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PAPER

Effects of residual antibiotics in groundwater on *Salmonella typhimurium*: changes in antibiotic resistance, *in vivo* and *in vitro* pathogenicity†

Berat Z. Haznedaroglu,[ab] Marylynn V. Yates,[c] Morris F. Maduro[d] and Sharon L. Walker*[a]

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An outbreak-causing strain of *Salmonella enterica* serovar Typhimurium was exposed to groundwater with residual antibiotics for up to four weeks. Representative concentrations (0.05, 1, and 100 μg L^{-1}) of amoxicillin, tetracycline, and a mixture of several other antibiotics (1 μg L^{-1} each) were spiked into artificially prepared groundwater (AGW). Antibiotic susceptibility analysis and the virulence response of stressed *Salmonella* were determined on a weekly basis by using human epithelial cells (HEp2) and soil nematodes (*C. elegans*). Results have shown that *Salmonella typhimurium* remains viable for long periods of exposure to antibiotic-supplemented groundwater; however, they failed to cultivate as an indication of a viable but nonculturable state. Prolonged antibiotics exposure did not induce any changes in the antibiotic susceptibility profile of the *S. typhimurium* strain used in this study. *S. typhimurium* exposed to 0.05 and 1 μg L^{-1} amoxicillin, and 1 μg L^{-1} tetracycline showed hyper-virulent profiles in both *in vitro* and *in vivo* virulence assays with the HEp2 cells and *C. elegans* respectively, most evident following 2nd and 3rd weeks of exposure.

Introduction

Salmonella enterica serovar Typhimurium is one of the most problematic food- and waterborne enteric pathogens in the world, with a high percentage of multiple antibiotic-resistant strains frequently isolated.[1] As a resilient pathogen, *Salmonella typhimurium* can cope well with a variety of stress conditions including temperature, osmolarity, and pH, both in its target host and in natural environments.[2,3] One of the most common matrices that *Salmonella* contaminates is groundwater systems,[4–8] frequently used as source water for direct consumption or vegetation watering practices. As a result, numerous cases of food and waterborne related *Salmonella* outbreaks are on the rise as indicated by recent reports from CDC.[9] Therefore, it is critically important to understand how the stress conditions imposed in groundwater environments affect the viability and pathogenicity of *S. typhimurium*.[10]

Pharmaceutical products are also major contaminants of aquatic systems[11–15] and the majority of the pharmaceutical products found in both surface and groundwater systems are antibiotics.[16–19] Although the resistance mechanisms of *S. typhimurium* against antibiotics are a widely explored topic,[20–24] there still exists a knowledge gap in overall bacterial response to antibiotics as an environmental stress condition and how exposure to low levels of antibiotic affect viability and pathogenicity.

[a]*Department of Chemical and Environmental Engineering, University of California, Riverside, CA, 92521, USA. E-mail: swalker@engr.ucr.edu; Fax: +1 951 827 5696; Tel: +1 951 827 6094*
[b]*Department of Chemical and Environmental Engineering, Yale University, New Haven, CT, 06520, USA*
[c]*Department of Environmental Sciences, University of California, Riverside, CA, 92521, USA*
[d]*Department of Biology, University of California, Riverside, CA, 92521, USA*

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Environmental impact

Salmonella enterica serovar Typhimurium is one of the most problematic enteric pathogens, with a high percentage of multiple antibiotic-resistant strains. As a resilient pathogen, it can cope well with a variety of stress conditions both in host and natural environments. Therefore, it is critical to understand how the stress conditions affect the viability and pathogenicity of *S. typhimurium*. This investigation found with the groundwater and antibiotic exposure, *S. typhimurium* can remain viable for long periods of exposure. Antibiotic susceptibility analysis and the virulence response of *Salmonella* were determined using human epithelial cells and soil nematodes. One notable observation was *S. typhimurium* exposed to residual amoxicillin and tetracycline showed hyper-virulent profiles in our virulence assays.

