Natural freeze-thaw cycles may increase the risk associated with \textit{Salmonella} contamination in surface and groundwater environments

Jennifer M. Rocarda, Bahareh Asadishad, Pamela Rose V. Samonte, Subhasis Ghoshal, Nathalie Tufenkji

\textit{Department of Chemical Engineering, McGill University, Montreal, Quebec, H3A 0C5, Canada}
\textit{Department of Civil Engineering, McGill University, Montreal, Quebec, H3A 0C3, Canada}

\textbf{Article info}

\textbf{Article history:}
Available online 2 November 2018

\textbf{Keywords:}
Bacteria transport
Salmonella
Freeze-thaw
Climate change
Public health risk
Groundwater contamination

\textbf{Abstract}

Groundwater contamination by bacteria poses a serious threat to our drinking water supplies. In cold climate regions, microorganisms introduced to upper soil layers by spreading of animal manure are subject to low temperatures and multiple cycles of freezing and thawing at the beginning of winter and during spring melt. We investigated the influence of temperature fluctuations around the freezing point, known as freeze-thaw (FT), on the inactivation rates, growth, and biofilm formation of a manure-isolated strain of \textit{Salmonella typhimurium}. Moreover, the effects of FT on the transport characteristics of \textit{S. typhimurium} in quartz sand were monitored in model porewater solutions of two different ionic strengths (IS: 10 and 100 mM KCl) and two different humic acid (HA) concentrations (1 and 5 mg/L). Increasing numbers of FT cycles were found to decrease the deposition of \textit{S. typhimurium} onto quartz sand and increase the percentage of detached cells in sand-packed column experiments. Based on the calculated bacterial attachment efficiencies, the predicted minimum setback distances between the location of water supply wells and manure spreading activities are higher when the effects of FT are taken into consideration. While FT treatment significantly affected cell viability (in the presence of HA), most cells were in a viable but non-culturable (VBNC) state with compromised ability to form biofilm. This investigation demonstrates the effects of spring temperature variations in upper soil layers on \textit{S. typhimurium} properties and the potential increased risk of bacterial contamination in representative aquifer environments in cold climate regions.

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1. Introduction

Although animal waste can be beneficial to the soil ecosystem by adding organic matter that improves the water holding capacity and by providing an economical source of nutrients for plant growth, it can also be a source of pathogen contamination (Brannan et al., 2000). Therefore, it is important to evaluate the survival and transport behavior of pathogenic microorganisms when animal manure is applied onto agricultural fields (Gerba and Smith, 2005). The majority of these pathogens are enteric in origin, and can cause waterborne diseases (Ewald, 1991). Some of the most common pathogenic microorganisms found in animal waste are \textit{Salmonella} spp., \textit{Vesinicia} spp., \textit{Vibrio cholerae}, \textit{Campylobacter jejuni} and pathogenic \textit{Escherichia coli} (Gerba and Smith, 2005; Sidhu and Toze, 2009).

\textit{Salmonella} spp. alone are responsible for infecting annually an estimated 88,000 people in Canada and 1.2 million people in the United States who consume food contaminated by animal waste or contaminated water (Jacobsen and Bech, 2012; Thomas et al., 2013). A study conducted in Canada from 2014 to 2016 found \textit{Salmonella} to be the sole pathogen independently associated with severe disease in children presenting for emergency care with gastroenteritis (Freedman et al., 2017).

\textit{Salmonella} has been identified as a direct source of contamination of produce and well water and is therefore of particular concern (Borchardt et al., 2003; Bennett et al., 2018; Li and Uyttendaele, 2018). Although not commonly associated directly with drinking water outbreaks, \textit{Salmonella} was chosen as a representative bacterium in this study because it may be present in animal waste and groundwater, while it presents a relatively lower biohazard in the laboratory as compared with the more pathogenic
Campylobacter or E. coli O157:H7.

Salmonella typhimurium is a Gram-negative, motile, rod-shaped bacterium approximately 0.7–1.5 μm in width and 2.0–5.0 μm in length (Bernard and Wormser, 2006). It has been shown to survive in cattle manure for 48 days and soil for 231 days (Greenberg, 1964).

In nature, microorganisms are able to modify their properties to adapt to their environment and survive (Mindock et al., 2001). Thus, environmental isolates may exhibit very different behaviors from laboratory strains (Mikkola and Kurland, 1988). For instance, Salmonella isolated from aquaculture environments was reported to exhibit increased survival properties and environmental Escherichia coli isolates were found to have a higher electrostatic charge, hydrophobicity and biofilm formation compared to lab cultures (Akinbowale et al., 2006; Yang et al., 2012). Many studies report the transport and survival behavior of Salmonella using laboratory strains (Kim and Surette, 2005; Dourou et al., 2009), but few studies have examined isolate properties (Menezes et al., 2010).

