cell membrane integrity.

The recovery of chlorine-exposed *E. coli* was determined by incubation in microcosms containing estuarine water (EMs) from nine sites (Figure 2). Single-factor analysis of variance performed on plate count data indicated that significant ($p < 0.05$) increases in culturable cell concentrations occurred over the 74-h incubation time in all EMs, including the control (Table 1). Single-factor ANOVAs, however, indicated that $R_{74}$ values of the EMs and the control were statistically different ($p < 0.05$) (Figure 3). Total and viable counts remained relatively constant over the 74-h incubation with the exception of slight increases in total counts for the EREM and viable counts in the CLEM sample (Table 1).

Bacterial recovery over a 74-h time period ($R_{74}$) was observed at varying degrees ranging from a factor of 2.8 to a factor of 50 (Figure 3, Table 1). DWEM exhibited the most extensive recovery, and APEM exhibited the least extensive recovery. With the exception of CLEM and APEM, increases in culturable cells were observed for all microcosms within the first 10 h of the experiment. No significant increases in culturable cells were observed after 24 h for APEM and DWEM and after 48 h for SREM and NWEM. No increases in CFUs were observed in any of the EMs following 74 h of incubation (*25*).

There was substantial variation in measured water-quality parameters between the microcosms with the exception of pH, which remained relatively neutral for all EMs (Table 2). Salinity ranged from 0 to 10.5 ppt. DOC and nitrate varied by nearly an order of magnitude ranging from 4.40 to 40.1 mg $L^{-1}$ and 0.03 to 0.29 mg $L^{-1}$, respectively. Ammonium concentrations varied by nearly 2.5 orders of magnitude, ranging from 0.041 to 19 mg $L^{-1}$. The EM with the highest concentrations of TDN, $NH_4^+$−N, and DOC was DWEM, demonstrating the direct influence of the Durham WWTF on the DWEM sample location. Nitrate was highest in OREM,

