Application of long amplicon propidium monoazide-PCR to assess the effects of temperature and background microbiota on pathogens in river water

Avid Banihashemi, Michele I. Van Dyke and Peter M. Huck

ABSTRACT

The decay rates of enteric waterborne pathogens were evaluated following the introduction of *Yersinia enterocolitica*, *Salmonella enterica*, *Campylobacter jejuni* and *Arcobacter butzleri* into river water at different temperatures (5, 15 and 25°C) for a period of 28 days. To improve the accuracy of the results a molecular viability assay, long amplicon propidium monoazide-polymerase chain reaction (PMA-PCR), was used to quantify the viable cell concentration and results from PCR with and without PMA were compared. As well, the effect of background microbiota was assessed for *Y. enterocolitica* and *S. enterica* by inoculating cells into sterile and non-sterile river water. Cell persistence was improved by up to 4 log for *Y. enterocolitica* and 4.5 log for *S. enterica* in sterile river water compared to natural river water, showing that the autochthonous biological activity in river water can accelerate the die-off of introduced bacteria. Results also showed that low temperature significantly improved the persistence of all four target bacteria in non-sterile river water. There was a more rapid decline in cell concentration in samples with PMA pretreatment; therefore using PMA-PCR analysis can provide more reliable data on viable/active enteric bacteria in aquatic microcosms and allows for improved assessment of pathogens in the environment.

Key words | *Arcobacter*, *Campylobacter*, *Salmonella*, temperature, water, *Yersinia*

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INTRODUCTION

Enteric pathogenic bacteria can be present in source waters used for drinking water and therefore are of concern for public health. Most enteric pathogens are not able to grow and replicate in water; however, they can survive for considerably long times in aquatic environments including surface waters, and therefore water can act as a route for their transmission (Hrudey & Hrudey 2004). Bacterial survival patterns in surface waters can be affected by different environmental factors including temperature, sunlight (UV), pH, salinity, nutrients and organic matter (e.g. Evison 1988; Wang & Doyle 1998; Obiri-Danso *et al.* 2001; Korajkic *et al.* 2013; Wanjugi & Harwood 2013; Pachepsky *et al.* 2014; Wanjugi *et al.* 2016). A better understanding of how environmental conditions affect pathogen longevity in water following point or non-point source contamination can offer valuable information for regulatory issues and health risk assessments.

To study the survival patterns of waterborne disease-causing bacteria, it is important to apply reliable enumeration methods. This is particularly important since many bacteria are able to enter a viable but non-culturable (VBNC) state and the potential formation of VBNC cells could influence pathogen survival rates in the environment (as reviewed by Oliver 2010; Hassard *et al.* 2016). The application of polymerase chain reaction (PCR)-based methods can address the issue of non-culturable bacteria, but such methods are not able to differentiate between live and dead cells or extracellular DNA that may be present in environmental aquatic

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systems. However, propidium monoazide (PMA) can be applied as a pretreatment to PCR to prevent amplification of dead cells with injured membranes or free DNA (Nocker *et al.* 2006). As shown in a previous study (Banihashemi *et al.* 2012), PMA pretreatment combined with long amplicon quantitative PCR (qPCR) could ensure that the false positive signal potentially originating from dead cells or extracellular DNA of *Salmonella enterica* and *Campylobacter jejuni* was not detected. Bae & Wuertz (2012) also showed that the application of PMA-qPCR could result in more realistic data for pathogen assessment and decay in water.

The present study aims to evaluate the longevity of enteric bacteria of concern in environmental (river) water using long amplicon PMA-qPCR analysis, with a particular focus on assessing the effects of temperature and naturally occurring microflora. Surface waters contain active bacterial communities that can affect the longevity of pathogens in water through processes including substrate competition, antagonism, and predation, and it is important to consider these effects when studying bacterial survival in the environment. It was previously shown that the presence of autochthonous microbiota can result in a more rapid decline in pathogen and/or indicator concentrations in natural water compared with disinfected water (Korhonen & Martikainen 1991; Korajkic *et al.* 2013; Wanjugi *et al.* 2016).