To reduce the risk of pathogen outbreaks, a better understanding of the effects of environmental conditions on the behavior and survival of naturally occurring microorganisms in representative aquifer media is required.

In Quebec, during the transition from winter to spring, pathogenic microorganisms may be subject to repeated freeze-thaw cycles. It has been shown that repeated fluctuations in temperature could decrease the survival of microorganisms to a greater extent than a constant cold temperature (Asadishad et al., 2013, 2014). For instance, survival rates of microorganisms such as Versinia entero- colitica, Bacillus subtilis, and Cryptosporidium parvum oocysts decrease after exposure to fluctuating cold temperatures (Asadishad et al., 2013, 2014; Kato et al., 2002). Similarly, Escherichia coli O157:H7 and Salmonella typhimurium experience loss in viability when the temperature of their holding environments varied in increments of ±4 °C and ±7 °C (Semenov et al., 2007). Temperature fluctuations may also affect bacteria transport behaviour by influencing their physicochemical properties such as cell membrane composition and surface chemistry or cell size. For instance, cold and freezing temperatures have an impact on the membrane and surface chemistry of bacteria (Mindock et al., 2001). However, different studies report contradictory results on the role of cold temperature on bacterial transport. For example, it has been demonstrated that cold temperature or FT events can lead to both enhanced (McCaulou et al., 1995; Stevik et al., 2004) or decreased transport of bacteria in the subsurface. Asadishad et al. (2013,2014) found that pre-exposure to temperature variations from 10 °C to −10 °C enhanced attachment of Versinia entero coli and Bacillus subtilis onto model aquifer grains. Stevik et al. (2004) found a reduction in attachment of Escherichia coli and Salmonella typhi murium when temperature was decreased from 20 °C to 3 °C during the transport experiments.

In representative aquifer environments, both temperature and dissolved organic matter affect the survival rate of microorganisms and are therefore important factors to incorporate in risk assessment studies of groundwater contamination (Avery et al., 2004; Chowdhury et al., 2015). Humic acids are the major organic constituents of surface soil and their concentrations can influence the transport and fate of microorganisms. For instance, the presence of humic acids reduced the attachment efficiency of Salmonella cells onto a model aquifer grain (glass bead) surface (Chowdhury et al., 2015) and significantly reduced their inactivation in terms of loss in culturability (Ramamurthy et al., 2014). In this latter study, the change in pH upon addition of NOM was not accounted for and may have contributed to the observed effect. The relative impacts of temperature fluctuations and humic acid levels on the risk of Salmonella groundwater contamination may however vary because of the high potential for attachment of colloids at air-water interfaces, particularly in unsaturated soils. Note that the graphical abstract represents only the saturated zone of the land spreading risk pathway as a worst case scenario.

A few studies have investigated the inactivation and transport behavior of microorganisms in the presence of dissolved organic matter (Johnson and Logan, 1996; Chowdhury et al., 2015) or in response to variable temperature (John and Rose, 2005), however, most studies have overlooked the combined effect of varying cold temperatures in the presence of dissolved organic matter (Chowdhury et al., 2015; Davies and Evison, 1991; Walker et al., 2006).

The aim of this study was to characterize the effect of cold temperatures and FT events typically encountered in southern Canada on the survival and transport behavior of S. typhimurium in model aquifer environments. A systematic laboratory investigation was conducted to evaluate how variations in temperature and dissolved organic matter content in porewater influence the survival and transport behavior of S. typhimurium in packed column experiments. The scope of this study does not include the effect of freeze-thaw on transport from topsoil to the water table but rather focuses on the risk of contamination through the water-saturated flow transport pathways in model aquifer material.

