Do aluminium concentrations in drinking water inhibit cultivation of *Escherichia coli*?

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ABSTRACT

In spite of reliance on *Escherichia coli* as an indicator of fecal pollution in water resources, including potable water, relatively little is known of its ecology and persistence. It is particularly important to be able to accurately detect *E. coli* presence and quantity in treated potable water because of potential human health effects from consumption of contaminated water. Presence of even a single *E. coli* in potable water can lead to significant consequences and costs for water utilities. Alum is frequently used as a coagulant in conventionally treated water, and is usually present in slight excess after treatment. We show here that *E. coli* can accumulate the Al^{3+} ions present at natural levels in potable water and in so doing become uncultivable. Thus, *E. coli*, and perhaps a number of other bacteria, present in potable water could readily escape detection on laboratory culture media. Chelation with Tiron of the Al^{3+} prior to exposure maintained the *E. coli* in a cultivable state. This phenomenon deserves further investigation in view of the reliance placed on *E. coli* as an indicator of fecal contamination.

Key words | alum, aluminium chelation, drinking water, *E. coli* cultivation

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INTRODUCTION

Escherichia coli is often used as the indicator of choice for monitoring fecal contamination of treated potable waters. Accurate detection of its presence and numbers during routine monitoring of drinking water is thus important to be able to assure public safety of the water supply. *E. coli* is well recognized as a gut commensal of warm-blooded animals but its survival/persistence in potable as well as in other water environments is not well understood. Survival is usually measured by in vitro cultivability, though some viable cells might not be cultivable (Grey & Steck 2001; Na *et al.* 2006; Liu *et al.* 2009; Sachidanandham & Gin 2009). Loss of cultivability is influenced by many biotic and abiotic factors (Unc & Springthorpe 2008), and varies from hours to months in different environments. In addition to its routine use as an indicator, *E. coli* presence and numbers are of interest for risk assessment and risk modelling.

doi: 10.2166/ws.2010.902

While there are many factors that can lead to *E. coli* decay in natural waters, its maximum persistence in any particular source water can be modelled by its suspension in sterile water with free chemical interchange of low molecular weight substances with the source water in question. We have previously used such an approach to examine microbial survival in situ (Springthorpe *et al.* 1993, 1996) and the survival of *E. coli* K12 spiked in parallel into sterile water from the Ottawa River (RW) and in sterile effluent from the filters (FEW) at a treatment plant in Ottawa that uses the Ottawa River as its sole water source (Springthorpe *et al.* unpublished results). In the latter case, much longer survival was apparent in the RW than in FEW. Since the FEW had not yet been disinfected at the treatment plant, this was a surprising observation. We speculated that the low nutrient content of the FEW following coagulation and settling might have been responsible for loss of cultivability, but when FEW

was enriched with an *E. coli* growth medium (Luria Bertani broth) to increase the carbon and energy sources, similar results were obtained. We also examined *E. coli* biofilms grown on plastic or metals supports in continuous-flow membrane reactors using sterile FEW or RW as the only sources of carbon and energy. After an initial biofilm formation in both RW and FEW, the original cultivable population in FEW decreased while the apparently viable total cell population remained constant.

Therefore, we supposed that one or more substances from the FEW might be responsible for the inhibition of *E. coli* cultivability and lack of continued biofilm formation, and that one of those candidates could be the residual aluminium present after the coagulation and filtration processes of water treatment (Bérubé & Soucy 2004). Aluminium ions are almost universally toxic (Garcidueñas & Cervantes 1996) though not usually dramatically so. Relatively little has been published about interaction of aluminium and bacteria, especially pathogens and indicators. *E. coli* is known to be vulnerable to aluminium at much higher concentrations than those used in this study (Guida *et al.* 1991). However, the key for aluminium toxicity to bacteria lies in its bioavailability. Since the removal of complexing humic acids and the lowered pH achieved by conventional water treatment processes contribute to keeping the residual aluminium remaining after coagulation in the free form (Gardner & Gunn 1995; Bérubé & Brûlé 1999), it could be readily accumulated by *E. coli* or other bacteria that might be present. As far as the authors are aware, there have been no publications relating aluminium exposure of *E. coli* to entering the VBNC state, though another toxic metal, copper, has been implicated in this regard (Grey & Steck 2001).