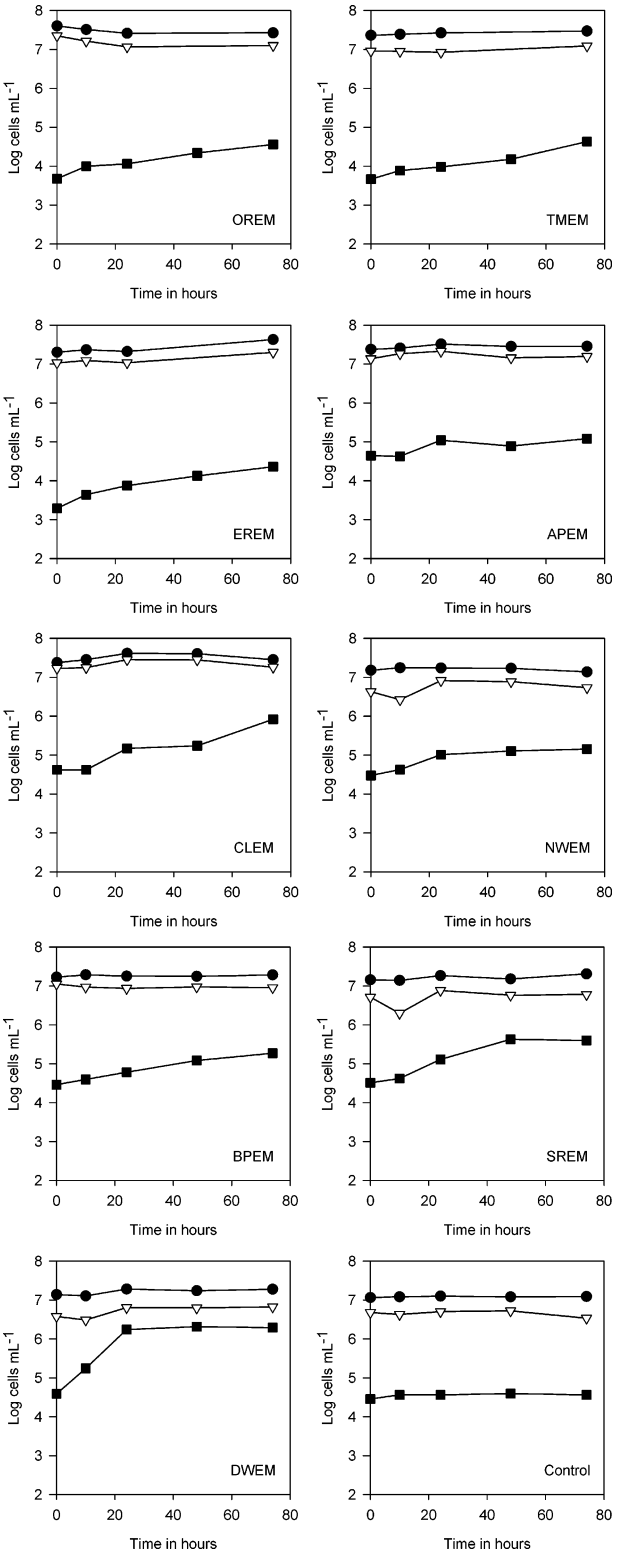


**FIGURE 2. Total (●), viable (△), and culturable (■) *E. coli* concentrations over the 74-h incubation period in nine replicate estuarine microcosms plus control.**

which is approximately 360 m downstream from DWEM. Nitrate concentrations could not be determined for TMEM, CLEM, or APEM due to sample matrix interference caused by elevated salinity; thus, $NO_3^-$−N data were not included in the statistical analyses. Phosphate concentrations were most elevated at TMEM and lowest at SREM, which were in close proximity to a WWTF and a WWTF/agricultural site, respectively.

**TABLE 1. Results of Single-Factor ANOVA Performed for Each Estuarine Microcosm[a]**

| | $R_{74}$ | | |
|---|---|---|---|
| estuarine microcosm identification | total direct count (*Bac*Light) | viable direct count (*Bac*Light) | plate count (mEndo LES agar) |
| OREM | 0.11 (0.69) | 0.13 (0.72) | **0.96 (5.7E-5)** |
| TMEM | −0.18 (0.37) | −0.25 (0.28) | **0.88 (8.3E-5)** |
| EREM | **0.32 (5.9E-3)** | 0.27 (0.090) | **1.07 (3.5E-4)** |
| CLEM | 0.07 (0.10) | **0.03 (3.0E-2)** | **1.30 (3.0E-7)** |
| APEM | 0.08 (0.66) | 0.06 (0.23) | **0.44 (5.0E-4)** |
| NWEM | −0.04 (0.82) | 0.10 (0.32) | **0.68 (5.5E-4)** |
| BPEM | 0.06 (0.79) | −0.09 (0.81) | **0.81 (1.0E-3)** |
| SREM | 0.15 (0.27) | 0.07 (0.14) | **1.09 (9.6E-3)** |
| DWEM | 0.14 (0.19) | 0.25 (0.19) | **1.70 (4.2E-8)** |
| control | 0.02 (0.59) | −0.14 (0.32) | **0.11 (5.8E-4)** |

[a] Bold values represent significant differences ($p < 0.05$) in cell concentrations following the 74-h incubation ($R_{74}$). *P*-values are in parentheses.

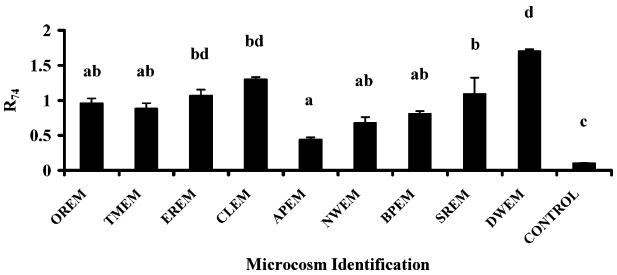


**FIGURE 3. Recovery of chlorine-treated *E. coli* in nine replicate estuarine microcosms plus the control after 74 h of incubation. Error bars represent the standard error of the mean. Similar letters denote statistically similar $R_{74}$ values.**

