CAMPYLOBACTER SURVIVAL IN GROUNDWATER: COMPARISON OF TWO STRAIN TYPES AND TWO GROUNDWATER TYPES

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ABSTRACT

Following the *Campylobacter* outbreak in Havelock North it was recognised that there was a lack of data on the survival of *Campylobacter* in groundwater. In particular, there is a lack of information on the survival of *Campylobacter* in different groundwater in terms of oxygen status. To close this knowledge gap we designed an experiment to measure the survival of an outbreak strain of *Campylobacter* and the type strain in an oxic and an anoxic groundwater.

A laboratory scale system was set up to investigate the survival of one of the Havelock North outbreak strains (HN16) and the laboratory type strain of *Campylobacter jejuni* (NCTC11351) in both an oxic and anoxic groundwater. The two groundwater types were held at 12°C (average temperature of local groundwater (Daughney and Reeves, 2002)) and the dissolved oxygen was maintained in both systems throughout the experiment. Samples were taken at specific time intervals over the period and analysed for *Campylobacter* using selective media.

The results show that *Campylobacter* can survive in groundwater for over two weeks. Differences were observed in the rate of die off between the two strains of *Campylobacter* studied. The outbreak strain of *Campylobacter* showed higher survival rate compared with the type strain of *Campylobacter* in anoxic groundwater. Initially (first 6 days), the two strains of *Campylobacter* died off at different rates, and were not significantly different between the types of groundwater, with die-off rates calculated as 0.055 – 0.084 days for HN16 and 0.288 – 0.289 days for NCTC 11351. After 6 days, the type of groundwater appeared to have an effect on the survival of both strains of *Campylobacter*. The outbreak strain continued to survive well in anoxic groundwater and showed a 1 log-drop in concentration after 16 days, die-off date of 0.0873 days. The type strain NCTC 11351 also showed slower die off in anoxic groundwater over days 6 to 16. It is interesting to note that there appeared to be a plateauing of the die off occurring in both strains of *Campylobacter* occurring in oxic groundwater. Looking at the overall survival rates for *Campylobacter* higher survival occurred in anoxic groundwater for both strains. *Campylobacter* is a microaerophillic microorganism which grows optimally in low oxygen levels. The low oxygen concentration (maintained at less than 2 mg/L) could play a role in the survival of *Campylobacter* in groundwater.

Comparative die off rates will be presented in anoxic and oxic groundwater for both strains. Implications for public health will be discussed.

KEYWORDS

***Campylobacter*, groundwater, oxic, anoxic, survival, transport, health, outbreak**

PRESENTER PROFILE

Louise Weaver is a microbiologist with expertise in groundwater and wastewater microbiology. Louise has been a part of ESR's Groundwater team for 10 years working on various projects from microbial pathogen transport to biofilm growth in groundwater. Louise is also a part of the CIBR (Centre for Integration Biowaste Research) group investigating low cost, sustainable wastewater treatment for optimised pathogen removal.

1 INTRODUCTION

Microbial pathogen survival within the environment can be variable and can depend on many criteria, including environmental conditions e.g. oxygen concentration, temperature, pH, sunlight etc. (Moriarty et al., 2012, Wilkes et al., 2011, Horswell et al., 2010, Haller et al., 2009, Sinton et al., 2007, Gerba and Keswick, 1981). Previously it has been assumed that survival of certain pathogenic bacteria such as *Campylobacter* is limited in the environment (Gilpin et al., 2009, Cook and Bolster, 2007, Cools et al., 2003, Thomas et al., 1999, Buswell et al., 1998, Korhonen and Martikainen, 1991, Weaver et al., 2016, Sinton et al., 2007). This is based on limited studies conducted in a variety of environments. Groundwater has been shown to enable prolonged survival of pathogenic organisms due to the absence of sunlight and relatively stable temperatures (Cook and Bolster, 2007). In other studies, however, survival has been lower in groundwater when compared with a sterile environment (e.g. sterilised groundwater or artificial groundwater) due to the presence of competing organisms and adverse conditions of pH and redox (Korhonen and Martikainen, 1991).

As the information available to date is conflicting and there appears to be no consistent data on survival of *Campylobacter* in groundwater and experiment was designed to study survival in controlled laboratory conditions. It was hypothesised that, due to *Campylobacter*s low tolerance to high oxygen levels, survival in oxic (dissolved oxygen (DO) levels over 5 mg per L) would be less than in an anoxic groundwater (DO levels below 2 mg per L).