The goal of this study was to understand the effects of stress induced by groundwater environments and antibiotic presence on the viability, pathogenicity, and antibiotic susceptibility of *S. typhimurium* for prolonged exposure periods. To fulfil this goal, artificial groundwater was prepared and the conditions were simulated with residual antibiotics. Due to persistence[25–28] and prevalence in the environment,[11,13–15] amoxicillin and tetracycline were selected as the candidate antibiotics. To represent a wide range of pristine and contaminated groundwater conditions, both low[15,29] and high[30] concentrations of antibiotics were used.[13,14] In addition to amoxicillin and tetracycline, a cocktail of antibiotics was prepared by mixing nine common antibiotics and spiked into the groundwater. Antibiotic susceptibility analysis of stressed cells, along with *in vitro* and *in vivo* virulence assays, were performed for *S. typhimurium* exposed to these combinations of antibiotics for up to four weeks. Results indicated that long-term exposure to groundwater supplemented with residual antibiotics may increase the virulence of *S. typhimurium* against human epithelial cells and nematodes. During the course of the study, bacteria remained mostly viable; however, they failed to cultivate. No further antibiotic resistance was gained in any of the tested isolates due to exposure to the tested antibiotics.

Materials and methods

Bacterial cell growth and preparation

Salmonella enterica serovar Typhimurium strain ST5383 used in this study was obtained from the Salmonella Genetic Stock Centre (SGSC) of University of Calgary, Alberta, Canada. *S. typhimurium* strain ST5383 is a wild-type strain originally isolated from an interprovincial outbreak that infected more than 1700 people.[31] *Salmonella* cells used during the course of study were cultured in Luria-Bertani (LB) broth, (Fisher Scientific, Fair Lawn, NJ) at 37 °C overnight, shaken continuously at 120 rpm. A refrigerated bench-top centrifuge (5804R; Eppendorf, Hamburg, Germany) equipped with fixed angle rotor (F-34-6-38; Eppendorf) was used to pellet the cells with an applied 3700 × *g* force for 15 min at 4 °C. Growth medium was decanted and the pellet was resuspended in 3 mM in prepared groundwater. The concentration of cell stock solution was determined by using a cell counting hemocytometer (Bürker-Turk, Germany) under a light microscope (Fisher Scientific).

Application of stress conditions

Bacteria cultured and harvested as described above were exposed to several antibiotics containing groundwater for 1 to 4 weeks. Groundwater solutions used in cell preparation and other experiments were prepared with de-ionized water (DIW) (Millipore, Billerica, MA) and reagent-grade salts (Fisher Scientific) with a slight modification to a previously used artificial groundwater recipe[32] by dissolving following in one liter of DIW: $CaCl_2 \cdot 2H_2O$ (36mg), $CaSO_4 \cdot 2H_2O$ (25mg), KNO_3 (20mg), $NaHCO_3$ (36mg), $Ca(NO_3)_2 \cdot 4H_2O$ (35mg), and $MgSO_4 \cdot 7H_2O$ (60mg). The pH of solutions was kept constant at 7.0 ± 0.2. The ionic strength (IS) of the solutions tested was 3 mM, typical for groundwater.[33,34]

The levels of antibiotics added to groundwater were 0.05, 1, and 100 μg L^{-1} ampicillin (MP Biomedicals, Solon, OH); 0.05, 1, and 100 μg L^{-1} tetracycline (MP Biomedicals), and a cocktail of 1 μg L^{-1} of each the following antibiotics: amoxicillin, streptomycin, gentamicin, sulfamethoxazole (MP Biomedicals), tetracycline, ampicillin, chloramphenicol, kanamycin (EMD Chemicals, Darmstadt, Germany), and penicillin (Fisher Scientific).

A bacterial suspension containing 10^7 cells mL^{-1} was exposed to the aforementioned stress conditions in 500-mL tissue culture flasks with phenolic caps (Corning, MA) to minimize gas-exchange, and wrapped with aluminum foil to minimize light exposure. The flasks were placed on orbital shakers, and mildly shaken (70 rpm) at room temperature for the desired time periods.

Viability and cultivability

At the end of exposure to groundwater with residual antibiotics, viability of the cells was determined by using the Live/Dead BacLight® kit (L-7012; Molecular Probes, Eugene, OR) according to the manufacturer's directions. Direct counting of the stained live and dead cells was done using an inverted microscope (IX70; Olympus, Japan) operated under a red/green fluorescence filter set (Chroma Technology Corp., Brattleboro, VT). Stressed organisms were also tested for loss of cultivability by spread plating serial dilutions of stressed cells on LB agar (Fisher Scientific) plates followed by overnight incubation at 37 °C. Colony forming units (CFUs) were enumerated the following day.