Long amplicon PMA-qPCR was used in this investigation to assess the persistence of enteric bacterial pathogens in river water, including representatives from *Yersinia, Salmonella*, *Campylobacter* and *Arcobacter*. Pathogens from each of these genera were previously shown to be present in rivers from the Grand River watershed (Cheyne *et al.* 2010; Van Dyke *et al.* 2010; Thomas *et al.* 2012; Banihashemi *et al.* 2015). *Yersinia* is a waterborne pathogenic bacterium that can survive for long periods, particularly in cold water (Colin 2006), and both *Salmonella* and *Campylobacter* are an important cause of waterborne disease worldwide (WHO 2011). *Campylobacter* is also recognized for readily forming a VBNC state and is therefore challenging to enumerate. *Arcobacter* has been recently reported as a possible health threat in source waters (Lee *et al.* 2012), and a previous study (Banihashemi *et al.* 2015) showed that high levels of viable *Arcobacter butzleri* were detected in water samples from the same river that was used in the current study (Grand River in Ontario, Canada).

The Grand River is typical of many drinking water sources, and water quality is affected by various activities such as farming, wildlife and urban discharges including runoff and treated wastewater. To study the effect of environmental factors on pathogen persistence trends in water from the Grand River, a strain from each of *Yersinia enterocolitica*, *S. enterica*, *C. jejuni* and *A. butzleri* was introduced separately into either untreated or autoclaved river water microcosms. Water samples were then incubated at three temperatures that represent seasonal differences expected in the river (5, 15 and 25°C) and sampled over a four week period. The experiments were conducted in laboratory incubators in the dark, and therefore the additional effect of sunlight on cell persistence was not assessed. To evaluate if the occurrence of dead cells or free DNA affected pathogen longevity patterns, cell concentrations were enumerated by PCR with and without PMA pretreatment.

MATERIALS AND METHODS

Bacterial cultures

S. enterica subsp. *enterica* ATCC 13311, *Y. enterocolitica* ATCC 9610, *C. jejuni* ATCC 35920, and *A. butzleri* ATCC 49616 were obtained from the American Type Culture Collection. *S. enterica*, *Y. enterocolitica*, and *A. butzleri* were grown on nutrient agar (BD Canada, Mississauga, ON) overnight at 37°C, and then inoculated separately into 100 mL of nutrient broth (BD Canada, Mississauga, ON) in 250 mL Erlenmeyer flasks. The cultures were grown overnight at 37°C without shaking. One mL of each culture was harvested by centrifugation at 12,000 × g for 5 min, and the cell pellet was resuspended in 10 mL of sterile phosphate buffered saline (PBS). *C. jejuni* was grown on Mueller Hinton blood agar (BD Canada, Mississauga, ON) at 42°C under microaerophilic conditions (CampyPak Plus System; BD Canada, Mississauga, ON) for 2–3 d. Using a sterile swab, colonies were suspended in 10 mL of sterile PBS. The cell concentration of each strain was then adjusted to 1×10^7 colony-forming units (CFU) mL^{-1} using PBS and was measured by direct microscopic cell count (Van Dyke *et al.* 2010).

Surface water collection and inoculation

A 10 L sample of river water was collected on 26 August, 2011 from a location on the Grand River north of Waterloo, Ontario, Canada. The sample was taken 2–3 m from the river edge and 10–20 cm below the surface in a fast-flowing area, then immediately placed on ice and transported to the laboratory. Turbidity of the water sample was 10.2 NTU with a temperature of 18.5°C at the time of collection. The river water was kept at 4°C and used within 24 h of collection. Five L was autoclaved at 121°C for 1 h and the remaining 5 L was not autoclaved, and then 500 mL samples from each were transferred into a series of 1 L sterile polypropylene bottles. *S. enterica* and *Y. enterocolitica* were inoculated separately into bottles to achieve a final concentration of 1×10^8 cells 100 mL^{-1}. One bottle of each strain in sterile or non-sterile water was placed in laboratory incubators at 5, 15, and 25°C and held stationary in the dark for 28 days. The same procedure was used to inoculate *A. butzleri* and *C. jejuni*; however, experiments involving these strains used water collected from the same sampling location on the Grand River on 9 October, 2011. The river water turbidity was 8.4 NTU and the temperature was 10.6°C at the time of collection. The persistence of *C. jejuni* and *A. butzleri* was only studied in non-sterile water.