2. Materials and methods

2.1. Cell culture preparation

A wild type strain of S. typhimurium, isolated from bovine manure in an agricultural site at Saint-Simon, Montérégie, Québec and supplied by IRDA (Institut de Recherche et de Développement en Agroenvironnement), was used as the model microorganism. The S. typhimurium (IRDA# 4.5.1i:1,2) stock cultures were stored in Lysogeny Broth (LB) with 30 % glycerol at −80 °C (Martinez et al., 2005). Bacteria were inoculated in sterile LB broth and grown overnight (18 hr) to stationary phase (37 °C, 140 rpm). For each experiment, 1 mL of the fresh overnight culture was used to inoculate 30 mL of fresh LB broth. Cultures were grown to exponential phase for 4 hr at the same conditions, harvested and washed by centrifugation (5,000g for 10 min) at 10 °C and re-suspended in 50 mL of electrolyte (1 mM, 10 mM, 100 mM KCl). To study the role of dissolved organic matter, in some experiments, the bacteria were suspended in 10 mM KCl containing 1 mg/L or 5 mg/L Suwannee River humic acid (HA) as a representative dissolved organic matter (International Humic Substances Society) without additional nutrients. More details are provided in Supporting Information.

2.2. Freeze-thaw treatment

Each FT cycle was 32-hr in total and the temperature profile comprised of four 8-hr time steps varying between 10 °C and −10 °C. Cell suspensions were prepared in seven 50 mL aliquots for different treatments: (1) “Before FT”: samples were tested after being maintained at 10 °C for 4 hr acclimatization; (2) “4 FT”: samples were exposed to 4 FT cycles; (3) “10 FT”: samples were exposed to 10 FT cycles; (4 and 5) “KCl”: samples were kept in electrolyte at 10 °C for 128 hr or 320 hr, the duration of 4 FT and 10 FT cycles respectively; (6 and 7) “LB”: samples were kept in LB broth (reference sample with no starvation) at 10 °C for 128 hr or 320 hr. This experimental matrix is similar to that used in our previous study (Asadishad et al., 2013). The reference samples that remained at a constant temperature of 10 °C, in electrolyte or in LB broth allow us to distinguish between the influence of FT and starvation on bacterial properties, respectively. Additional details are provided in Supporting Information.
2.3. Bacterial transport experiments

Transport experiments were performed inside chambers maintained at 10 °C, which is representative of groundwater temperature in southern Canada (Lesage et al., 1990; Castro and Tufenkji, 2007). Bacterial suspensions were injected into a glass column packed with granular quartz sand having a mean size of 256 μm (US standard mesh size –50/+70) (Sigma-Aldrich). Prior to conducting bacterial transport experiments, the column was equilibrated by injecting 6 pore volumes (PVs) of background electrolyte solution (KCl). The rate of injection was 0.8 mL/min (equivalent to a Darcy velocity of 14.7 m/d) for all column experiments. To establish breakthrough curves, the bacterial suspensions (at a concentration \( C_0 \)) were injected for 17 PVs followed by a cell-free electrolyte solution for 3 PVs. The concentration of the effluent \( (C) \) was measured in real time by UV-visible spectroscopy (Agilent HP8453) at a wavelength of 600 nm using a 1 cm flow-through cell (Mitzel and Tufenkji, 2014). Finally, to study the strength of bacterial attachment to the sand grains, a cell-free electrolyte at a lower ionic strength (1 mM KCl) was injected into the column and the extent of bacterial detachment was quantified. More details regarding the column experiments are provided in the Supporting Information.

2.4. Cell characterization

The culturability of the cells was evaluated by counting colony forming units (CFUs) on LB-agar plates. The viability of the cells was evaluated by assessing membrane integrity using the BacLight Live/Dead kit (Invitrogen). The electrophoretic mobility (EPM) and hydrodynamic diameter of the bacteria were measured using dynamic light scattering (DLS) at 10 °C using cells suspended in KCl before and after FT treatment (ZetaSizer Nano ZS, Malvern) (Castro et al., 2010). Details are provided in the Supporting Information. The “KCl” and “LB” control data of the cell characterization experiments are presented in Tables S1 to S7.

2.5. Biofilm and growth assays

Biofilm formation and growth curves were measured for each temperature treatment using a microtitre plate model (Merritt et al., 2005; O’Toole et al., 1999). To quantify the amount of biofilm formation and the amount of bacterial growth before and after temperature treatment, the absorbance of solubilized crystal violet was measured using a microplate reader at 570 nm (TECAN Infinite M200 Pro, Switzerland) (Karaca et al., 2013). Details are provided in the Supporting Information.