Our hypothesis was that free aluminium species present in FEW can inhibit growth of or kill the cellular population of *E. coli* K12. To test this hypothesis we examined the effects of natural levels of aluminium present in drinking water on the cultivability of *E. coli* K12 cells in the absence and presence of an aluminium chelator. Among the numerous possible aluminium chelators (Robinson 1993; Yokel 2002), Tiron (4,5-dihydroxy-1,3-benzene-disulfonic acid) was selected because it presents high affinity and efficiency to chelate aluminium at low concentration (Cevette & Orvig 1990).

METHODS

Preparation of stationary phase bacterial cells

One *E. coli* K12 colony from Luria Bertani agar (LBA) was inoculated into 5 mL of Luria Bertani broth (LB) and then incubated overnight on an orbital shaker at 37°C. An aliquot (5 mL) of this culture was diluted into 500 mL of LB and incubated for 48 h with magnetic agitation at room temperature (24 ± 1°C). Three hundred mL of the culture was centrifuged at 6,000 × *g* for 10 min. and the sedimented cells washed twice with the same volume of 0.85% sodium chloride solution (normal saline) and then with the same volume of deionized distilled water pH 5.5 (ddH_2O) before resuspension in 300 mL of ddH_2O. Ten mL aliquots of this washed suspension were placed into each of 24 sterile dialysis tubes; 6 tubes were used in each of the four sterile reactors.

Experimental reactors

Four 2.2 L constant volume semi continuous membrane reactors were continuously fed with fresh 200 mL/h RW (2 reactors) and FEW (2 reactors). The input water at 24 ± 1°C from separate reservoirs, with or without different Tiron concentrations was filtered through on-line 0.2 μm sterile hollow fibre filters. Filter Effluent Water (FEW) is the processed River Water (RW) after the coagulation and filtration and before the chloramine treatment in the Water Treatment Plant. Both types of water were collected fresh every 24 h from the Lemieux Island Water Treatment Plant, Ottawa, ON. The sterile dialysis tubes with 10 mL of stationary phase cellular suspension were inserted in a special holder inside the reactors and incubated for several days or weeks according to the running time of the experiment. At selected times, one tube from each reactor was taken for chemical and microbial analysis.

Analysis

The cellular suspension and any potentially attached biofilm inside the sampled dialysis bags was harvested by massaging the closed dialysis tube using sterile gloves. The end of the dialysis tube was then snipped and

the contents were poured aseptically into a sterile acid-washed 50 mL conical centrifuge tube. An aliquot (1 mL) was used immediately for bacterial analysis of total and viable cell numbers as well as cultivable cells. Viable cells were counted by epifluorescence microscopy using the BacLight staining kit (Molecular Probes, Inc). Total cell numbers were counted using acridine orange staining. For each sample time point and for each of these staining methods two specimens were prepared for microscopy and 5 random fields were photographed from each to do the cell counts. The picture area and in consequence the volume was standardized. The number of cells counted per field was always > 50. Cultivable cell numbers were obtained from serial dilutions plated on trypticase soy agar. All cell counts, (totalcells, viable cells and colony forming units) were performed at least in duplicate with the means shown in the graphical data. The standard error was always equal to or less than 10%. Statistical analysis and data processing used Excel Software (Microsoft Corporation, USA); XLstat, v 7.5.2 (Addinsoft, NY, USA) and Origin Pro v8 (Origin Lab Corporation, MA, USA). The remaining cell suspensions (usually 9 mL) were pelleted at 6,000 × g for 10 minutes and the supernatants were transferred separately to additional acid washed sterile tubes and stored at − 20°C for aluminium analysis. Total aluminium concentrations were determined in acid digested pelleted cells (95°C, 2 h in $HNO_3/H_2O_2/HF$, diluted to 3.5% HNO_3) and cell supernatants (acidified with 0.2% HNO_3) by inductively coupled plasma mass spectrometry–ICPMS (Bérubé 2004).