The experiment presented was the first experiment to our knowledge comparing survival of *Campylobacter* in two groundwater types. There were limitations to the experiment, such as no adjustment was made to the pH of each of the groundwater at the start of the experiment. In addition, due to equipment failure the experiment was stopped after 16 days. Subsequent experiments are underway to extend this experimental period.

2 METHODS

2.1 INOCULUM PREPARATION

After the recent *Campylobacter* outbreak in Havelock North, New Zealand, a strain of *Campylobacter* which was found in the source of the drinking water reservoir and also present in clinical samples was isolated and used for this survival study. This outbreak strain was characterised by molecular methods at ESR and called HN16. To compare the survival of the outbreak strain with another *Campylobacter* strain a type strain, NCTC 11351 was used.

To prepare the *Campylobacter* inoculates 16 x 50ml centrifuge tubes each containing 50mls Exeter broth (Fort Richard), were inoculated with a single colony of *Campylobacter* HN16 or type strain NCTC 11351 grown on Exeter plates (Fort Richard). The tubes were incubated at 37°C in 10% CO_2 for 4 hours and then transferred to 42°C in 10% CO_2 for 40 hours.

The broths were then spun at 5,000g for 10 minutes and washed 3 times in phosphate buffered saline (PBS) centrifuging between washes. The final pellets were combined and made up to a total volume of 40mL with PBS to create each inoculum.

2.2 EXPERIMENTAL DESIGN

Four sterile 5 L glass jars were used for the experiments (Figure 1). Fitted lids were used to contain the atmospheric conditions within the jars. The lids contained probes for pH, dissolved oxygen (DO) and temperature. An air lock was used to regulate the pressure within the jars. Nitrogen gas was fed into the anoxic jars to maintain an anaerobic environment. A sampling port was placed in the lid with a glass rod to sample from a set height within each jar. The jars were contained within a covered (to prevent light entering) cooled platform shaker for the duration of the experiments. The temperature was set to maintain groundwater temperature at 12-14°C during the experiments. This is the typical temperature of New Zealand groundwater (Daughney and Reeves, 2002). pH, DO and temperature were recorded at 15 minute intervals throughout the experiment using a multi-probe meter (Hach HQ40d, Hach NZ, Auckland, New Zealand).

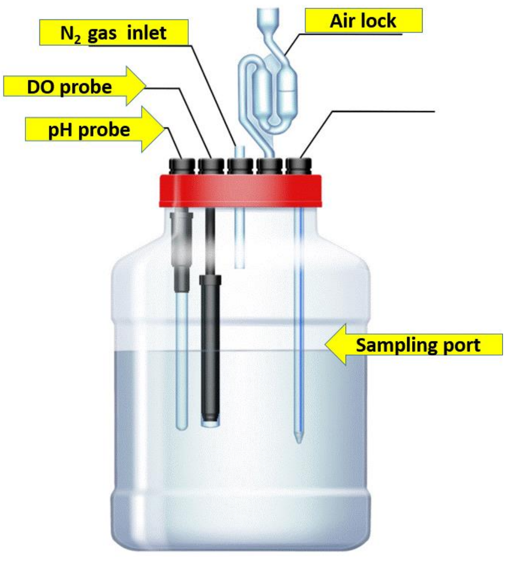

Figure 1: Set up of jars for Campylobacter survival experiment.

2.3 GROUNDWATER COLLECTION

Anoxic groundwater was collected from a local well and returned to the laboratory within 2 hours. The well was purged before the sample was taken with a pump into a sterile 5 L container (pre filled with nitrogen gas). The samples were contained within chilly bins to maintain a stable temperature prior to starting the experiments. Oxic groundwater was taken on site directly into a sterile 5 L container. Each groundwater type was collected on day 1 (time zero) of the experiment and subsamples were sent to an external laboratory for analysis of the water chemistry (Hill Laboratories, Christchurch, New Zealand). The water chemistry of each of the groundwater at the time of sampling is presented in Table 1.

Each of the groundwater were aseptically added to the glass jars, two for oxic and two for anoxic groundwater. The anoxic jars were pre filled with nitrogen gas.

Table 1: Water chemistry measurements taken at the time of sampling. Note, both groundwater types were brought to 12°C before the start of the experiment.