3. Results and discussion

3.1. The deposition of S. typhimurium decreases with FT treatment

The transport and deposition behavior of S. typhimurium was evaluated over the range of solution chemistries for the control (before FT) cells and for cells exposed to 4 or 10 FT cycles. Bacterial retention onto sand grains decreased as the number of FT cycles increased in all solution chemistries (Fig. 1). The extent of bacterial attachment was greater at higher IS before FT. For example, the percentage of bacterial retention \( (1 - \frac{C}{C_0}) \) was 23 % ± 0.2 and 35 % ± 0.3 for the cells without treatment (Before FT) in 10 and 100 mM KCl, respectively (Fig. 1a and b). \( \frac{C}{C_0} \) was quantified by integrating the area under the breakthrough curves (BTCs). The percentage of bacterial retention decreased to 4 % ± 1.7 after 10 FT in both 10 and 100 mM KCl. The trend observed in bacterial retention after exposure to FT in Salmonella was not in agreement with that previously observed in our laboratory using Bacillus subtilis and Yersinia enterocolitica (Asadishad et al., 2013, 2014), whereby those organisms exhibited higher retention on sand after exposure to FT. Because S. typhimurium and Y. enterocolitica only rarely cause outbreaks in well water users, it could be inferred that both pathogens have low mobility in representative aquifer environments under all climatic conditions, including FT.
dichotomy of high transport potential of *S. typhimurium* after freeze–thaw might be explained by the difference in type of isolates in our studies, where *S. typhimurium* was isolated from manure and *Y. enterocolitica* is a laboratory strain.

To take into account the starvation condition experienced by bacteria in groundwater environments, we conducted column experiments with cells that had been maintained at 10 °C in electrolyte (no nutrients) for 128 hr and 320 hr. Results shown in the Supporting Information (Fig. S1) reveal that starvation is not responsible for the observed bacterial transport patterns.

In a set of parallel experiments, the electrolyte was amended with dissolved HA to determine the effect of dissolved organic matter in representative aquifer environments on the transport behavior of bacteria exposed to FT. The extent of bacterial attachment onto sand grains in the presence of HA also decreased after FT treatment (Fig. 1c and d). For example, the percentage of cells retained onto sand before FT treatment was 30 ± 0.3 and 85 ± 0.4 in 10 mM KCl amended with 1 mg/L and 5 mg/L HA, respectively, and these values decreased to 10 ± 1.5 and 22 ± 2.2 after 10 FT treatment, respectively.

Interestingly, an increase in bacterial retention onto sand was observed in the presence of 5 mg/L HA when compared to pure electrolyte without HA. This observation is contrary to the findings of some other studies of bacterial transport in the presence of dissolved organic matter (Knapp et al., 1998; Yang et al., 2012). For instance, Foppen et al. reported that the presence of humic acid decreased the attachment of *Escherichia coli* ATCC 25922 onto goethite-coated sand (Foppen and Schijven, 2006). Park and Kim also showed that the attachment of *E. coli* ATCC 11105 onto iron-coated sand decreased with increasing humic acid concentration (Park and Kim, 2009). Abudalo et al. found that the attachment of *Cryptosporidium parvum* oocysts to ferric oxyhydroxide-coated quartz sand decreased as the concentration of fulvic acid increased (Abudalo et al., 2010). One explanation for the differences in the bacterial retention results could be related to differences in the granular media used. In the other studies (Abudalo et al., 2010; Park and Kim, 2009; Foppen and Schijven, 2006), the sand was coated with clays or metal oxides which would be masked by the humic acid, resulting in decreased bacterial retention. Another factor that may have led to the observed differences in bacterial retention could be the aggregation of cells in the presence of HA in this study, which in turn could lead to physical straining of the bacteria in the porous medium (Petosa et al., 2013). This is supported by the larger hydrodynamic diameter (2.2 ± 0.1 μm) measured for bacteria in 10 mM KCl + 5 mg/L HA compared to HA-free solutions of 10 mM KCl (1.2 ± 0.2 μm) (Table 1). Aggregation would also lead to an increase in contact frequency with grain surfaces due to an increase in gravitational settling and interception (Tufenkji and Elimelech, 2004).

The average cell size changed significantly as a result of FT treatment in 100 mM KCl, with hydrodynamic diameters of 1 ± 0.2 μm before FT and 0.7 ± 0.2 μm after 10 FT under the nutrient condition (Table 1) (p < 0.05). Similarly, the average cell size was smaller after FT treatment when cells were suspended in 10 mM KCl (with or without HA) (p < 0.05). The observed decrease in cell sizes after FT treatment may have influenced the single-collector contact efficiency as well as any potential role of straining in bacterial retention.