Antibiotic susceptibility analysis

Antibiotic susceptibility tests were employed to assess the changes in *S. typhimurium*'s resistance to 11 antimicrobial substances commonly used by the U.S. National Antimicrobial Resistance Monitoring System (NARMS). Tests were performed in compliance with the Clinical and Laboratory Standards Institute/National Committee for Clinical Laboratory Standards (CLSI/NCCLS)[35] for the following antimicrobial agents (numbers in parentheses denote the amount of antimicrobial substance impregnated in 6 mm disks): amikacin (30 μg), amoxicillin-clavulanic acid (30 μg), cefoxitin (30 μg), ceftriaxone (30 μg), ceftiofur (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), tetracycline (30 μg), trimethoprim-sulfamethoxazole (25 μg). Antibiotic susceptibility analyses were performed in triplicate.

In vitro invasion assays

Human epithelial cell line HEp2 was obtained from American Type Culture Collection (CCL-23; ATCC, Manassas, VA) and cultured in Eagle's Minimum Essential Medium (ATCC) supplemented with 10% fetal bovine serum (ATCC), and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO). Epithelial cells were incubated at 37 °C under 5% CO_2 atmosphere.

Invasion of stressed *S. typhimurium* into epithelial cells was quantified with slight modifications to a protocol as described elsewhere.[36] Briefly, a monolayer of HEp2 cells was grown until confluence in 24-well plates (Corning, Corning, NY), and was subsequently inoculated with 10^5 *Salmonella* cells and incubated for 2 h at 37 °C to allow for internalization. Following the

incubation, each well was washed three times with phosphate buffered saline (PBS) to remove unbound bacteria. Bacteria that were bound to the epithelial cells, but had not been internalized, were killed by applying fresh growth medium containing penicillin and gentamicin (5 and 100 μg mL^{-1} respectively); the plates were then incubated for 2 h at 37 °C. Following incubation, cells were washed with PBS, treated with trypsin-EDTA complex (ATCC), and lysed with 1% Triton-X100 (Fisher). The lysates were spread onto LB agar plates and incubated for 18 h at 37 °C. The CFUs were counted to quantify the number of *S. typhimurium* that had successfully invaded the monolayer of epithelial cells. Invasion assays and CFU enumeration were performed in triplicate.

Caenorhabditis elegans maintenance and *in vivo* virulence assays

C. elegans strain SS104 [*glp-4* (*ts*)], a temperature-sensitive mutant of nematode worms that produces progeny at 15 °C but not at 25 °C,[37] was obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Saint Paul) and used for virulence assays. Worms were maintained by slight modifications to standard procedures described elsewhere.[38] Briefly, worms were grown on modified nematode growth medium (NGM) (US Biological, Swampscott, MA) [with 0.35% peptone instead of 0.25%, and supplemented with uracil (Fisher Scientific) (2 g L^{-1} final concentration)] plates at 15 °C, and fed with nonpathogenic *E. coli* strain OP50 as previously described.[39]

Virulence assays were performed based on similar studies described in literature.[40–42] Fifteen synchronized worms in larval stage L4 were transferred to fresh NGM plates seeded with 10 μL of stressed *S. typhimurium* cells (from the antibiotics containing groundwater flasks) mixed with *E. coli* OP50 (1 : 100 ratio respectively), and maintained at 25 °C. The number of viable worms was counted daily and reported as the number of survivors until all fifteen worms were killed. Worms that were sessile and unresponsive to touch were considered to be dead. For each stress condition, virulence assays were performed in triplicate. The control group was only fed with *E. coli* OP50.

Statistical analysis of data

Changes in the diameters of antibiotic inhibition zones and the number of CFUs of *S. typhimurium* infecting HEp2 cells were statistically compared to the control groups using unpaired Student's t-test using Minitab® Version 14 (State College, PA). Differences between control and stressed *Salmonella* cells were considered to be significant at 95% confidence interval ($P < 0.05$).