Measurement	*Oxic groundwater*	*Anoxic groundwater*
Temperature (°C)	18.0	12.8
pH	7.9	7.12
Dissolved oxygen (DO) mg L^{-1}	7.3	0.1
Dissolved oxygen (DO) %	78.0	1.0
Conductivity µS cm	113.0	176.0
Oxidative reductive potential (ORP) mV	212.0	66.5
Total organic carbon (TOC) g m^{-3}	0.6	0.7
Total biochemical oxygen demand ($TBOD_5$) g O_2 m^{-3}	<2	<2
Total alkalinity g m^{-3} as $CaCO_3$	50	72
Total suspended solids g m^{-3}	<3	5
Total dissolved solids g m^{-3}	67	107
Total volatile solids g m^{-3}	<3	<3
Total phosphorous g m^{-3}	0.006	0.026
Phosphate g m^{-3}	0.034	0.036
Total nitrogen g m^{-3}	0.20	<0.11
Nitrate-N g m^{-3}	<0.002	<0.002

2.4 INOCULATION

To each jar, *Campylobacter* inoculum, prepared as described above, was added. One anoxic and one oxic groundwater was inoculated with HN16 strain and one anoxic and one oxic groundwater with the type strain NCTC 11351. The initial concentration of *Campylobacter* in each jar were 10^5 CFU per mL. To keep the *Campylobacter* in suspension during the experiment, the jars were gently shaken using a platform shaking incubator.

2.5 ANALYSIS

2.5.1 MICROBIAL ANALYSIS

Samples (5 mL) of the groundwater from each jar was taken aseptically at set time points over the experimental period. Samples were taken using a sterile 20 mL syringe connected to a tube that fitted to the glass sampling tube held within each jar. An initial 20 mL of sample was taken and discarded to flush the tubing. Subsequently a 5 mL sample was taken and placed in a pre-labelled sterile P35 bottle. Samples were then serially diluted in sterile peptone water to give a dilution series from 10^{-1} to 10^{-4}.

Samples for each dilution analysed were tested in triplicate by filtering the samples through 0.22 µM pore size filters (EZGSWG474, mixed cellulose ester, Merck, Germany). Filters were placed onto CampyCount agar plates (prepared by Fort Richards, New Zealand using Brilliance™ CampyCount dehydrated media, Oxoid, UK). Plates were incubated inverted in air tight boxes with microaerophilic gas generation packs (Oxoid™ Campygen™, ThermoScientific, USA). The plates were incubated at 37°C for 4±1 hours and 44°C for 44±4 hours. After incubation, typical (red) colonies were enumerated on plates. Plates with between 20-80 colonies on the plates were used to calculate end concentration of *Campylobacter* in samples per mL, taking into account the dilutions used.

2.5.2 STATISTICAL ANALYSIS

A first order die-off rate constant was calculated from regression model to evaluate the change in *Campylobacter* concentration over time. Comparison of the survival between the *Campylobacter* strains and survival of each strain between groundwater types was calculated with a t test on normalized data (SigmaPlot, Version 12.5).

3 RESULTS

During the experiments the levels of dissolved oxygen were maintained in each of the jars. Table 2 shows the average measurements in each of the jars with the range measured in parentheses. The temperature in all the jars were maintained between 12 and 14°C. The pH in the oxic and anoxic jars were different but were maintained to the levels that were measured in the two groundwater types before commencing the experiments. The DO were also maintained at the level that was measured in the two groundwater types.

After day 16 of the experiment the refrigeration unit failed on the platform shaking incubator and so the experiment had to be stopped at this point.

Table 2: Measurements taken in the jars during the experiment. Results show the average with range in parentheses.