On the basis of simple linear regression analysis, the parameters most able to describe the observed variation in $R_{74}$ were DOC ($R^2 = 0.762$) > $NH_4^+$−N ($R^2 = 0.702$) > TDN ($R^2 = 0.621$) > salinity ($R^2 = 0.366$) > $PO_4^{-3}$ ($R^2 = 0.0826$) where only TDN, $NH_4^+$, and DOC were statistically significant. Multiple regression analysis on all combinations of parameters indicated that the two-parameter model which included $NH_4^+$ and salinity had the highest adjusted $R^2$, lowest root-mean-square error, highest *F*-statistic, and lowest *p*-value of all the models tested (Table 3). At first glance this seems counterintuitive given that DOC was the most strongly correlated to $R_{74}$ in the single-parameter model; however, close inspection of the correlation matrix (Table 4) shows that $NH_4^+$ was strongly correlated with both TDN and DOC; therefore, it is not possible to determine with any degree of certainty the unique effect DOC or $NH_4^+$ has on $R_{74}$.

## Discussion

Our results show that following exposure to chlorine, concentrations of culturable *E. coli* can increase significantly over a 3-day period if incubated in microcosms containing estuarine water. Chlorine-treated *E. coli* cells were exposed to a broad range of nutrient concentrations which led to a wide range of bacterial recovery. In general, recovery increased as nutrients and DOC increased and salinity decreased, but even at low nutrient and carbon concentrations *E. coli* recovery was observed. The role of nutrients in the resuscitation of VBNC bacteria has been observed in several studies. Rockabrand et al. (*8*) found that after the chlorination of wastewater effluent a 1000-fold decrease in coliform concentration took place. However, with supplementation of a nutrient (0.1% tryptone) to the chlorinated wastewater/cell suspension pre-chlorination coliform con-

**TABLE 2. Water-Quality Parameters and $R_{74}$ Values Associated with Replicate Estuarine Microcosms[a]**

| estuarine microcosm identification | $R_{74}$ | environmental parameter | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | pH | salinity (ppt) | TDN (mg $L^{-1}$) | $NO_3^-$—N (mg $L^{-1}$) | $NH_4^+$—N (mg $L^{-1}$) | DOC (mg $L^{-1}$) | $PO_4^{-3}$ (mg $L^{-1}$) |
| OREM | 0.96 | 7.4 | 0.7 | 0.87 | 0.29 | 0.12 | 7.01 | 0.028 |
| TMEM | 0.88 | 6.2 | 5.5 | 2.22 | ND | 0.16 | 8.38 | 0.203 |
| EREM | 1.07 | 7.3 | 0.0 | 0.45 | 0.19 | 0.045 | 7.53 | 0.007 |
| CLEM | 1.30 | ND | 0.2 | 1.04 | ND | 0.40 | 7.91 | 0.052 |
| APEM | 0.44 | ND | 10.5 | 0.33 | ND | 0.067 | 4.40 | 0.027 |
| NWEM | 0.68 | 7.5 | 0.1 | 0.36 | 0.13 | 0.041 | 6.16 | 0.004 |
| BPEM | 0.81 | 7.4 | 5.9 | 0.27 | 0.03 | 0.062 | 4.97 | 0.005 |
| SREM | 1.09 | 7.6 | 0.7 | 0.49 | 0.14 | 0.14 | 7.75 | 0.001 |
| DWEM | 1.70 | 7.8 | 1.3 | 30.15 | 0.16 | 19.0 | 40.1 | 0.088 |
| control | 0.11 | 6.9 | 0.0 | ND | ND | ND | ND | ND |

[a] Nutrient concentrations represent the average of two replicate samples. ND: not determined.