Results

Viability and cultivability of *Salmonella typhimurium*

Changes in the percent viability and cultivability of *S. typhimurium* exposed to antibiotic-containing groundwater were determined with respect to time and reported in Fig. 1 and 2, respectively. As can be seen from Fig. 1, the percent viability of the control group that was not exposed to antibiotics, *i.e.*, week 0, ranged from 92% to 100%. As anticipated, the percentage of viable cells decreased gradually as cells were exposed to antibiotic-containing groundwater for longer time periods (2 and 3 weeks) (Fig. 1). By the end of week 4, the percent viabilities of cells exposed to amoxicillin were 38%, 41%, and 34% for concentrations of 0.05, 1, and 100 μg L^{-1}, respectively. The percent viabilities of the cells exposed to tetracycline were 37% and 32% for concentrations of 0.05 and 1 μg L^{-1}, respectively. At higher tetracycline concentrations, *i.e.*, 100 μg L^{-1}, the percent

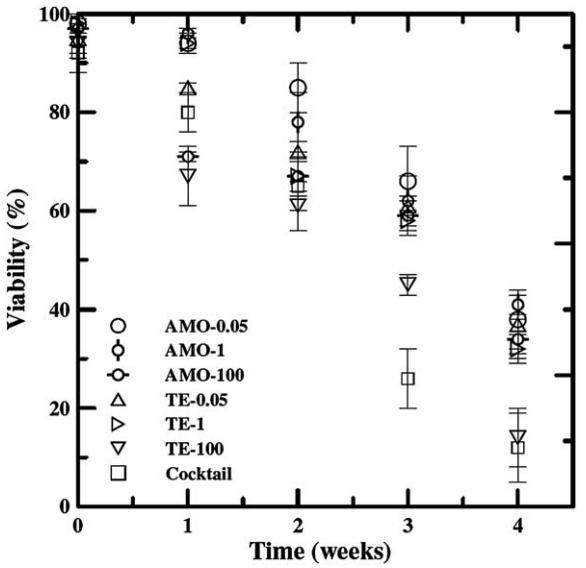

Fig. 1 Changes in percent viability of *S. typhimurium* exposed to antibiotics-containing AGW with respect to time and compared to control group. The abbreviations in the legend refer to the tested antibiotics (AMO for amoxicillin, and TE for tetracycline). The numbers in the legend refer to the concentrations of tested antibiotics in μg L^{-1}. Error bars indicate the standard deviation of three replicates.

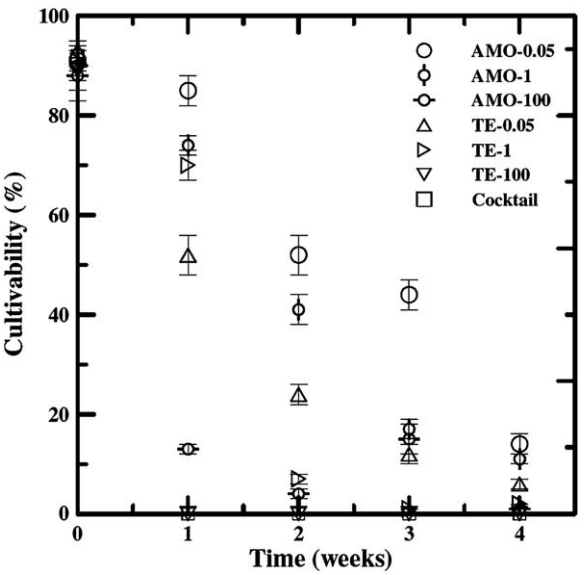

Fig. 2 Changes in percent cultivability of *S. typhimurium* exposed to antibiotics-containing AGW with respect to time and compared to control group. The abbreviations in the legend refer to the tested antibiotics (AMO for amoxicillin, and TE for tetracycline). The numbers in the legend refer to the concentrations of tested antibiotics in μg L^{-1}. Error bars indicate the standard deviation of three replicates.

viability was much lower (14%). The lowest percent viability (12%) was observed in batch exposed to the cocktail of antibiotics at the end of week 4.