At each sampling date, one 30 mL sample was taken from each 1 L bottle and transferred to a 50 mL sterile centrifuge tube and pelleted at 9,000 × g for 20 min. The supernatant was removed, and each pellet resuspended in 1 mL sterile PBS and transferred to a 1.5 mL microcentrifuge tube. The samples were centrifuged again at 12,000 × g for 5 min to achieve a final concentrated pellet for each sample. The pellet from each tube was then resuspended in 400 μL of sterile PBS. Each 400 μL sample was divided into two 200 μL samples, one of which was treated with PMA and the other with no PMA treatment as described below.

PMA pretreatment

Solid PMA (phenanthridium 3-amino-8-azido-5-[3-(diethylmethylammonio)propyl]-6-phenyl dichloride) was purchased from Biotium Inc. (Hayward, CA, USA), and a 4 mM stock solution was prepared in 20% (v/v) dimethyl sulfoxide (DMSO). The stock solution was transferred to 1.5 mL light-impermeable microcentrifuge tubes and stored at −20°C. PMA (15 μM final concentration) was added to one of the 200 μL sub-samples of concentrated water. The optimal PMA concentration was previously determined by Banihashemi *et al.* (2012), and the effectiveness of PMA in suppressing the PCR signal from dead cells or free DNA in water from the Grand River was confirmed by Banihashemi *et al.* (2015). After PMA addition, the cell suspension was mixed well by vortexing, followed by incubation in the dark for 5 min with constant mixing by inversion. The sample tubes were then placed on ice to avoid excessive heating and exposed to a 500 W halogen lamp (NOMA Lightgen) for 10 min at a distance of 20 cm with the caps open. All the tubes (with and without PMA treatment) were then centrifuged at 12,000 × g for 5 min and supernatant was discarded. The pellets were resuspended in 1 mL guanidium thiocyanate (GITC) buffer (5 mol L^{-1} GITC, 0.1 mol L^{-1} EDTA [ethylenediaminetetraacetic acid; pH 8.0], 5 g L^{-1} N-laurylsarcosine) and transferred to a −80°C freezer before DNA extraction.

DNA extraction and PCR

Concentrated river water samples suspended in GITC extraction buffer were mixed by inversion for 1 h at room temperature followed by purification using the Qiagen DNeasy tissue kit (Mississauga, ON, Canada) as described by Cheyne *et al.* (2009). After column purification (as described by the manufacturer), samples were eluted in 100 μL of AE buffer (150 × concentrated from the river water) and stored at −80°C until analysis.

PCR primers previously described by Banihashemi *et al.* (2012) were used for *C. jejuni* (JH23F and JH2534R, 1512 bp product, *cpn70* gene) and *S. enterica* (Sal-1614-F and Sal-1614-R, 1614 bp product, *invA* gene). Primers for *Y. enterocolitica* and *A. butzleri* were designed in this study using Beacon Designer 7.7 software (Bio-Rad, Mississauga, ON, Canada) and sequence data obtained from the National Center for Biotechnology Information (NCBI). Sequence alignments were carried out using ClustalW multiple alignment programme (Thompson *et al.* 1994) and refined using JalView alignment editor (Clamp *et al.* 2004). *Y. enterocolitica* primers were Yers-1213-F (5′-GGGAAGTAGTTTACTACTTTGCC-3′) and Yers-1213-R (5′-TGTGGTCCGCTTGCTCTC-3′) targeting

a 1213 bp segment of the 16S rRNA gene. *A. butzleri* primers were Arco-1415-F (5′-ACGAAGAATGTCTCTGGAACTC-3′) and Arco-1415-R (5′-GGAACTTATGTTACTCCTG-GAATG-3′) targeting a 1415 bp segment of the *rpoB* gene. Primers were obtained from Sigma-Genosys (Oakville, ON, Canada).