RESULTS AND DISCUSSION

We report here on experiments using natural levels of aluminium in filtered effluent and the river water source from the Lemieux Island Water Purification Plant in Ottawa, Canada. The first experiment used a stationary phase suspension of *E. coli* K12 incubated in parallel in FEW and RW (2 reactors each) as described. In FEW, the population showed a decrease in cultivability as compared with total cell numbers (Figure 1A) or total and cultivable cells in RW (Figure 1B). While mean cultivable cells were notably different at days 7 (8.2%) and 14 (10.9%), this difference was only statistically different at 28 days ($n = 4$, $P < 0.0001$, $\alpha = 0.05$). This was in spite of the total aluminium concentration being higher in RW (200 μg/L) than in FEW (120 μg/L). However, it was previously determined that in FEW most Al was free as Al^{3+} whereas in RW most Al was already complexed (Gardner & Gunn 1995; Bérubé & Brûlé 1999). Thus, the viability decrease seemed to be related to the increase in the aluminium accumulated in the sedimented cells held in FEW (Figure 1A).

Before working with Tiron to relieve inhibition of the cultures, it was important to determine that Tiron itself

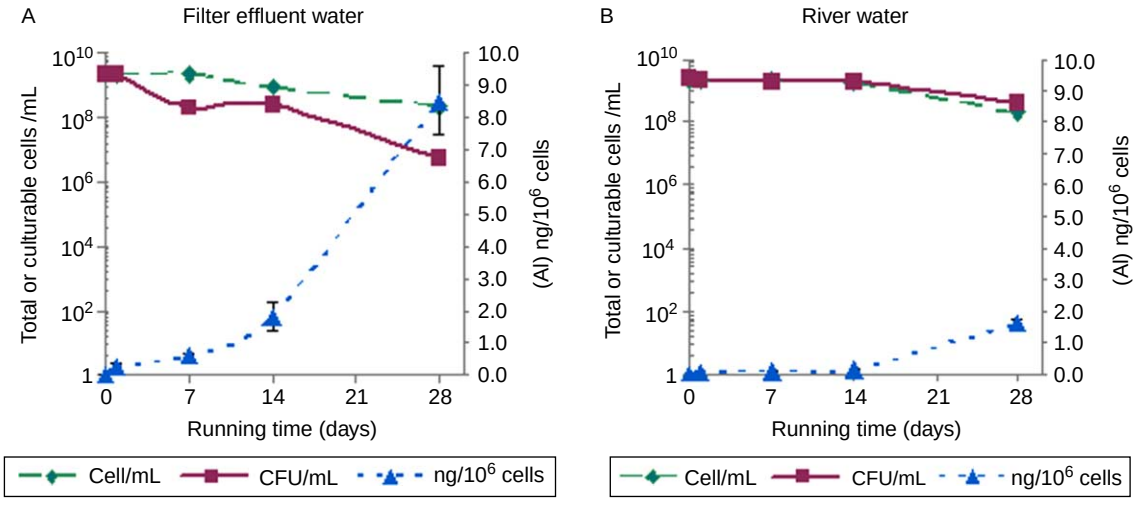

Figure 1 | Total cells per mL, cultivable cells (CFU) and accumulated Al (ng per million cells) for *E. coli* K12 incubated in FEW (panel A) and Ottawa River (panel B).

caused no growth culture inhibition. Inhibitory effects were seen only above 100 mM; we were working with only low concentrations in the order of 1 to 50 μM. It was also apparent that at some concentrations, slight stimulation of culture growth by Tiron was observed (not shown), perhaps by chelating inhibitor(s) within the culture medium.