During the course of study, the loss of cultivation in *S. typhimurium* cells was also monitored and reported as percent cultivability (Fig. 2). The initial (week 0) cultivability percentages were very close to the percentages of determined viability; however, significant differences were observed between percent viability and cultivability values starting at the end of week 1 and persisting through the end of week 4. The cells exposed to a low concentration of amoxicillin were 85% and 74% cultivable (for 0.05 and 1 μg L^{-1} respectively); however, only 13% cultivability was observed for cells exposed to the 100 μg L^{-1} of amoxicillin. In the case of tetracycline-exposed cells, the cultivability was 52% and 70% for 0.05 and 1 μg L^{-1}, respectively. In the case of high concentration of tetracycline exposure (100 μg L^{-1}), none of the cells were capable of being cultivated by the end of week 1. This was also observed for the cells exposed to the cocktail of antibiotics (Fig. 2). Similar to the trend observed in percent viability measurements, the cultivability of cells in the presence of amoxicillin decreased during the exposure period of week 2 and week 3. The cells exposed to the high concentration of tetracycline and the cocktail of antibiotics failed to cultivate following weeks 2, 3 and 4. By the end of week 4, percent cultivability was relatively low for the rest of the conditions: 14% and 11% (for 0.05 and 1 μg L^{-1} amoxicillin exposure, respectively), 1% (for 1 μg L^{-1} amoxicillin exposure), as well as 6% and 2% (for 0.05 and 1 μg L^{-1} tetracyclin exposure, respectively). No growth was observed after 4 weeks of exposure to either 100 μg L^{-1} tetracycline or the cocktail of antibiotics (Fig. 2).

Antibiotic susceptibility analysis

The full names and abbreviations of all antibiotics used for the susceptibility tests are presented in SI Table S1, along with the resistance breakpoints indicating the thresholds of susceptible, intermediate resistance, and resistant levels.[35] As further indication and confirmation of low levels or no cultivability in bacteria exposed to high concentrations of antibiotics (exposure to 100 μg L^{-1} of amoxicillin and tetracycline, and the cocktail of antibiotics), no bacteria were observed on the antibiotic susceptibility plates and therefore these tests were not performed.

Table S2 shows the measured diameters of the zones of inhibition for *S. typhimurium* exposed to groundwater with residual amoxicillin (0.05 μg L^{-1}). Week 0 data denote the susceptibility profile of the control group consisting of the cells that were not exposed to antibiotic stress. As can be seen in Table S2, the control group of *S. typhimurium* strain used in this study (ST5383) was susceptible to all 11 antibiotics tested. As cells were exposed to a low concentration of amoxicillin (0.05 μg L^{-1}), subtle increases were observed in the size of the inhibition zone. These increases were statistically significant for only a few cases as indicated in Table S2. Similarly, exposure to groundwater with 1 μg L^{-1} amoxicillin did not change the susceptibility profile of the control group for any of the antibiotics tested. As can be seen in Table S3, the zone diameters increased for the majority of the cells during the exposure period from week 1 to week 4.

Antibiotic susceptibility of cells exposed to tetracycline-containing groundwater resulted in similar trends to the amoxicillin exposure. The inhibition zone diameters are given in Table S4 and Table S5 for tetracycline concentrations of 0.05 and 1 μg L^{-1}, respectively. The results indicate that cells exposed to tetracycline-containing groundwater sustained their susceptibility to the tested antibiotics for the duration of week 1 to week 4 for either concentration of tetracycline.

In vitro invasion assays

The number of *S. typhimurium* cells (denoted as CFUs) that can successfully invade human epithelial cell cultures (HEp2) following the exposure to antibiotic-containing groundwater were presented in Table 1. Prior to the groundwater exposure (week 0) 258 CFUs from the control group invaded HEp2 cells. At the end of week 1, the CFUs decreased significantly to 192 for cells exposed to tetracycline (0.05 μg L^{-1}), and 128 and 64 for cells exposed to amoxicillin (0.05 and 100 μg L^{-1}, respectively) ($P < 0.05$). However, cells exposed to amoxicillin and tetracycline (both 1 μg L^{-1}) resulted in the number of CFUs that could invade HEp2 cells to increase significantly to 448 and 384, respectively ($P < 0.05$). None of the cells exposed to the cocktail of antibiotics and high concentration of tetracycline (100 μg L^{-1}) were able to invade HEp2 cells for the remaining duration of the study. By the end of week 2, the number of CFUs has dramatically increased to 2667 and 1600 for cells exposed to 0.05 and 1 μg L^{-1} amoxicillin, respectively. The number of CFUs that can invade HEp2 cells was 107 for 100 μg L^{-1} amoxicillin-exposed cells, which is higher than week 1 results but still less than the control group. Among the cells exposed to 1 μg L^{-1} tetracycline, none of them were able to invade the epithelial cells starting from week 1 to the end of the study. At the end of week 3, the number of CFUs from amoxicillin-exposed cells (0.05 and 1 μg L^{-1}) was significantly higher than the control group; however, it was less than week 2 exposed cells. By the end of the study, the number of CFUs that could successfully invade the epithelial cells was less than those of the control group, except for cells exposed to the high concentration of amoxicillin (100 μg L^{-1}).