qPCR amplification was performed using Ssofast EvaGreen Supermix (Bio-Rad) and a Bio-Rad iCycler iQ Real-Time PCR Detection System. Each 50 μL reaction contained 20 μL of DNA template, 400 nmol of each primer, and 1× EvaGreen supermix. The PCR amplification conditions for the *Salmonella invA* gene fragment were as follows: one cycle at 95°C for 3 min; 50 cycles at 95°C for 30 s, 53°C for 30 s, 72°C for 1.5 min; and one cycle at 72°C for 10 min. Amplification conditions for the other three primer sets were the same except the annealing temperature was 46°C for *C. jejuni*, and 58°C for both *Y. enterocolitica* and *A. butzleri*. PCR product specificity was confirmed by melting curve analysis using a ramping rate of 0.5°C/10 s from 55 to 95°C. The presence of PCR inhibitors was done using an external *luxB* amplification method as described by Cheyne *et al.* (2010) and PCR inhibition was not detected in any samples. Each qPCR assay produced standard curves with R^2 values of 0.99 or greater and with efficiency values between 90 and 109%. DNA amplification was always observed for qPCR reactions containing 10 cells, which indicated that the detection limit was less than 10 cells per reaction (equivalent to 30 cells [1.5 log] 100 mL^{-1} in the river water). DNA extracted from uninoculated river water stored at 5°C was also tested at the end of the experiment using PCR without PMA treatment. Results showed that naturally occurring pathogens were below the detection limit of the assay.

Each sample was analysed by PCR in duplicate. Each PCR run included duplicate standard curves and negative PCR controls. The standard curves were produced using DNA extracted from pure cultures of the target bacteria. *C. jejuni* standard curves were prepared as described by Van Dyke *et al.* (2010). *Y. enterocolitica*, *S. enterica* and *A. butzleri* were grown overnight at 37°C in nutrient broth (BD Canada, Mississauga, ON) followed by staining and direct fluorescence microscopic cell count enumeration as described by Van Dyke *et al.* (2010). The Qiagen DNeasy tissue kit was then used for DNA purification and the final standard solutions were prepared by serial dilution in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and stored at −80°C.

Data analysis

Cell concentrations measured by qPCR were log transformed and each data point was plotted as the average log cells 100 mL^{-1} versus incubation time (days). Error bars on each symbol represent the 95% confidence interval of duplicate PCR measurements. Decay curves of log transformed data were calculated by regression analysis using GInaFiT (Geeraerd and Van Impe Inactivation Model Fitting Tool; Geeraerd *et al.* 2005). The model that best fits the data was selected based on decay rate coefficients, root mean squared error and R^2 values. A biphasic inactivation model (Cerf 1977) was found to be the best fit for all the river water data sets, but for certain data sets from autoclaved river water (as shown on Table 1) a log linear inactivation model (Bigelow & Esty 1920) was applied. The model decay curves were used to calculate decay rate coefficients, R^2 values and 1 log decimal reduction times (T_{90}) (Table 1). Multivariate analysis of variance (ANOVA) statistical analysis of experimentally measured bacterial concentrations was used to determine if there was a significant difference in cell persistence among temperatures based on the entire data sets including results from all sampling days. Any statistical probability equal to or less than $\alpha = 0.05$ was considered significant.

RESULTS AND DISCUSSION

Effect of temperature on pathogen persistence in river water

The decay of pathogens following inoculation into natural (untreated) river water was assessed by regression analysis. Results showed that all strains exhibited a biphasic decay pattern, with a more rapid reduction in cell concentration in the first week, followed by a lower rate of reduction in the subsequent 3 weeks (Figures 1–4). *Y. enterocolitica, S. enterica*, *C. jejuni* and *A. butzleri* cell concentrations measured by qPCR were compared at three different temperatures (5, 15, and 25°C), and results show that incubation at lower