**TABLE 3. Best-Fit Models for Each Model Type (i.e., number of included parameters) Based on Multiple Regression Analyses for All Possible Combinations of Parameters**

| no. of params | params | $R^2$ | adjusted $R^2$ | RMSE | *F*-statistic | *P*-value |
|---|---|---|---|---|---|---|
| 1 | DOC | 0.762 | 0.728 | 0.190 | 22.41 | 0.0021 |
| 2 | $NH_4$, salinity | 0.904 | 0.871 | 0.131 | 28.11 | 0.000896 |
| 3 | TDN, $NH_4$, salinity | 0.905 | 0.849 | 0.142 | 15.96 | 0.00541 |
| 4 | TDN, $NH_4$, $PO_4$, salinity | 0.908 | 0.815 | 0.157 | 9.83 | 0.0240 |
| 5 | TDN, $NH_4$, DOC, $PO_4$, salinity | 0.921 | 0.789 | 0.168 | 6.99 | 0.0702 |

**TABLE 4. Correlation Matrix for $R_{74}$ and Water-Quality Parameters[a]**

| | $R_{74}$ | log (DOC) | log ($NH_4^+$—N) | log (TDN) | salinity |
|---|---|---|---|---|---|
| log (DOC) | **0.873**[c] | | | | |
| log ($NH_4^+$—N) | **0.838**[c] | **0.940**[d] | | | |
| log (TDN) | **0.788**[b] | **0.953**[d] | **0.947**[d] | | |
| salinity | −0.605 | −0.377 | −0.195 | −0.205 | |
| log ($PO_4^{-3}$) | 0.287 | 0.423 | 0.523 | 0.660 | 0.236 |

[a] Correlations considered significant are in bold. [b] Significant at $\alpha = 0.05$ level. [c] Significant at $\alpha = 0.01$ level. [d] Significant at $\alpha = 0.001$ level.

centrations were observed within 9 h. Walsh and Bissonnette (*9*) observed that following chlorination enterotoxigenic *E. coli* were able to regain culturability on m-FC agar because of nutrients that accumulated from residual growth media during the chlorination experiment. In contrast to our study, however, no attempts were made in either of these studies to associate specific water-quality parameters with bacterial recovery.

Simple and multiple regression analyses indicated that DOC and/or $NH_4^+$ was the strongest determinant in bacterial recovery in our microcosms. Because DOC, TDN, and $NH_4^+$ were so strongly correlated, however, it was impossible to determine whether carbon, nitrogen, or a combination of the two was primarily responsible for the observed increases in $R_{74}$. The presence of both nitrogen and carbon sources has been shown to increase bacterial survival. LeChevallier et al. (*26*) reported that organic carbon can favor extended coliform survival within drinking water systems. Boualam et al. (*27*) showed that DOC concentrations as low as 2.5 mg $L^{-1}$ were enough to maintain the culturable concentrations of five coliform species in eutrophied water for 96 h, while lower DOC concentrations were associated with a loss of bacterial culturability. Craig et al. (*28*) found that high organic content enhances *E. coli* survival in sediments. Henis et al. (*29*) observed a 1000-fold increase in culturable cells in 48 h with the addition of amino acids and phosphate to lake water microcosms. Given the low C:N ratios in heterotrophic bacteria (*30*) and findings that additions of glucose to estuarine and seawaters can increase $NH_4^+$ uptake (*31*, *32*), it is likely that carbon and nitrogen are both required in sufficient quantities to initiate bacterial recovery.

Although not statistically significant by itself, multiple regression analyses demonstrated the importance of the inverse correlation between salinity and $R_{74}$ observed in our EMs. Salinity at relatively high concentrations has been shown to be a major influence on the survival (*33*−*35*) and culturability (*36*) of *E. coli* and coliform bacteria in general. Šolić and Krstulović (*35*) showed that at a salinity of 10 ppt fecal coliforms in the absence of light out-survived fecal coliforms exposed to 35 ppt under similar conditions. Although decreases in survival were significant over a 24-h period, fecal coliform survival at 10 ppt was much greater than that at 35 ppt.