In vivo virulence assays

To confirm the virulence trends of stressed *S. typhimurium* cells against HEp2 cells, *in vivo* virulence assays were performed with *C. elegans* and the results are presented in Fig. 3. Worms that were fed with *E. coli* OP50 and *S. typhimurium* cells not exposed to stress conditions were referred as the control group. As can be seen in Fig. 3, the control group died in 17 days. When *E. coli* OP50 was mixed with *S. typhimurium* cells exposed to amoxicillin (for concentrations of 0.05, 1 and 100 μg L^{-1}) and tetracycline (1 μg L^{-1}) for the durations of one to four weeks, worms died in a shorter period of time than the control group. *S. typhimurium* exposed to tetracycline (for concentrations of 0.05 and 100 μg L^{-1}) and the cocktail of antibiotics killed the worms in 16 and 17 days, respectively, which is very similar to the control group (data not shown). *S. typhimurium* cells exposed to 1 μg L^{-1} tetracycline for one week were able to kill worms in 13 days (Fig. 3). The cells exposed to tetracycline (1 μg L^{-1}) for two weeks or later resulted in very similar results to the control group as well (data not shown; $P > 0.05$).

Table 1 Number of *S. typhimurium* CFUs infecting HEp2 cells exposed to antibiotics containing groundwater with respect to time and compared to control group

Stress conditions	Week 0	Week 1	Week 2	Week 3	Week 4
Amoxicillin (0.05 μg L^{-1})		**128 ± 18**[a]	**2667 ± 269**	**720 ± 83**	213 ± 45
Amoxicillin (1 μg L^{-1})		**448 ± 56**	**1600 ± 127**	**480 ± 57**	**107 ± 39**
Amoxicillin (100 μg L^{-1})		**64 ± 12**	**107 ± 18**	**400 ± 38**	**320 ± 49**
Tetracycline (0.05 μg L^{-1})	258 ± 34	**192 ± 24**	0	0	0
Tetracycline (1 μg L^{-1})		**384 ± 28**	320 ± 34	**320 ± 42**	**53 ± 17**
Tetracycline (100 μg L^{-1})		0	0	0	0
Cocktail (1 μg L^{-1})		0	0	0	0

[a] Numbers in bold denote the changes in the CFUs are statistically significant compared to the control group.

S. typhimurium cells exposed to low concentrations of amoxicillin-containing groundwater were the fastest killers of the worms as an indication of hyper-virulence.[43–45] It took 10, 7, 9, and 11 days to kill all the worms after one, two, three, and four weeks of exposure to 0.05 μg L^{-1} amoxicillin, respectively. For 1 μg L^{-1} amoxicillin-exposed cells, worms were dead in 11, 8, 10, and 12 days (week 1, 2, 3, and 4 exposure, respectively). Exposure to a high concentration of amoxicillin (100 μg L^{-1}) and tetracycline (1 μg L^{-1}) showed similar results in terms of the days required to kill all worms.

Discussion

It is widely accepted that human pathogenic bacteria enter the viable but nonculturable (VBNC) state when exposed to harsh environmental conditions.[46–48] However, it is a matter of