	HN16		Type strain	
	Oxic	**Anoxic**	**Oxic**	**Anoxic**
DO mg L^{-1}	9.742 (8.671 – 10.6)	0.139 (0 – 0.835)	9.888 (8.884-10.575)	0.408 (0 – 1.736)
Temperature (°C)	12.8 (12.6 – 13.2)	12.5 (12.3 – 12.9)	13.3 (13.0 – 13.6)	12.4 (12.2 – 12.7)
pH	7.2 (6.9 – 7.5)	8.5 (8.0 – 8.8)	7.6 (7.4 – 7.9)	8.1 (7.9 – 8.4)

The results presented demonstrate that there are differences in the survival of the two *Campylobacter* strains tested and also differences in survival of *Campylobacter* HN16 depending on groundwater type. Figure 2 shows the average concentration of *Campylobacter* strains in groundwater types over time. The results presented are average of three replicates. Over the whole experimental period survival of *Campylobacter* HN16 was greatest in anoxic groundwater, and only a 1 log reduction was observed (Figure 2), equating to a 79.6% survival after 16 days (Table 3). The die-off rate of *Campylobacter* HN16 in anoxic groundwater was calculated to be 0.0873 days and T_{90} 6.85 days (Table 4).

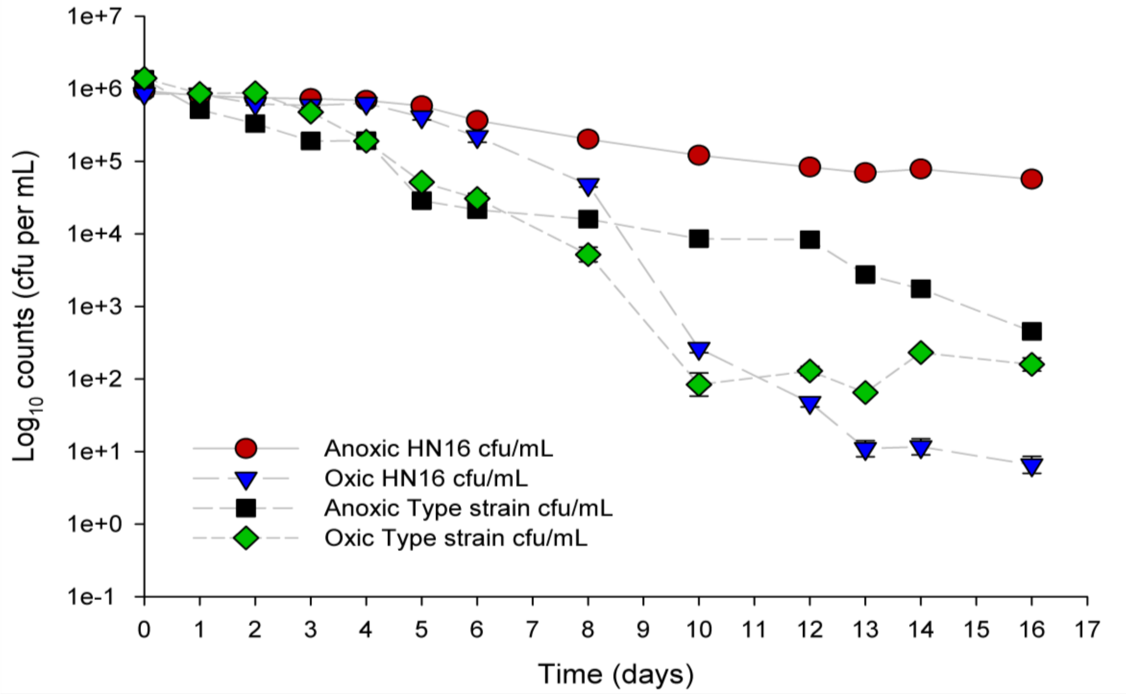

Figure 2: Campylobacter outbreak strain (HN16) and type strain NCTC 11351 survival in anoxic and oxic groundwater. The symbols are average (mean) counts (n = 3), lines are the standard error of the mean.

Interestingly, *Campylobacter* HN16 showed highest die off (4 log reduction) compared to the type strain NCTC 11351, in oxic groundwater. After 16 days only 14.2% survival was observed (Table 3). The die-off rate for *Campylobacter* HN16 in oxic groundwater was calculated as 0.391 days, T_{90} 1.52 days (Table 4).

The type strain NCTC 11351 gave similar survival over the whole experimental period, independent of groundwater type (Figure 2). A 4 Log reduction was observed after 16 days in both groundwater types (Table 3), with survival percentage calculated as 36.1% in oxic groundwater and 43.3% in anoxic groundwater.

Table 3: Mean Campylobacter (Log CFU per mL) during the experiment and percentage survival of each strain in both groundwater types.