Most field and laboratory studies have shown that die off, not recovery, is common for *E. coli* when placed into unamended aquatic environments (*36*−*42*). In fact, the presence of *E. coli* in an aquatic environment is often assumed to be a sign of recent fecal contamination due to the expectation that *E. coli* do not survive well, let alone increase in numbers, outside their host (*15*). The relatively large increases in culturable cells observed in our unamended estuarine microcosms, therefore, suggest that resuscitation, rather than regrowth, may have occurred in our study. However, given that culturable *E. coli* concentrations were at least 2 orders of magnitude below the total direct counts it was impossible to detect an increase in total cell concentrations with increases in culturable cell concentrations. Therefore, we cannot state with any certainty whether the observed increases in culturable cells were due to resuscitation, regrowth, or a combination of the two. Regardless of the mechanism, the increase of culturable bacteria within estuarine water may lead to a public health risk through ingestion of contaminated surface water or the consumption of contaminated shellfish.

Estuarine microcosms composed of source water with high concentrations of nutrients and organic carbon, such as those associated with WWTF effluent (DWEM and TMEM) and agricultural runoff (SREM and CLEM), all exhibited $R_{74}$ values close to, or in excess of, 1.0. That is, chlorine-exposed *E. coli* inoculated in these EMs responded with at least an

order of magnitude increase in culturable cells over a 74-h time period. These data support the hypothesis of Rockabrand et al. (*8*) that discharge of chlorinated cells to waters in which elevated nutrients are present may result in significant increases in the number of culturable cells. On the basis of the data presented in the current study and findings by Rockabrand et al. (*8*), it is necessary to consider both the bacterial populations associated with WWTF effluent as well as the nutrient conditions into which effluent is discharged. Because advanced treatment designed to remove nutrients from WWTF effluent is utilized infrequently, effluent is likely a source of nutrients capable of stimulating a recovery response (*16*−*18*). It is not surprising, therefore, that *E. coli* recovery was greatest in DWEM, where DOC and nutrient concentrations were extremely high due to the fact that this water sample was collected immediately downstream of a WWTF. In fact, nutrient concentrations for DWEM were nearly identical to concentrations previously observed in the effluent of this WWTF (*43*), indicating that WWTF effluent may be capable of stimulating the recovery and survival of bacteria in nearby aquatic environments, especially where dilution is minimal.

We recognize that the chlorine concentrations and contact times used in this study are less than what is typically reported by WWTFs, however, the levels and contact times are well within the range reported in studies looking at chlorine-induced bacterial injury (*3*, *5*, *6*, *44*, *45*). Given our results from an earlier study suggesting that discharge of chlorine-injured bacteria by WWTFs is a common occurrence in the Seacoast region of New Hampshire (*43*), we wanted to establish conditions representative of a worst-case scenario, i.e., conditions in which culturability is greatly reduced with minimal reductions in cell viability. Furthermore, detection limitations required that we inoculate our microcosms with concentrations of culturable cells in excess of those found in disinfected sewage. Given that recovery was defined in relative terms, we do not see this as being a major limitation to our findings. The recovery of even a small population of bacteria in estuarine water could cause unacceptable levels of microbial contaminants in aquatic systems.

While the environmental parameters associated with the estuarine water samples were adequate to elicit the recovery of *E. coli* following removal from chlorine stress, it is unclear how additional environmental stresses would affect a cell's ability to survive, regrow, and/or reacquire culturability. For example, all experiments were conducted using pure cultures of *E. coli*, yet estuarine water contains a diversity of microorganisms. The presence of these microorganisms can result in predation and/or the inability of *E. coli* to effectively compete for nutrients and carbon sources (*16*, *46*−*48*), thus severely limiting the potential for *E. coli* to recover. It is also possible that other factors controlled in this study, such as temperature (*35*, *49*, *50*) and solar radiation (*51*), could reduce or eliminate bacterial recovery all together, indicating the need for further research to evaluate the true potential for bacterial recovery in estuarine waters. These limitations notwithstanding, the extensive recovery observed in our study indicates that chlorine-treated bacteria may pose a greater threat to water quality and public health than previously thought and that the regular monitoring of wastewater effluent may underestimate the true impact on estuarine water quality.