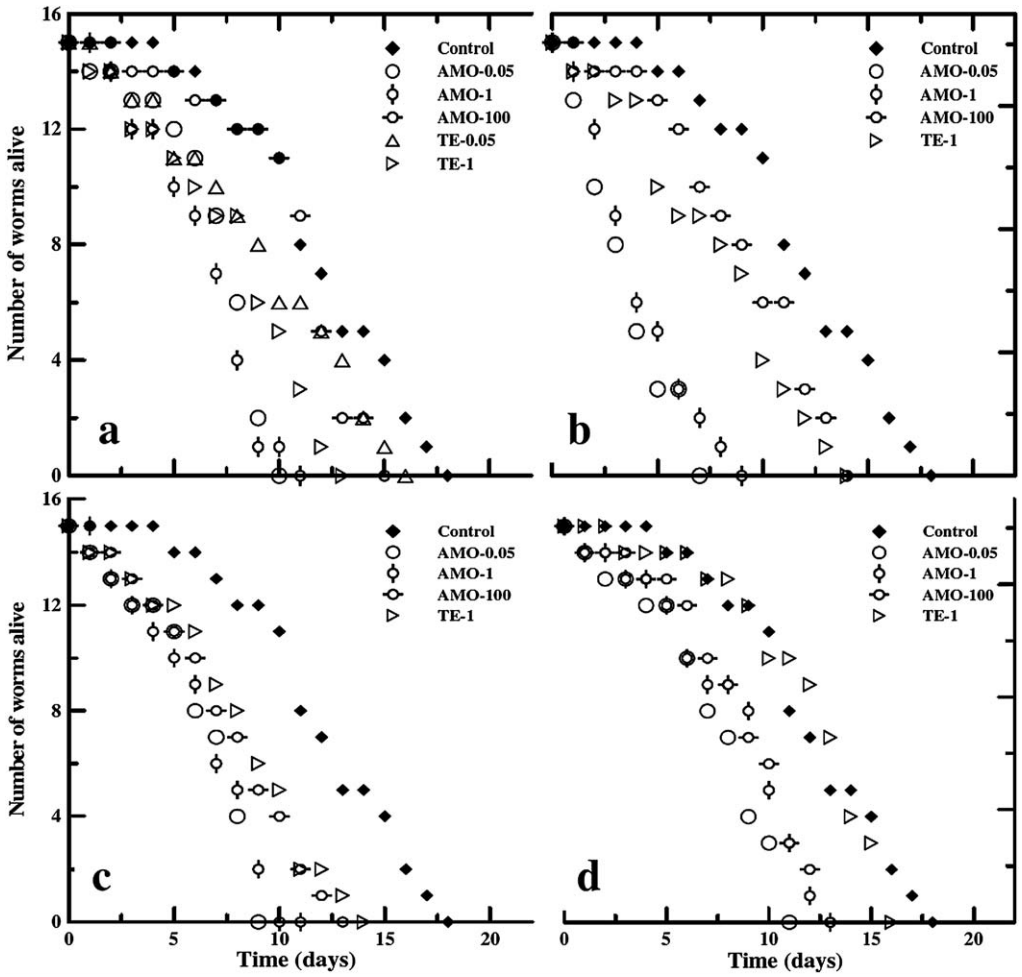

Fig. 3 Number of *C. elegans* remaining alive when fed with *S. typhimurium* exposed antibiotic-containing groundwater for one week (a), two weeks (b), three weeks (c), and four weeks (d). The abbreviations in the legend refer to the tested antibiotics (AMO for amoxicillin, and TE for tetracycline). The numbers in the legend refer to the concentrations of tested antibiotics in μg L^{-1}. All experiments were performed in triplicates, error bars are not shown for clarity purposes.

continuous debate whether or not VBNC cells pose a risk to human and animal health.[48–52] Therefore, both percent viability and cultivability values were determined (Fig. 1 and 2, respectively). Results have shown that although the cells remain viable, they may not be cultivable at all (*e.g.*, exposure to 100 μg L^{-1} tetracycline and cocktail of antibiotics from week 1 to week 4) or cultivable at low levels [*e.g.*, exposure to 100 μg L^{-1} amoxicillin from week 1 to week 4 and lower concentrations of tetracycline (0.05 and 1 μg L^{-1}) from week 2 to week 4]. Overall, the results showed that the majority of the cells exposed to antibiotics for up to two weeks may remain structurally intact[53] (as determined by viability); however, they fail to cultivate. As the exposure time to antibiotics is prolonged, both the percent viability and cultivability decreases to minimal levels (Fig. 1 and 2, respectively).

The antibiotic susceptibility tests could not be performed for the cells that failed to cultivate [tetracycline (100 μg L^{-1}), and cocktail of antibiotics]. This was also the case for cells exposed to groundwater with the high concentration of amoxicillin (100 μg L^{-1}), due to the low cultivability (Fig. 2). Antibiotic susceptibility tests were successfully performed for cells exposed to groundwater with tetracycline (0.05 and 1 μg L^{-1}), even though they showed similar cultivability (especially after week 2) to cells exposed to groundwater with high concentration of amoxicillin (100 μg L^{-1}). This may be due to the fact that different media were used for the cultivability (LB agar) and antibiotic susceptibility (Mueller-Hinton agar) tests, resulting in different growth characteristics.