Bacterial zeta potentials were evaluated from EPM measurements conducted over the range of experimental conditions and are presented in Table 1. The results indicate that bacteria were negatively charged in all aquaeous chemistries. The absolute magnitude of the cell zeta potential tended to decrease with an increase in IS of KCl. Zeta potentials of clean sand are also negative at these conditions (−30 mV and −5 mV for 10 and 100 mM KCl, respectively) at pH 6.5, as reported elsewhere (Redman et al., 2004). In 10 mM and 100 mM KCl, the absolute zeta potential of bacterial cell surface charge did not change significantly after 4 FT (from −27 to −28 mV for 10 mM KCl and −22 to −26 mV for 100 mM KCl) (p > 0.05) but decreased to values lower than its initial value after 10 FT (p < 0.05). If classical DLVO (Derjaguin-Landau-Verwey-Overbeek) interactions were controlling the bacterial transport behavior, we would expect the cells exposed to 10 FT to exhibit higher retention than the untreated cells (Before FT); however, inspection of Fig. 1 reveals that this is not the case even for electrolyte solutions containing HA. Thus, bacterial zeta potential data suggest that factors other than purely electrostatic forces were involved in controlling the transport behavior of FT-treated *S. typhimurium*. It is likely that changes to the cell membrane properties (e.g., membrane fluidity, integrity of pilus and flagella) contributed to the different transport characteristics after being subjected to freeze-thaw cycles that involve rapid changes in temperature as well as changes in salinity associated with salt exclusion during freezing. Our prior work with a model bacterial strain showed that motility, flagellar activity, and the stiffness of the cell-silica bond were influenced by FT cycles (Asadishad et al., 2014).

For each experimental condition, the bacterial cell-sand attachment efficiency (\(a_{pc}\)) was calculated using the following equation (Yao et al., 1971).

\[
a_{pc} = \frac{2d_c}{3(1-\theta)L} \ln \left( \frac{C}{C_0} \right)
\]

(1)

where \(d_c\) is the mean grain diameter, \(\theta\) is the bed porosity, \(L\) is the packed-bed length, and \(C_0\) is the single-collector contact efficiency calculated using the Tufenkji-Elimelech equation (Tufenkji and Elimelech, 2004). Values of \(C/C_0\) in Eq. (1) were obtained by integration of the BTCs.

Overall, bacteria attachment efficiencies decreased after FT treatment (Table 2). For instance, the attachment efficiency decreased by 72 ± 0.2 and 95 ± 1.7 after 4 and 10 FT in 10 mM KCl, respectively. Moreover, in the presence of HA, the attachment efficiency decreased by 28 ± 0.3 % and 51 ± 1.5 after 4 and 10 FT cycles, respectively (when compared to no FT).

The calculated attachment efficiencies (Table 2) were used to estimate setback distances between drinking water wells and manure spreading activities (i.e., the length of the packed porous

| IS of KCl (mM) | Cell diameter (μm) | Zeta potential (mV) |
|---------------|--------------------|---------------------|
|               | 10 | 10 | 10 | 100 |
| 1 mg/L HA     | 1.5 ± 0.1 | 2.2 ± 0.1 | 1.2 ± 0.2 | 1.0 ± 0.2 | -8.0 ± 0.4 | -20.0 ± 0.3 | -27.2 ± 0.8 | -21.6 ± 0.8 |
| 5 mg/L HA     | 0.7 ± 0.0 | 0.9 ± 0.1 | 0.5 ± 0.0 | 0.9 ± 0.1 | -8.4 ± 1.3 | -4.5 ± 0.7 | -28.2 ± 0.8 | -26.2 ± 0.9 |
| 0 mg/L HA     | 0.8 ± 0.1 | 0.7 ± 0.2 | 0.9 ± 0.0 | 0.8 ± 0.2 | -10.2 ± 0.7 | -6.8 ± 1.1 | -22.9 ± 0.3 | -15.0 ± 0.9 |
medium required for 99.9% (3-log) removal of bacteria in the pore fluid was calculated using the Tufenkji-Elimelech equation by setting $C/C_0 = 0.001$. Our results demonstrate that the estimated setback distance would be greater after FT treatment. Hence, manure management practices need to take into consideration the effect of cold temperature variations on the transport behavior of pathogens.

In this study, cell suspensions stored in the same electrolyte at $10^\circ C$ for an equivalent amount of time (i.e., 128 hr and 320 hr) without FT treatment, exhibited no change in zeta potential (Tables S1 and S2). Also, the mean cell hydrodynamic diameter of starved cells was $0.8 \pm 0.0 \mu m$ after 320 hr in 100 mM KCl (Table S1). However, 320 hr LB samples kept at constant $10^\circ C$ (no starvation) exhibited the same cell size ($0.7 \pm 0.3 \mu m$). The results in Tables 1 and S1 suggest that variations in cell size and surface charge were not contributing factors in the observed bacterial retention following FT treatment at higher IS.