Table 1 | Decay rate coefficients (kmax1 and kmax2) and calculated decimal reduction times for a 1 log (T_{90}) decrease in cell concentration in river water (A) or autoclaved river water (B) based on regression curve analysis

		kmax1 (days^{-1})		kmax2 (days^{-1})		T_{90} (days)[a]		R^2	
Strain	**Temp °C**	**−PMA**	**+PMA**	**−PMA**	**+PMA**	**−PMA**	**+PMA**	**−PMA**	**+PMA**
A. River water									
Yersinia enterocolitica	5	0.50	1.76	0.27	0.35	4.7	1.3	0.98	0.99
	15	1.21	2.30	0.34	0.24	1.9	1.0	0.98	0.96
	25	2.05	3.51	0.16	0.08	1.1	0.7	0.91	0.96
Salmonella enterica	5	0.54	1.02	0.10	0.15	4.3	2.4	0.93	0.97
	15	4.04	3.09	0.10	0.05	1.2	0.8	1.00	0.97
	25	5.89	5.43	0.06	0.06	0.4	0.4	0.97	0.98
Campylobacter jejuni	5	0.55	0.78	0.00	0.16	4.2	2.9	1.00	1.00
	15	2.51	2.65	0.12	0.16	0.9	0.8	0.99	1.00
	25	3.65	3.73	0.15	0.08	0.6	0.6	0.99	1.00
Arcobacter butzleri	5	1.02	1.34	0.17	0.26	2.3	1.7	0.99	0.98
	15	3.82	5.45	0.12	0.10	0.6	0.4	1.00	0.98
	25	5.67	5.40	0.14	0.10	0.4	0.4	0.98	0.99
B. Autoclaved river water									
Yersinia enterocolitica	5	0.05	1.23	NA[b]	0.00	44	2.0	0.60	0.83
	15	0.10	1.11	NA	0.07	23	2.7	0.92	0.70
	25	0.13	1.16	NA	0.29	18	2.5	0.91	0.94
Salmonella enterica	5	0.10	0.30	NA	0.02	22	8.2	0.63	0.86
	15	0.03	0.08	NA	NA	72	29	0.08	0.58
	25	0.07	0.11	NA	NA	34	21	0.58	0.65

A biphasic reduction model was applied except where indicated.
[a]T_{90} values were calculated based on the first decay phase for biphasic inactivation curves.
[b]NA, not applicable as a log linear decay model best fit the data set.

temperatures resulted in improved persistence in river water. The decay rates during the first phase (kmax1) increased with temperature for all strains, in samples measured both with and without PMA pretreatment (Table 1). Because of this, the T_{90} (time for a 1 log reduction in cell concentration) calculated for the first phase of decay were shorter as temperatures increased. In general, there was a greater difference in kmax1 and T_{90} between 5 and 15°C compared with the difference between 15 and 25°C, and this effect was more pronounced in samples analysed without PMA pretreatment. During the second phase of decay (typically following day 7), the decay rate constants were smaller and less affected by incubation temperature. At day 28, *Y. enterocolitica*, *C. jejuni* and *A. butzleri* incubated at 15 and 25°C all had an overall 6 log decrease in cell concentration, but for *S. enterica* the final cell concentrations were higher (overall 4 log reduction).

Other researchers have observed similar trends related to temperature for bacterial pathogen survival or persistence in surface waters (McCambridge & McMeekin 1980; Evison 1988; Korhonen & Martikainen 1991; Terzieva & McFeter 1991; Wang & Doyle 1998, Obiri-Danso *et al.* 2001; Van Driessche & Houf 2008). Pachepsky *et al.* (2014) surveyed a large number of published data sets on *Salmonella* survival in marine and freshwater, and found that overall there was an increase in inactivate rate with an increase in temperature. Results from the current study show that, in general, the decay profiles of the more closely related strains *C. jejuni* and *A. butzleri* were similar, but that *Arcobacter* die-off was more rapid compared with *Campylobacter*. This suggests that the higher concentrations of *A. butzleri* compared with *C. jejuni* shown by previous data in the Grand River (Banihashemi *et al.* 2015) were not due to differences in cell persistence, but likely higher inputs from point or non-point sources.