Results of the antibiotic susceptibility tests were quite unexpected (Tables S2–S5). As bacteria are exposed to antibiotics in different aquatic, terrestrial, and host habitats, it is inevitable that resistance is gained to those antibiotics.[11,24,54–57] However, there was no increased amoxicillin resistance induced by exposure to this antibiotic condition during the prolonged duration of this study. On the contrary, the cells showed increased susceptibility (Tables S2, S3). This observation was also confirmed with tetracycline exposure: the susceptibility of cells exposed to 0.05 and 1 μg L^{-1} of tetracycline-containing groundwater showed increased susceptibility to tetracycline (Tables S4, S5). This may be explained by the following mechanisms. First, the concentration of the antibiotics tested might be too low (0.05 and 1 μg L^{-1}) to induce resistance in the bacteria. This hypothesis could have been confirmed with the cells exposed to high concentrations of antibiotics; however, the tests failed as mentioned earlier. Secondly, although both tetracycline[25–27] and amoxicillin[28] are quite resistant to degradation, they might have been degraded in the groundwater with time[11,16,58] and resulted in a lesser or insignificant level of activity.[59]

Usage of human epithelial cell lines to test the *in vitro* virulence of *Salmonella* is a quite common and effective way of analyzing the degree of pathogenicity.[60–63] However, *in vitro* virulence assays may not fully represent the internal habitat of a living organism. Therefore, the commonly-studied soil nematode *C. elegans*[64] was utilized to test the *in vivo* virulence of *S. typhimurium*[40–42,65–68] exposed to antibiotic-containing groundwater as a complement to *in vitro* assays. *In vitro* virulence assays have shown that exposing *S. typhimurium* to antibiotic-containing groundwater may increase the virulence of the bacteria as indicated by a greater number of cells entering the host epithelial cell lines (Table 1). This was more evident for cells exposed to a low concentration of amoxicillin during weeks 2 and 3. The changes in CFUs involved in invasion were not pronounced for week 1 and week 4 under the low concentration amoxicillin condition. The cells exposed to high (100 μg L^{-1}) and low (0.05 and 1 μg L^{-1}) (after week 1) concentrations of tetracycline and the cocktail of antibiotics did not invoke virulence against HEp2 cells as described earlier.

In vivo virulence assays were in agreement with the *in vivo* virulence assays: the worms were killed in a shorter amount of time with *S. typhimurium* exposed to a low concentration of amoxicillin for two and three weeks as compared to the control group (Fig. 3b, c). Cells exposed to stress conditions for four weeks were still virulent to *C. elegans*; however, the days required to kill the whole population was longer than those exposed for one to three weeks (Fig. 3).

The reason for increased virulence at exposure periods of two and three weeks may be that the cells were becoming accustomed to the exposed stress conditions during the first week and eventually adjusted their metabolism to the groundwater environment during the following weeks. This has been proven with K^+, Ca^{2+} and Mg^{2+} ions (all present in groundwater), known promoters of increased virulence of *S. typhimurium*.[69–74] The decreased virulence observed in week 4 is possibly due to cells starting to die and lose their infectious characteristics as indicated by viability and cultivability data (Fig. 1, 2). This is in agreement with another study that has shown that *S. typhimurium* stressed with UV-C light and seawater may lose its cultivability and virulence, whereas it remained structurally intact and viable.[75]

Conclusions

In this study, evidence that long-term exposure (up to approximately one month) to groundwater supplemented with several antibiotics may induce a viable but nonculturable (VBNC) state in *Salmonella typhimurium* was obtained. It has been also shown that *S. typhimurium* remains viable in groundwater up to four weeks. During the course of the study, *S. typhimurium* exposed to low-concentration amoxicillin-containing groundwater for two and three weeks showed hyper-virulence against human epithelial (HEp2) cells and nematodes (*C. elegans*) as shown by both *in vitro* and *in vivo* virulence assays, respectively. Enhanced virulence was also observed to be due to exposure to groundwater with a representative concentration of tetracycline. Exposure of *S. typhimurium* to amoxicillin- and tetracycline-containing groundwater up to four weeks did not induce any resistance against these antibiotics among the bacteria. These results suggest that *S. typhimurium* in groundwater contaminated with trace antibiotics may remain viable and may show increased virulence to either human and/or animal hosts.

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