	Oxic groundwater				**Anoxic groundwater**			
	HN16		**Type strain**		**HN16**		**Type strain**	
Time (days)	Log CFU per mL	% survival	Log CFU per mL	% survival	Log CFU per mL	% survival	Log CFU per mL	% survival
0	5.93	100.0	6.15	100.0	5.98	100.0	6.14	100.0
1	5.93	99.9	5.94	96.6	5.91	98.8	5.72	93.1
2	5.79	97.6	5.95	96.8	5.88	98.3	5.52	90.0
3	5.77	97.3	5.68	92.5	5.87	98.1	5.31	86.5
4	5.80	97.8	5.28	85.9	5.85	97.8	5.29	86.1
5	5.62	94.6	4.72	76.8	5.77	96.5	4.47	72.8
6	5.36	90.2	4.50	73.2	5.56	93.1	4.35	70.9
8	4.68	78.9	3.74	60.8	5.32	88.9	4.21	68.7
10	2.43	41.0	1.99	32.3	5.09	85.1	3.94	64.3
12	1.69	28.4	2.12	34.5	4.93	82.4	3.93	64.0
13	1.07	18.0	1.82	29.5	4.85	81.1	3.45	56.2
14	1.09	18.4	2.37	38.5	4.89	81.9	3.26	53.1
16	0.85	14.2	2.22	36.1	4.76	79.6	2.66	43.3

To compare the statistical significance of differences in survival between the two strains in each groundwater types the results over the 16 days of the experiment were compared. In oxic groundwater, after 16 days, there was not a significant difference in the survival of HN16 outbreak strain compared to type strain NCTC 11351 ($t = -1.79$, $df = 11$, $p = 0.088$). In anoxic groundwater, after 16 days, there was a significant difference in the survival of HN16 compared with NCTC 11351 ($t = 7.67$, $df = 11$, $p = <0.005$). In addition, over the whole experimental period there were significant differences between the survival of both the outbreak strain and the type strain in oxic compared with anoxic groundwater: HN16, $t = 8.45$, $df = 11$, $p = < 0.005$; NCTC 11351, $t = 3.71$, $df = 11$, $p = 0.001$.

When the results were studied more closely, it appeared that there was a difference in the survival characteristics depending on the time. When looking at the first 6 days (Table 3 & Figure 3), the outbreak strain showed no significant die off whereas type strain NCTC 11351 showed a 1-2 log die off, in both types of groundwater. For this part of the experiment there appeared to be no effect from the groundwater type on *Campylobacter* survival. When statistically analysed it was found that there was no significant difference in the survival of either *Campylobacter* strains in the two types of groundwater ($p = > 0.05$). There were significant differences between the two strains, however, with the outbreak strain HN16 present in significantly higher concentration in both groundwater types after 6 days ($p = < 0.001$).