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## Literature Cited

(1) Boulos, L.; Prevost, M.; Barbeau, B.; Coallier, J.; Desjardins, R. Live/Dead *Bac*Light: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *J. Microbiol. Methods* **1999**, *37*, 77−86.

(2) DuPreeze, M.; Kfir, R.; Coubrough, P. Investigation of injury of coliforms after chlorination. *Water Sci. Technol.* **1995**, *31*, 115−118.

(3) Lisle, J.; Pyle, B.; McFeters, G. A. The use of multiple indices of physiological activity to access viability in chlorine disinfected *Escherichia coli* O157:H7. *Lett. Appl. Microbiol.* **1999**, *29*, 42−47.

(4) Singh, A.; Yeager, R.; McFeters, G. A. Assessment of in vivo revival, growth and pathogenicity of *Eschericia coli* strains after copper- and chlorine-induced injury. *Appl. Environ. Microbiol.* **1986**, *52*, 832−837.

(5) Tryland, I.; Pommepuy, M.; Fiksdal, L. Effect of chlorination on $\beta$-D-galactosidase activity of sewage bacteria and *Escherichia coli*. *J. Appl. Microbiol.* **1998**, *85*, 51−60.

(6) Camper, A. K.; McFeters, G. A. Chlorine injury and the enumeration of waterborne coliform bacteria. *Appl. Environ. Microbiol.* **1979**, *37*, 633−641.

(7) LeChevallier, M. W.; Cameron, S. C.; McFeters, G. A. New medium for improved recovery of coliform bacteria from drinking water. *Appl. Environ. Microbiol.* **1983**, *45*, 484−492.

(8) Rockabrand, D.; Austin, T.; Kaiser, R.; Blum, P. Bacterial growth state distinguished by single-cell protein profiling: does chlorine kill coliforms in municipal effluent? *Appl. Environ. Microbiol.* **1999**, *65*, 4181−4188.

(9) Walsh, S.; Bissonnette, G. Survival of chlorine-injured enterotoxigenic *Escherichia coli* in an in vitro water system. *Appl. Environ. Microbiol.* **1989**, *55*, 1298−1300.

(10) Terzieva, S.; McFeters, G. A. Survival and injury of *Escherichia coli*, *Campylobacter jejuni*, and *Yersinia enterocolitica* in stream water. *Can. J. Microbiol.* **1991**, *37*, 785−790.

(11) Troussellier, M.; Bonnefont, J.; Courties, C.; Derrien, A.; Dupray, E.; Gauthier, M.; Gourmelon, M.; Joux, F.; Lebaron, P.; Martin, Y.; Pommepuy, M. Responses of enteric bacteria to environmental stresses in seawater. *Oceanologica Acta* **1998**, *21*, 965−981.

(12) Bogosian, G.; Bourneuf, E. A matter of bacterial life and death. *EMBO Rep.* **2001**, *21*, 770−774.

(13) Bogosian, G.; Morris, P.; O'Neil, J. A mixed culture recovery method indicates that enteric bacteria do not enter the viable but nonculturable state. *Appl. Environ. Microbiol.* **1998**, *64*, 1736−1742.

(14) Rozen, Y.; Belkin, S. Survival of enteric bacteria in seawater. *FEMS Microbiol. Rev.* **2001**, *25*, 513−529.

(15) Winfield, M. D.; Groisman, E. A. Role of host environments in the lifestyles of *Salmonella* and *Escherichia coli*. *Appl. Environ. Microbiol.* **2003**, *69*, 3687−3694.

(16) Lim, C.-H.; Flint, K. P. The effects of nutrients on the survival of *Escherichia coli* in lake water. *J. Appl. Bacteriol.* **1989**, *66*, 559−569.

(17) Watkins, W.; Cabelli, V. Effect of fecal pollution on *Vibrio parahaemolyticus* densities in an estuarine environment. *Appl. Environ. Microbiol.* **1985**, *49*, 1307−1313.