Figure 2 shows data from the four reactors when parallel reactors were run for FEW and RW with and without 50 μM Tiron. Total cells, as well as viable cell number, differ little if at all in FEW and RW with or without Tiron, Figure 2A and B. The proportion of live and dead cells was about the same from the start to the end of the experiment (~50%). Because of that only the total and viable cells (BacLight) are shown. In FEW, the cell population accumulates Al initially with little loss of viability or cultivability up to about day 14, with an average accumulation rate of 25 pg/10^6 cells/day. At this point, aluminium starts to appear in the cell supernatant and the titre of cultivable cells drops by $> 4 \log_{10}$ whereas the viability drops by only $\sim 0.8 \log_{10}$, Figure 2C. However, it is clear that when Tiron is included in the input water, the cultivability pattern of the *E. coli* in the FEW with Tiron (Figure 2C) is similar to that in RW with or without Tiron, (Figure 2D). The increased Al in the supernatant in FEW without Tiron (Figure 2C) might be due at least in part to cell leakage or death since it coincides with a drop in the cell-associated Al and a drop in culturable cells. However, microscopic observations of the cells stained with BacLight show little evidence of dead cells. Panels E and F show pH levels during the experiment; the reason for differences in FEW in the presence of Tiron is unknown.

Careful observations of viable cells show a reduced cell size after 28 running days. Reduction in cell size has often been reported following starvation (Morita 1990). The reason for the slight rise in Al in the cells in RW towards the end of the experiment (Figure 2D) is unknown but might be due to Tiron pulling Al of the walls of the dialysis sacs. Since the authors are very aware of the sensitivity of aluminium chemistry to the pH, pH measurements were made on a daily basis as the reservoirs feeding the reactors were changed. There was essentially no difference in pH of the output RW over that of the incoming water, though the pH varied during the experiment. For FEW there was a pH change between the input water and output water, especially after day 10, however, since this was identical between reactors with and without Tiron, it is believed to be independent of Tiron and aluminium chemistry and is likely to be more related to the physiological status of the cells.

There are obvious difficulties in working with the day-to-day levels of aluminium present in drinking water since these levels change continually, and it is not possible to know in advance of the sample collection what the level of aluminium is. So, it is better to speak of a range (i.e. 1–6 μM Al for this FEW). Data in Figure 2 was obtained with a relatively high level of Tiron (50 μM) that clearly neutralizes all aluminium ions present. Believing that the concentration of aluminium was decreasing (during summer months), we decided to do a titration experiment with different levels of Tiron and FEW alone to try and capture the full range of response from no Tiron to a Tiron level that was stoichiometrically equivalent to the aluminium ions present and including a Tiron level that might be in excess of the aluminium. Tiron concentrations were set at 0, 0.5, 1.0 and, 5.0 μM, respectively, in each of the four reservoirs feeding the reactors. This data is shown in Figure 3. Significant losses in cultivability were observed without and with the two lowest Tiron concentrations, after 35 and 42 days; data was compared by ANOVA and a Tukey Multiple Comparison tests to analyze the differences between groups with a confidence interval of 95%, P ranged from > 0.019–0.045 when compared with 5 μM Tiron (Figure 3A). With 5 μM Tiron, the preservation of cultivability was much clearer; approximate time in days for 1 log loss in cultivability were ~ 8 (0 Tiron), ~14 (0.1 μM Tiron), ~17 (0.5 μM Tiron), and ~ 22 (5.0 μM Tiron), respectively.. This is reflected in the lowest accumulation of aluminium in the cells and contrasts with when Tiron was absent or very low (Figure 3B). We believe that the 5 μM Tiron was approximately stoichiometrically equivalent to the Al in the FEW since the Al in the cells did not exceed Al in the output from the reactor (Figure 3B) whereas in the presence of the two lower Tiron concentrations the amount of Al in the cells was essentially identical to when there was no Tiron present at all (Figure 3B). The nature of the aluminium binding molecules remains to be determined, though proteins in the membranes or periplasmic space are obvious potential targets.