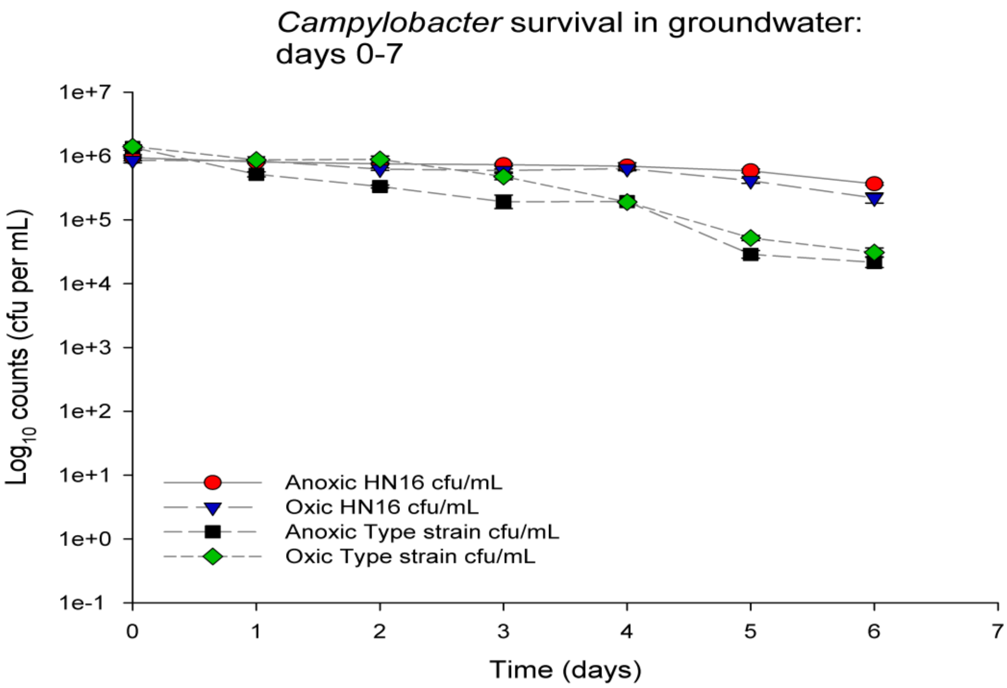

Figure 3: Initial survival of outbreak strain (HN16) and type strain of Campylobacter in groundwater. Figure shows days 0 to 7.

After day 7, to the end of the experiment, the type of groundwater appeared to have an effect on the survival of *Campylobacter* (Table 3 & Figure 4). Both strains of *Campylobacter* survived better in anoxic groundwater. The outbreak strain showed less than 1 log reduction in anoxic groundwater whereas over 4 log reduction was seen in oxic groundwater. The type strain showed a 2 log reduction in both groundwater types (Figure 4). It is interesting to note that there was a difference in the die off rate between groundwater types for the type strain. In oxic groundwater the type strain appeared to stabilise after day 12 and no significant die off was observed after this time.

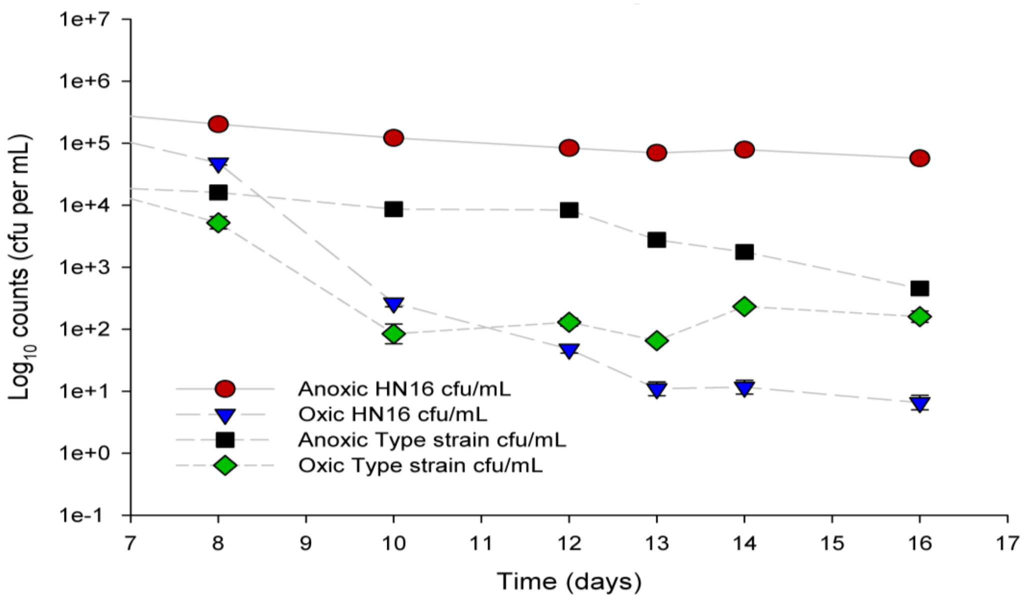

Figure 4: Survival of outbreak strain (HN16) and type strain of Campylobacter in groundwater. Figure shows days 7 to 17.

Table 4: Die off rates, and removal rates for Campylobacter strains HN16 and Type strain in both groundwater types. T90 is equivalent to a 1 Log removal, T99 to 2 Log removal and T99.9 to 3 Log removal.