(18) Lopez-Torres, A.; Prieto, L.; Hazen, T. Comparison of the in situ survival and activity of *Klebsiella pneumoniae* and *Escherichia coli* in tropical marine environment. *Microbial Ecol.* **1988**, *15*, 41−57.

(19) Lisle, J.; Broadway, S.; Prescott, A.; Pyle, B.; Fricker, C.; McFeters, G. Effects of starvation on physiological activity and chlorine disinfection resistance in *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* **1998**, *64*, 4658−4662.

(20) APHA. *Standard methods for the examination of water and wastewater*, 19th ed.; American Public Health Association: 1995.

(21) Hoben, H.; Somasegaran, P. Comparison of the our, spread and drop plate methods for the enumeration of *Rhizobium* spp. in inoculants made from presterilized peat. *Appl. Environ. Microbiol.* **1982**, *44*, 1246−1247.

(22) Grey, B.; Steck, T. Concentrations of copper thought to be toxic to *Escherichia coli* can induce the viable but nonculturable condition. *Appl. Environ. Microbiol.* **2001**, *67*, 5325−5327.

(23) Merriam, J.; McDowell, W. H.; Currie, W. S. A high-temperature catalytic oxidation technique for determining total dissolved nitrogen. *Soil Sci. Soc. Am. J.* **1996**, *60*, 1050−1055.