### 3.2. The release of *S. typhimurium* cells from sand may increase following FT treatment

The results of bench-scale column studies showed that the risk of *S. typhimurium* contamination could increase with FT. It is also of interest to consider the impact of disturbances such as snowmelt or surface runoff on bacterial transport and on detachment of cells from sediment grain surfaces. Cells attached to aquifer or sediment grain surfaces can detach and re-mobilize. To better predict the transport behavior of *S. typhimurium* under disturbed conditions, we modeled porewater changes by injecting an electrolyte solution at lower IS after obtaining a complete BTC. Lowering the IS of the pore fluid gives rise to more repulsive electrostatic interactions between the bacterial cells and the sand grains which may cause release of attached cells from the secondary energy minimum (Tufenkji, 2007). After injecting 1 mM KCl to the packed columns that had been previously equilibrated at 10 mM KCl or 100 mM KCl or 10 mM KCl + 5 mg/L HA, we observed a distinctive peak for released bacteria (Fig. 2a,b,d). In general, the percentage of detached cells was higher for bacteria that had been exposed to FT. However, for 10 mM KCl + 1 mg/L HA condition, the bacteria before and after FT treatment were detached to the same extent when 1 mM KCl was injected to the column.

### 3.3. *S. typhimurium* become viable but non-culturable (VBNC) after FT treatment

Viability (as characterized by cell membrane integrity) and culturability were measured for cells acclimatized at $10^\circ C$ for 4 hr

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**Table 2**

|                | Setback distance $(m)$ | Setback distance $(m)$ | Setback distance $(m)$ | Setback distance $(m)$ |
|----------------|------------------------|------------------------|------------------------|------------------------|
|                | $\alpha$               | $\alpha$               | $\alpha$               | $\alpha$               |
| 10 mM KCl      | $3.6 \times 10^{-4}$   | $4.0 \times 10^{-4}$   | $4.3 \times 10^{-4}$   | $2.4 \times 10^{-7}$   |
| Before FT      | 2 m                    | 1.8 m                  | 1.7 m                  | 0.3 m                  |
| After 4 FT     | $1.0 \times 10^{-4}$   | $1.5 \times 10^{-4}$   | $3.1 \times 10^{-4}$   | $4.6 \times 10^{-4}$   |
| After 10 FT    | $1.7 \times 10^{-5}$   | $2.3 \times 10^{-5}$   | $2.1 \times 10^{-4}$   | $3.1 \times 10^{-4}$   |
| 100 mM KCl     |                        |                        |                        |                        |
| 10 mM KCl + 1 mg/L HA | $3.6 \times 10^{-0}$ | $4.0 \times 10^{-0}$ | $4.3 \times 10^{-0}$ | $2.4 \times 10^{-3}$ |
| Before FT      | 2 m                    | 1.8 m                  | 1.7 m                  | 0.3 m                  |
| After 4 FT     | $1.0 \times 10^{-4}$   | $1.5 \times 10^{-4}$   | $3.1 \times 10^{-4}$   | $4.6 \times 10^{-4}$   |
| After 10 FT    | $1.7 \times 10^{-5}$   | $2.3 \times 10^{-5}$   | $2.1 \times 10^{-4}$   | $3.1 \times 10^{-4}$   |
| 10 mM KCl + 5 mg/L HA | $3.6 \times 10^{-0}$ | $4.0 \times 10^{-0}$ | $4.3 \times 10^{-0}$ | $2.4 \times 10^{-3}$ |

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**Fig. 2.** Detachment curves obtained by injecting a lower IS solution after BTCs of *S. typhimurium* through clean quartz sand at $10^\circ C$ in (a) 10 mM KCl (b) 100 mM KCl (c) 10 mM KCl + 1 mg/L HA and (d) 10 mM KCl + 5 mg/L HA before FT treatment (- - -), after 4 FT (- O -), and after 10 FT (- - -). Detachment curves were identical for two replicate experiments.
with no exposure to FT (Before FT) and for cells exposed to 4 and 10 FT using BacLight Live/Dead and CFU assays, respectively. A cell is considered viable if the cell membrane is intact while a cell is considered culturable if it also has the ability to divide and replicate. A VNBC cell cannot divide or replicate although it remains alive, but could recover these abilities given environmental conditions that would restore its metabolic activity.