Oxic groundwater						**Anoxic groundwater**				
Experimental range (days)	**Die-off rate (k, day^{-1})**	**R^2**	**T90 (days)**	**T99 (days)**	**T99.9 (days)**	**Die-off rate (k, day^{-1})**	**R^2**	**T90 (days)**	**T99 (days)**	**T99.9 (days)**
HN16										
0 to 3	0.062	0.873	9.65	10.62	10.71	0.0369	0.890	16.21	17.83	17.99
0 to 6	0.084	0.807	7.08	7.79	7.86	0.055	0.809	10.80	11.88	11.98
0 to 10	0.291	0.712	2.04	2.25	2.27	0.089	0.910	6.71	7.39	7.45
0 to 12	0.357	0.818	1.66	1.83	1.84	0.093	0.944	6.42	7.07	7.13
0 to 14	0.401	0.899	1.48	1.63	1.64	0.091	0.963	6.60	7.26	7.32
0 to 16	0.391	0.916	1.52	1.67	1.68	0.0873	0.965	6.85	7.53	7.60
Type strain										
0 to 3	0.138	0.873	4.46	4.91	4.96	0.268	0.963	2.29	2.52	2.54
0 to 6	0.288	0.928	2.14	2.35	2.37	0.289	0.934	2.12	2.33	2.36
0 to 10	0.396	0.928	1.55	1.71	1.72	0.225	0.927	2.73	3.00	3.03
0 to 12	0.382	0.949	1.61	1.77	1.79	0.192	0.898	3.19	3.51	3.54
0 to 14	0.343	0.940	1.80	1.97	1.79	0.188	0.939	3.27	3.59	3.62
0 to 16	0.309	0.913	1.99	2.19	2.21	0.193	0.954	3.18	3.50	3.53

4 DISCUSSION AND CONCLUSIONS

After 16 days, in anoxic groundwater 10^4 CFU per mL *Campylobacter* HN16 were cultured. Whereas the type strain NCTC 11351, in the anoxic groundwater, only 10^2 CFU per mL were culturable. In oxic groundwater only 10^1 CFU per mL of *Campylobacter* HN16 remained. Even though it has been shown that there is a significantly lower concentration of *Campylobacter* HN16 it must be considered with the fact that it has been estimated that as few as 10-100 cells could cause illness. There is also the difference seen between the two strains tested here indicating a possible difference between other strains of *Campylobacter* in groundwater. We are aiming to extend our experimentation to other outbreak strains of *Campylobacter jejuni* as well as other *Campylobacter* that can cause disease in humans. Previous studies have demonstrated that there is a difference in survival of *Campylobacter jejuni* strains in drinking water (Cools et al., 2003). This study also indicated a difference in the survival rate depending on the origin of the *Campylobacter jejuni*.

The die-off rate of the type strain NCTC 11351 (0.19 – 0.31 days, T_{90} 1.9 – 3.2 days) was consistent with other studies in faecal material in the environment. Gilpin et al. (2009) found that T_{90} of *Campylobacter* species in cowpats in pasture was 2.2 days. The prolonged survival of both strains (most marked in the outbreak strain HN16) in anoxic groundwater could be related to the lower oxygen levels as *Campylobacter* is a microaerophilic organism that survives better in lower oxygen levels. Previously, Cook and Bolster (2007) found that dissolved organic carbon also had an effect on *Campylobacter* survival in groundwater. This study still found low survival of *Campylobacter* (less than 14 days) and significantly lower than the indicator organism *E. coli* which survived for over 400 days. The two groundwater types were similar in terms of organic loading, temperature and only varied in their dissolved oxygen concentration. This points to the higher levels of oxygen in the oxic groundwater being a factor in limiting the survival of both *Campylobacter* strains. Previously, Buswell et al. (1998) found that oxygenation of mesocosms affected *Campylobacter* survival. They did point to significant differences occurring between different strains of *Campylobacter* in their survival at different oxygen levels. This could also be seen in the survival differences seen between the two strains observed in the oxic groundwater tested in this research.

There have been indications that *Campylobacter* forms a stable non-culturable state (viable but not culturable, VBNC) in the environment. Zhao et al. (2017) reports that *Campylobacter* has been proved to enter a VBNC state in food under conditions of low temperature, and nutrient or oxygen rich conditions. Although, not investigated in this research it could be a mechanism that *Campylobacter* could use to remain viable in the environment longer than previously thought. More research is needed in the area of groundwater to establish is VBNC plays a role in *Campylobacter* transport and survival.

The overall implications of these results are that *Campylobacter* could survive for substantial periods of time in groundwater. The importance of these results are seen when considering the potential for transport of viable *Campylobacter* in groundwater. Although most groundwater used for drinking water is required to be over a year to be used without treatment, there is the possibility for breakthrough of *Campylobacter* in certain circumstances. For example, over use of groundwater can lead to drawdown effects in wells which, could mean drinking water is younger than previously estimated to be.

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