(24) Sugimura, Y.; Suzuki, Y. A high-temperature catalytic oxidation method for the determination of non-volatile dissolved organic carbon in seawater by direct injection of liquid sample. *Mar. Chem.* **1988**, *24*, 105−131.
(25) Bromley, J. M. Evidence for the resuscitation of chlorine-induced viable but nonculturable *Escherichia coli* in estuarine environments. M.S. Thesis, University of New Hampshire: Durham, NH, 2003.
(26) LeChevallier, M. W.; Schulz, W.; Ramon, L. Bacterial nutrients in drinking water. *Appl. Environ. Microbiol.* **1991**, *57*, 857−862.
(27) Boualam, M.; Mathieu, L.; Fass, S.; Cavard, J.; Gatel, D. Relationship between coliform culturability and organic matter in low nutritive waters. *Water Res.* **2001**, *35*, 1−9.
(28) Craig, D. L.; Fallowfield, H. J.; Cromar, N. J. Use of microcosms to determine persistence of *Escherichia coli* in recreational coastal water and sediment and validation with *in situ* measurements. *J. Appl. Microbiol.* **2004**, *96*, 922−930.
(29) Henis, Y.; Gurijala, K. R.; Alexander, M. Factors involved in multiplication and survival of *Escherichia coli* in lake water. *Microbial Ecol.* **1989**, *17*, 171−180.
(30) Goldman, J. C.; Dennett, M. R. Growth of marine bacteria in batch and continuous culture under carbon and nitrogen limitation. *Limnol. Oceanogr.* **2000**, *45*, 789−800.
(31) Kirchman, D. L.; Keil, R. G.; Wheeler, P. W. Carbon limitation of ammonium uptake by heterotrophic bacteria in the subarctic Pacific. *Limnol. Oceanogr.* **1990**, *35*, 1258−1266.
(32) Sherr, B. F.; Sherr, E. B.; Andrew, T. L.; Fallon, R. D.; Newell, S. Y. Trophic interactions between heterotrophic protozoa and bacterioplankton in estuarine water analyzed with selective metabolic inhibitors. *Mar. Ecol. Prog. Ser.* **1986**, *32*, 169−179.
(33) Gauthier, M.; Munro, P.; Mohajer, S. Influence of salts and sodium chloride on the recovery of *Escherichia coli* from seawater. *Curr. Microbiol.* **1987**, *15*, 5−10.
(34) Munro, P.; Gauthier, M.; Breittmayer, M.; Bongiovanni, J. Influence of osmoregulation processes on starvation survival of *Escherichia coli* in seawater. *Appl. Environ. Microbiol.* **1989**, *55*, 2017−2024.
(35) Solic, M.; Krstulovic, N. Separate and combined effect of solar radiation, temperature, salinity and pH on the survival of faecal coliforms in seawater. *Mar. Poll. Bull.* **1992**, *24*, 411−416.
(36) Xu, H.; Roberts, N.; Singleton, F.; Atwell, R.; Grimes, D. J.; Colwell, R. R. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in estuarine and marine environments. *Microbial Ecol.* **1982**, *8*, 313−323.
(37) Bordalo, A. A.; Onrassami, R.; Dechsakulwatana, C. Survival of faecal indicator bacteria in tropical estuarine waters (Bangpakong River, Thailand). *J. Appl. Microbiol.* **2002**, *93*, 864−871.
(38) Hood, M. A.; Ness, G. E. Survival of *Vibrio cholerae* and *Escherichia coli* in estuarine waters and sediments. *Appl. Environ. Microbiol.* **1982**, *43*, 578−584.
(39) Klein, T. M.; Alexander, M. Bacterial inhibitors in lake water. *Appl. Environ. Microbiol.* **1986**, *52*, 114−118.
(40) McFeters, G. A.; Stuart, D. G. Survival of coliform bacteria in natural waters: field and laboratory studies with membrane-filter chambers. *Appl. Microbiol.* **1972**, *24*, 805−811.
(41) Nasser, A. M.; Zaruk, N.; Tenenbaum, L.; Netzan, Y. Comparative survival of *Cryptosporidium*, coxsackievirus A9 and *Escherichia coli* in stream, brackish and sea waters. *Water Sci. Technol.* **2003**, *47*, 91−96.
(42) Wang, G.; Doyle, M. P. Survival of enterohemorrhagic *Escherichia coli* O157:H7 in water. *J. Food Protection* **1998**, *61*, 662−667.
(43) Bolster, C. H.; Jones, S. H.; Bromley, J. M. *Evaluation of effects of wastewater treatment discharge on estuarine water quality*; New Hampshire Department of Environmental Services: 2004.
(44) Calabrese, J. P.; Bissonnette, G. K. Improved membrane filtration method incorporating catalese and sodium pyruvate for detection of chlorine-stressed coliform bacteria. *Appl. Environ. Microbiol.* **1990**, *56*, 3558−3564.
(45) Singh, A.; Yu, F.-P.; McFeters, G. A. Rapid detection of chlorine-induced bacterial injury by the direct viable count method using image analysis. *Appl. Environ. Microbiol.* **1990**, *56*, 389−394.
(46) Gonzalez, J.; Iriberri, J.; Egea, L.; Barcina, I. Characterization of culturability, protist grazing, and death of enteric bacteria in aquatic ecosystems. *Appl. Environ. Microbiol.* **1992**, *58*, 998−1004.
(47) Gonzalez, J.; Iriberri, J.; Egea, L.; Barcina, I. Differential rates of digestion of bacteria by freshwater and marine phagotrophic protozoa. *Appl. Environ. Microbiol.* **1990**, *56*, 1851−1857.
(48) Korhonen, L. K.; Martikainen, P. J. Survival of *Escherichia coli* and *Campylobacter jejuni* in untreated and filtered lake water. *J. Appl. Bacteriol.* **1991**, *71*, 379−382.
(49) Faust, H.; Aotaky, A.; Haragadon, M. Effect of physical parameters on the in situ survival of *Eschericia coli* MC-6 in an estuarine environment. *Appl. Environ. Microbiol.* **1975**, *30*, 800−806.
(50) Gauthier, M. Environmental parameters associated with the viable but nonculturable state. In *Nonculturable Microorganisms in the Environment*; Colwell, R. R., Grimes, D. J., Eds.; ASM Press: Washington, D.C., 2000.
(51) Davies, C. M.; Evison, L. Sunlight and the survival of enteric bacteria in natural waters. *J. Appl. Bacteriol.* **1991**, *70*, 265−274.

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