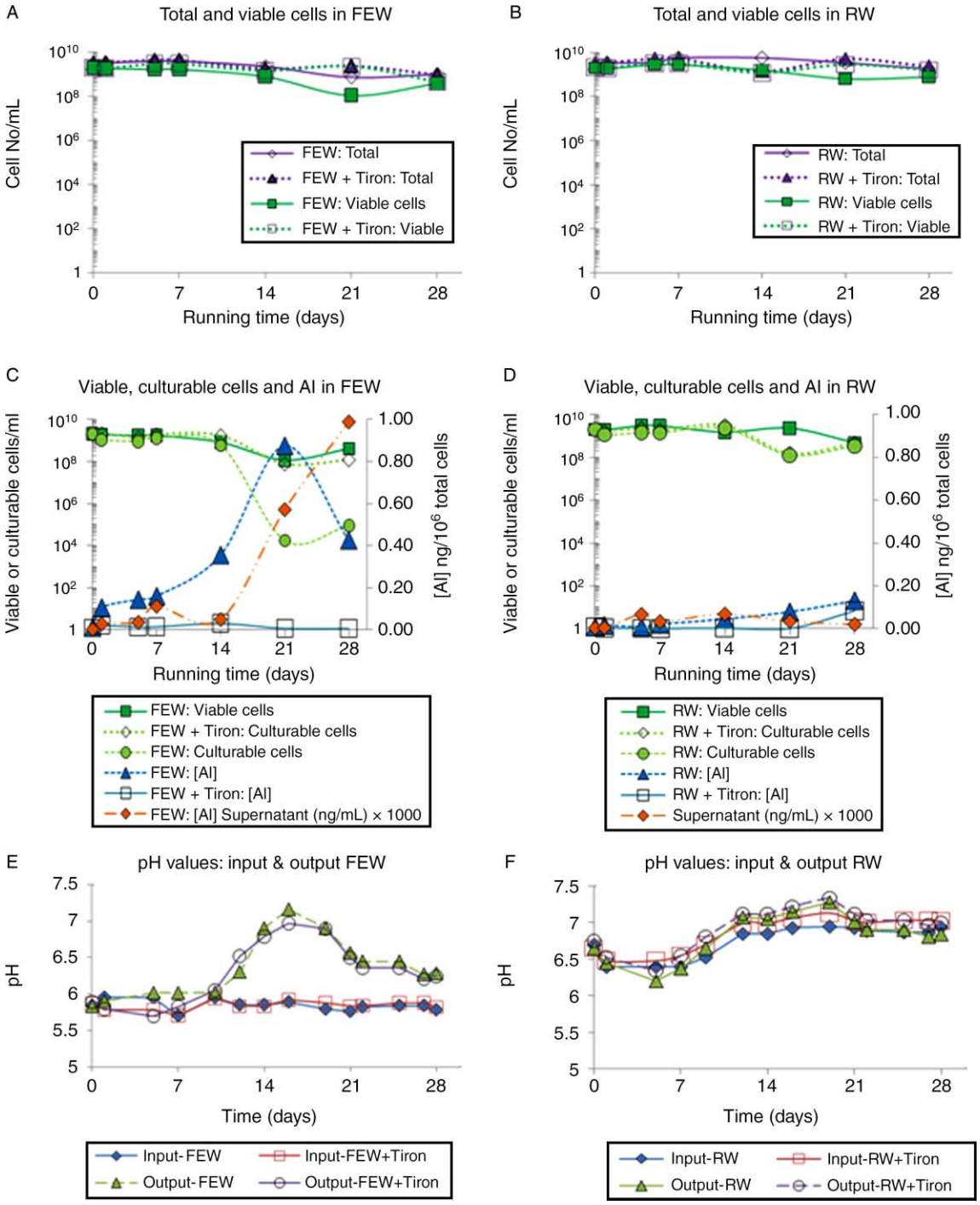

Figure 2 | Effect of Tiron (50 μM) on the viability and cultivability of stationary phase *E. coli* cell suspension in FEW and RW held at ambient temperature 24 ± 1°C. The standard error in estimation of total, viable and cultivable cell concentrations was less than or equal to 10%.