The application of a PMA-PCR viability assay in this study could provide more accurate data on the persistence and longevity of bacterial pathogens in water, because it was less

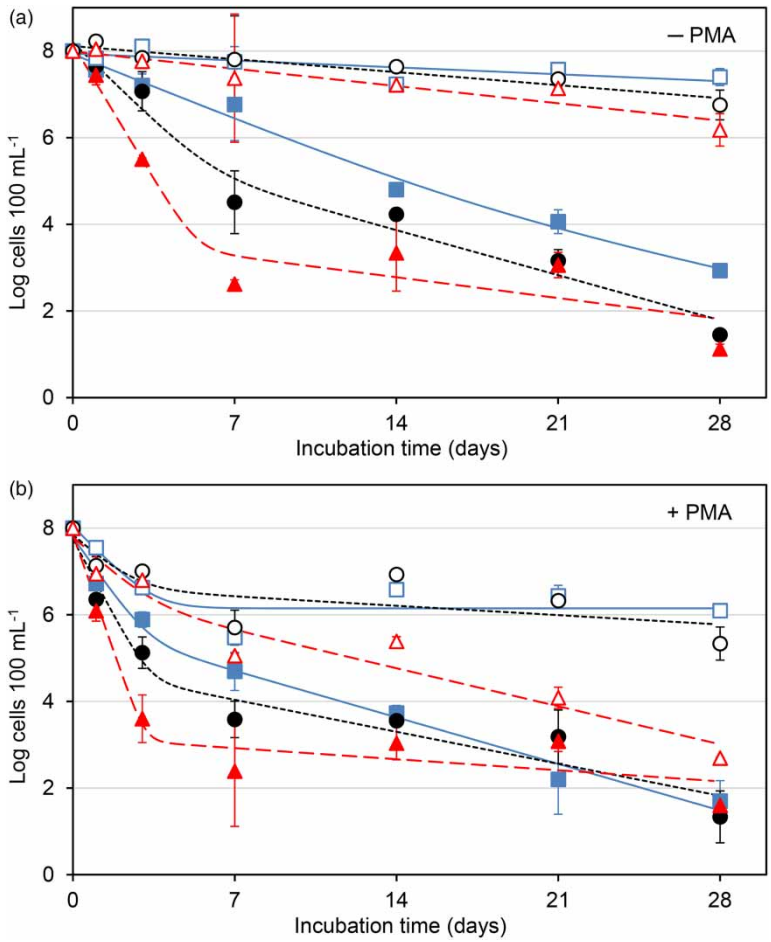

Figure 1 | Survival of *Yersinia enterocolitica* in river water (closed symbols) and autoclaved river water (open symbols) incubated at 5°C (■ □), 15°C (● ○), or 25°C (▲ △) and quantified by qPCR without (a) or with (b) PMA pretreatment. Error bars represent the 95% confidence interval of duplicate PCR measurements. Lines show the calculated model decay curves.

affected by VBNC cells or false positive signals from dead cells. The difference in persistence between viable (with PMA) and total (no PMA) cells was clearly evident in our results. For *Y. enterocolitica* in river water, there was a more rapid decline and higher kmax1 and T_{90} values at all three temperatures in samples with PMA pretreatment compared with those without PMA (Table 1). For *S. enterica* and *C. jejuni*, the effect of PMA pretreatment on results was only observed at 5°C, with little or no difference between total and viable cell concentrations or decay rate values at higher temperatures (15 and 25°C). *A. butzleri* showed higher kmax1 values in samples with PMA treatment at 5 and 15°C but not at 25°C. These results suggest that particularly at low temperatures the PMA-PCR viability assay can more reliably measure cell persistence in environmental samples.

To further evaluate the effect of temperature and PMA treatment on persistence trends in river water, a statistical comparison using a multivariate ANOVA was performed between pairs of temperatures (5 and 15°C, 15 and 25°C). Results in Table 2 show that both viable (with PMA) and total (no PMA) cell persistence were significantly affected by temperature, as the calculated $F_{observed}$ values for almost all paired comparisons (between each of the two temperatures) exceeded the $F_{critical}$ value of 4.75, with the exception of *S. enterica* and *A. butzleri* at higher temperatures (between 15 and 25°C). Overall results of the statistical analysis showed that the differences between temperatures were considerably smaller for viable compared with total cells. Although this should be further studied, these results suggest that the effect of temperature on the