As presented in Fig. 3a, the culturability of the cells significantly decreased from 2 × 10^6 CFU/mL to 3 × 10^5 and 7 × 10^5 CFU/mL after 4 and 10 FT cycles in 10 mM KCl, respectively (p < 0.05). The same trend was observed in 100 mM KCl, 10 mM KCl + 1 mg/L HA and 10 mM KCl + 5 mg/L HA (Fig. 3b,c,d). The Live/Dead assay results showed only 4% reduction in viability after 10 FT cycles in both 10 and 100 mM KCl indicating that S. typhimurium cell membranes were resistant to FT temperature fluctuations in 10 mM and 100 mM KCl (Fig. 3a and b). Thus, after FT treatment, the cells became less culturable but were still intact (as measured by the BacLight Live/Dead assay). The reduction in viability was more pronounced in model groundwater containing HA. HA is known to lower the pH of the environment. In this study, the addition of 5 mg/L HA to 10 mM KCl decreased the solution pH to 6.0 (initially pH 6.5). The significant decrease in cell viability from 95 ± 3% and 85 ± 3% before FT treatment to 60 ± 4% and 48 ± 4% after 10 FT in 10 mM KCl + 1 mg/L HA and 10 mM KCl + 5 mg/L HA (Fig. 3c and d), respectively, may be related to the slightly more acidic environment (p < 0.05). In this study, S. typhimurium survival uncertainty ranges between 1.2% under FT conditions and between 0.2% at constant temperature. Y. enterocolitica survival uncertainty ranges between 2.4% under FT conditions and between 0.1% at constant temperature (Asadishad et al., 2013). For B. subtilis, survival uncertainty ranges between 2-7% under FT conditions and between 0-1% at constant temperature (Asadishad et al., 2014). In light of the review of uncertainty ranges reported in the previously published datasets (above mentioned), it seems freeze-thaw may increase the overall survival uncertainty ranges of pathogen survival, however this increase is minimal.

The culturability of the bacteria in electrolyte with HA was lower than the culturability in electrolyte without HA in all treatments (Before FT, after 4 and 10 FT). For example, in 10 mM KCl + 5 mg/L HA the number of culturable bacteria decreased from 9 × 10^6 CFU/mL ± 3.9 × 10^6 (before FT) to 1.3 × 10^5 CFU/mL ± 6.9 × 10^4 (after 10 FT). In all other conditions (10 mM KCl, 100 mM KCl and 10 mM KCl + 1 mg/L HA), the culturability of the cells after 4 FT was lower compared to 10 FT. This may be related to the fact that the cells acclimatized to the FT treatments and became more resistant to the cold variations after 4 FT. Additional studies are needed to better understand the S. typhimurium FT stress response to predict whether the bacteria can build FT resistance.

In this study, cell suspensions stored in the same electrolyte at 10°C for the same amount of time but without FT treatment, showed no significant reduction in viability or culturability (p > 0.05) (Tables S3–S6). Therefore, the loss of culturability of the cells can be directly attributed to the temperature variations and FT exposure.

The analysis of S. typhimurium viability revealed their unexpected resistance to FT in electrolyte without HA. The reduction in viability was more pronounced in model groundwater containing HA which suggests that further investigation into the effect of dissolved organic matter on Salmonella growth and survival is needed.

3.4. S. typhimurium forms less biofilm, but has the potential to recover and re-grow after FT

To evaluate the effect of cold temperature and FT treatment on the survival strategy of S. typhimurium, biofilm formation and