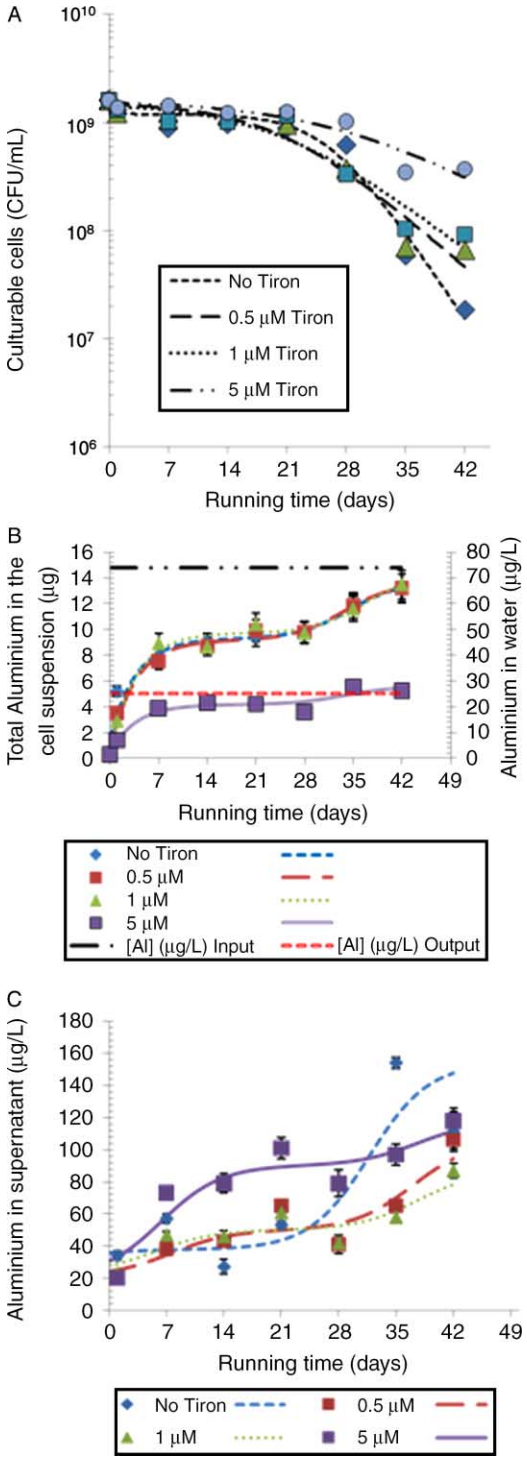

Figure 3 | Relationship between *E. Coli* cultivable cells (panel A), Al accumulation in the cells (panel B) and Al concentration in the supernatant (panel C) as a function of the input Tiron concentration during the reactor run with FEW.

The aluminium accumulation patterns in the supernatants change significantly as the Tiron concentration increases (Figure 3C). These changes goes from a sigmoidal behaviour (no Tiron present) with a latency phase in which there is no increase in the aluminium concentration where the cells accumulate aluminium without loss of cultivability, to a saturation hyperbolic behaviour (5 μM Tiron), transiting through biphasic behaviour at sub-stoichiometric Tiron concentrations. Supernatant Al was highest in the reactor with 5 μM Tiron but presumably did not accumulate to high levels since it can pass freely through the dialysis tubing and be lost in the reactor flow.

During the experiments we also measured the total aluminium concentration in the reactor input and output. Continuous uptake into the cells, and perhaps attachment to the reactor apparatus, resulted in significant drops in aluminium concentration across the reactor (30–60%). Attempts to perform a mass balance indicated that approximately 63% and 98% of the input aluminium could be accounted for in the absence and presence of excess Tiron, respectively. Of the aluminium recovered in the absence of Tiron, approximately 70% was cell-associated.

During these experiments, there was always the potential for aluminium to become precipitated inside or outside cells. However, there is no evidence from the experiments as to whether this was happening or not. Experiments were conducted at or close to pH levels associated with minimum aluminium solubility, but the concentrations used were low and solubility might have been facilitated by the presence or a wide range of organic molecules in RW or associated with the cells in the FEW reactors.

Aluminium is the most abundant metal on earth. It is ubiquitous and found in all solutions unless deliberately excluded. To avoid experimental anomalies, we worked with only indigenous aluminium levels in RW and FEW. Such aluminium concentrations varied temporally making it difficult to repeat experiments or to predict the concentrations that we would want to see in them. We nevertheless found that the water collected daily during experiments had concentrations of the same order of magnitude. In spite of these difficulties, all results are consistent with aluminium having a significant effect on the cultivability of *E. coli*, and potentially other bacteria as well. In preliminary

experiments we observed a similar loss of cultivability for a *Pseudomonas* strain held under identical conditions, but no further experiments were performed for that strain. The aluminium chelator, Tiron, was highly effective at quenching the effect of aluminium. As well, our demonstration of low Tiron toxicity to *E. coli* suggests further testing for its use in culture media. However, preliminary experiments attempting to resuscitate non-cultivable *E. coli* by inclusion of Tiron in culture media were not very successful.