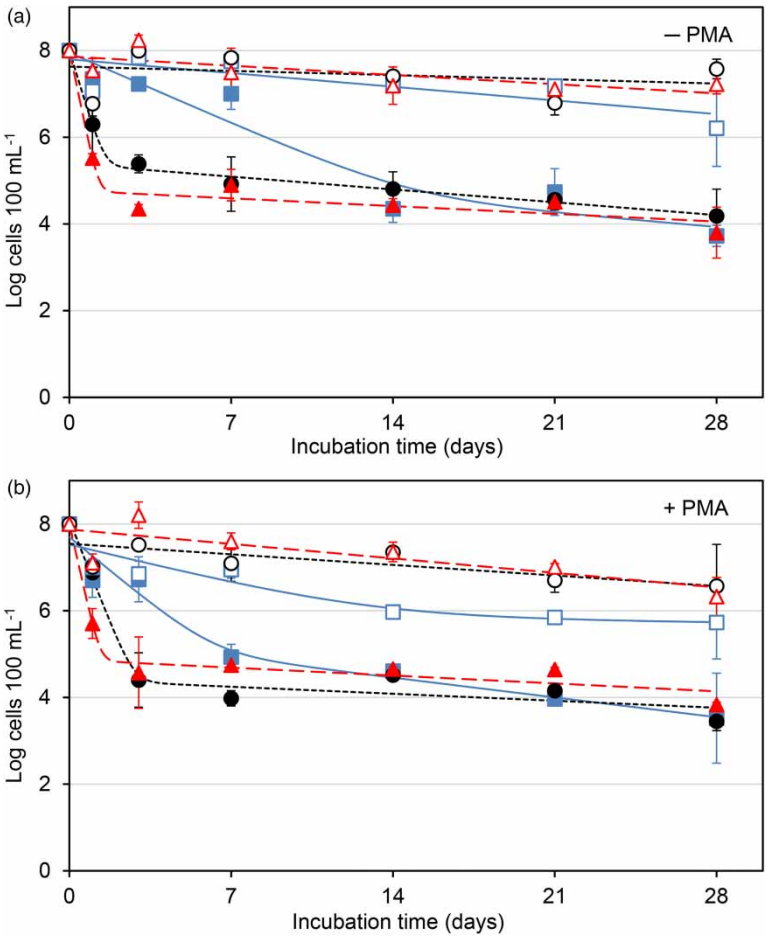

Figure 2 | Survival of *Salmonella enterica* in river water (closed symbols) and autoclaved river water (open symbols) incubated at 5°C (■ □), 15°C (●○), or 25°C (▲△) and quantified by qPCR without (a) or with (b) PMA pretreatment. Error bars represent the 95% confidence interval of duplicate PCR measurements. Lines show the calculated model decay curves.

persistence of bacteria may be overestimated using some methods that cannot differentiate viable and dead cells.

Effect of natural river water microflora on pathogen persistence

The effect of background microbiota on cell persistence was assessed for *Y. enterocolitica* and *S. enterica* by inoculating each strain separately into samples of sterile and non-sterile river water. Sterile river water was prepared by autoclaving to ensure inactivation of all biological systems. While it is possible that heating the samples may have caused some changes in the water, this method was selected to ensure inactivation of all microbial groups including viruses. In addition, owing to the high level of particles in the river water, large volume filtration would be difficult.

Results show that the persistence of *Y. enterocolitica* (Figure 1) and *S. enterica* (Figure 2) was considerably improved in the absence of background microorganisms at all three temperatures, and this effect was apparent for both viable (with PMA pretreatment) and total (without PMA pretreatment) bacteria. For both strains, samples analysed by qPCR without PMA pretreatment showed a log linear decay curve in sterile water with no effect of temperature (Table 1), and cell concentrations after 28 days decreased by less than 2 log units. Pathogen decay curves in sterile river water tended to have lower R^2 values (Table 1), possibly because the low decay rates were more affected by small differences in observed values.

Samples from sterile river water measured with PMA pretreatment (indicating viable cells) showed higher inactivation rates compared with the total cell count (no PMA).