![Fig. 3. Effects of freeze-thaw (FT) on S. typhimurium culturability (columns) on LB agar plates. The viability (cell membrane integrity) (●) of S. typhimurium cells in the column influent and effluent at different time points was measured using Live/Dead assay at 10°C in (a) 10 mM KCl (b) 100 mM KCl (c) 10 mM KCl + 1 mg/L humic acid (HA) and (d) 10 mM KCl + 5 mg/L HA before FT treatment, after 4 FT, and after 10 FT. Viability results represent mean values ± SD for two replicate experiments and CFU/mL results represent mean values ± SD for three independent experiments. Asterisks indicate a statistically significant difference of results when compared to Before FT and control conditions (128 hr and 320 hr in KCl or LB) which was determined using Student’s t-test (**: p < 0.05).]
planktonic growth potential were evaluated. Overall, after FT, biofilm formation decreased, and hence, cells were more likely to remain in planktonic state. Biofilm formation decreased by 69\% \pm 2 after 4 FT cycles (Fig. 4a). Reference controls were used to measure the effect of starvation and FT. The control cell suspensions that remained in LB at 10 °C with no FT for 128 hr (duration of 4 FT cycles) and for 320 hr (duration of 10 FT cycles) resulted in the same amount of biofilm formation as the control samples before FT. The same result was observed for cell suspensions in 10 mM KCl after 320 hr at 10 °C. However, after 128 hr in KCl at 10 °C, biofilm formation decreased by 62\% \pm 3. This particular behavior indicates that S. typhimurium was affected by starvation conditions after a duration corresponding to 4 FT cycles. However, after 320 hr in KCl, the biofilm formation increased suggesting that starvation conditions did not affect biofilm formation after 10 FT. The increase in biofilm formation observed in the LB or KCl controls may be attributed to cell acclimatization.

A similar trend was observed in planktonic growth patterns of S. typhimurium. Planktonic growth decreased after 4 FT cycles and then increased after 10 FT (relative to 4 FT). Overall, the extent of planktonic growth decreased after FT treatment (Fig. 4). The growth rates before and after FT treatment remained the same (Fig. 4b), however, the lag phase and growth yields in LB broth at 37 °C were affected. Planktonic growth started 50 min and 250 min later after 10 FT and 4 FT, respectively. A clear understanding of the processes governing S. typhimurium FT stress responses is needed to understand whether the cells harbor FT or starvation resistance.

Analysis of planktonic growth of S. typhimurium at 10 °C before and after FT treatment reveals the ability of bacteria to recover and grow during ‘thaw’ temperature periods. After 10 FT, planktonic growth is reduced by 70 \% \pm 0.5 (Fig. S2) but cells are still alive. It only takes a small number of pathogenic cells to present a risk of contamination. For instance, Salmonella cells can transfer into fresh produce through direct contamination of seedling or rainfall-induced splashes that may contaminate the edible part of the plant (Jacobsen and Bech, 2012). The presence of 5 mg/L HA inhibited the growth of S. typhimurium at 10 °C. As described above, this could be caused by the increase in acidity (pH without HA = 6.5, pH with HA = 6.0) induced by HA but further studies need to be done to confirm the pH sensitivity of S. typhimurium.

Although FT exposure had little effect on cell membrane integrity, most cells became viable but non-culturable (VBNC) and their biofilm formation decreased. Moreover, during thawing, at 10 °C, S. typhimurium cells could recover from their VBNC state.

The presence of HA was found to significantly impair S. typhimurium culturability and to increase bacterial retention in model groundwater environments (p < 0.05). Further investigation needs to be done to understand how the presence of HA may impact Salmonella behavior in the environment. This is especially important because the chosen HA concentrations for this study were relatively low to represent saturated zone conditions. In future studies, it may be of interest to examine the impact of higher concentrations of dissolved organic matter, which may be more characteristic of manure land spreading sites.

Furthermore, this study shows that S. typhimurium cells have little retention and high survival rates after experiencing FT cycles which makes it an important organism to be further investigated. Future work needs to analyse the virulence characteristics of S. typhimurium cells isolated from manure that have survived prolonged exposure to cold temperature and repeated FT.

4. Conclusions

- Subjecting S. typhimurium to increasing numbers of freeze-thaw cycles led to increased bacterial transport in saturated quartz sand and increased the likelihood of remobilization of retained cells.
- To protect groundwater consumers from pathogen contamination for private and public water supply wells, the setback distances between the location of water supply wells and manure spreading activities necessary to provide three log pathogen removal in uncoated sand could exceed 40 m depending on local aquifer conditions.
- It is also noteworthy that the transport behavior of Salmonella after FT exposure was in direct contrast with that previously observed in our laboratory using Bacillus subtilis and Yersinia enterocolitica. This outcome points to the need for further studies with different pathogenic bacteria, and particularly with strains indigenous to manure-amended soils in an effort to understand the effects of environmental conditions on the risks for source water contamination.

**Conflict of interest**

The authors declare no conflict of interest.
Acknowledgements

This research was financially supported by the Fonds Québecois de la Recherche sur la Nature et les Technologies, the Natural Sciences and Engineering Research Council of Canada, and the Canada Research Chairs program. The authors acknowledge Caroline Côté, Institut de Recherche et de Développement en Agroenvironnement (IRDA), for providing indigenous Salmonella strains.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wroa.2018.10.002.

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