In this study, it is clear that *E. coli* is accumulating aluminium and that the first phase of accumulation is quite rapid. The main issue is whether the *E. coli* that cannot be cultivated after aluminium exposure are indeed mostly viable as indicated by the BacLight stain, or whether they are dead, but with intact membranes. Clearly accumulation of a toxic element such as aluminium places a stress on the organism. The coincidence of loss of cultivability with an increase in the aluminium content of the supernatant is interesting and suggests the possibility that either the cells become non-viable and release aluminium, presumably complexed, or the cells deliberately release the bound aluminium through membrane vesicles as has been reported to occur under stress (McBroom & Kuehn 2007) for gram negative bacteria. In the event of the latter, the probability is that after the vesicle release the cells are viable but non-cultivable (VBNC). Such a VBNC state has been reported to occur after exposure to another toxic metal–copper (Grey & Steck 2001).

The numbers of *E. coli* used for these experiments greatly exceed anything likely to be found in drinking water. However, is it likely that the same phenomenon would occur for any low numbers of *E. coli* found and retained in filters, or other parts of the treatment plant where they can be exposed to a free aluminium residual in water following alum coagulation? Or in other words, can accumulation of aluminium mask the presence of *E. coli* and limit its utility as an indicator of contamination in the treatment and distribution of water? The aluminium concentrations here are very low and several days are required for sufficient aluminium to be accumulated to see a marked effect on *E. coli* cultivability. On the other hand, the flow rate used in the experiments was very much less than the exposure conditions for bacterial cells in biofilms in drinking water systems. If we consider an experiment running for 30 days, the cells would be exposed to only 144 L of FEW, and, diffusion is relatively slow in apparatus of the dimensions we used. In a real treatment plant, the volume of water and amount of residual aluminium that might contact *E. coli* cells could be considerably greater, and therefore loss of cultivability could occur very rapidly. On the other hand, is the rate of exposure important to whether the cells can survive in a VBNC state or be killed? This is unknown but a slower exposure might allow for intercellular signalling to occur or adaptation with greater ensuing cell survival.

CONCLUSIONS

Our experiments demonstrate a positive answer to the title question: the aluminium as found in drinking water can induce a stage in the *E. coli* population with loss of cultivability, but it is not clear whether it is identical to what is usually referred to as VBNC. This observation raises a number of additional questions about the use of *E. coli* as an indicator and of whether other potential indicators and or pathogens behave similarly. Clearly, those opportunistic pathogens that grow well in drinking water are much less sensitive to the presence of aluminium. Further investigations could address numerous questions. Among these, can aluminium account for or contribute to the differences in apparent survival of *E. coli* in different water matrices, or in acidic soils? Can the liberation of free aluminium by acidogenic bacteria or fungi contribute to certain bacteria losing cultivability and entering a VBNC state? What happens when *E. coli* is in a mixed population biofilm, can it be protected from the presence of aluminium by the other organisms present?

An alternative explanation for our results could be that even BacLight is not a suitable means for distinguishing live and dead cells. Monitoring dead cells is comparatively easy, but 'live cells' could be intact and non-viable. Other means of measuring viability might be necessary to prove a VBNC state exists. The ultimate proof, however, is if the cells can be resuscitated, and we continue to try and achieve this. So far, it is clear that some regrowth can be observed but in very small colonies that do not show typical behaviour on chromogenic media although they can be confirmed as

E. coli. In view of the importance of assessing viability of indicator and pathogenic bacteria in drinking water, we consider that the influence of aluminium accumulation deserves further investigation.

ACKNOWLEDGEMENTS

The assistance of Barry Duchene, Ian Douglas and other staff of the Lemieux and Britannia water treatment plants is gratefully acknowledged.

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