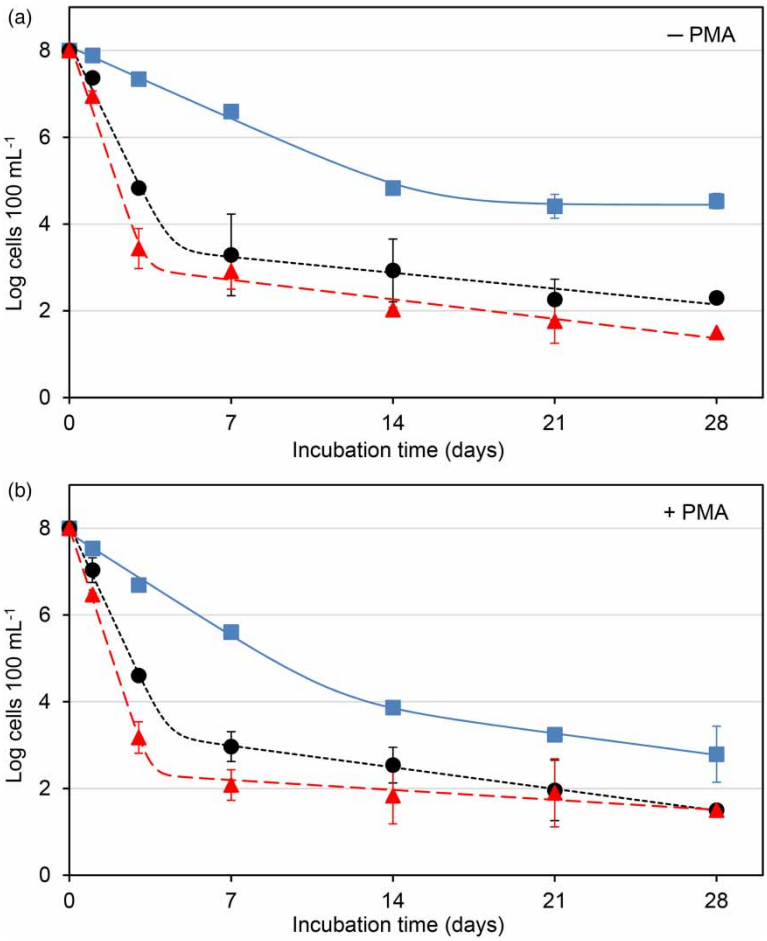

Figure 3 | Survival of *Campylobacter jejuni* in river water incubated at 5°C (■), 15°C (●), or 25°C (▲) and quantified by qPCR without (a) or with (b) PMA pretreatment. Error bars represent the 95% confidence interval of duplicate PCR measurements. Lines show the calculated model decay curves.

For *S. enterica* there was only a small increase in decay rate constants for viable cells, but for *Y. enterocolitica* this difference was greater. *Y. enterocolitica* analysed by PCR with PMA pretreatment followed a biphasic decay curve similar to those measured in non-sterile water. The rate constant (kmax1) and T_{90} values for *Y. enterocolitica* during the first phase of decay were similar at all temperatures, but during the second phase there was a higher decay rate (kmax2) at 25°C (0.29 $days^{-1}$) compared with 5 and 15°C (0.00 and 0.07 $days^{-1}$, respectively).

Overall, results suggest that the cell reduction rate was influenced by the activity of background microflora, with higher persistence values in sterile compared with non-sterile water. Other studies have also shown that the survival or persistence of bacterial pathogens and indicator strains (*E. coli*, *Enterococcus*) was similarly affected by the presence of background microflora (Flint 1987; Korhonen & Martikainen 1991; Kersters *et al.* 1996; Wang & Doyle 1998; Ramalho *et al.* 2001; Korajkic *et al.* 2013; Wanjugi & Harwood 2013; Wanjugi *et al.* 2016). Background microorganisms can include protozoan grazers and competing or predatory bacteria, and Wanjugi *et al.* (2016) found that predation had a greater effect compared with competition in reducing *E. coli* concentrations in a natural river water environment.

There are several reasons why temperature might influence pathogen inactivation rates and have a greater effect in non-sterile compared with sterile water. Background microflora including protozoan grazers and competing bacteria have been reported to be more active at higher temperatures (McCambridge & McMeekin 1980; Sherr *et al.* 1988). Also, river water with high microbial activity

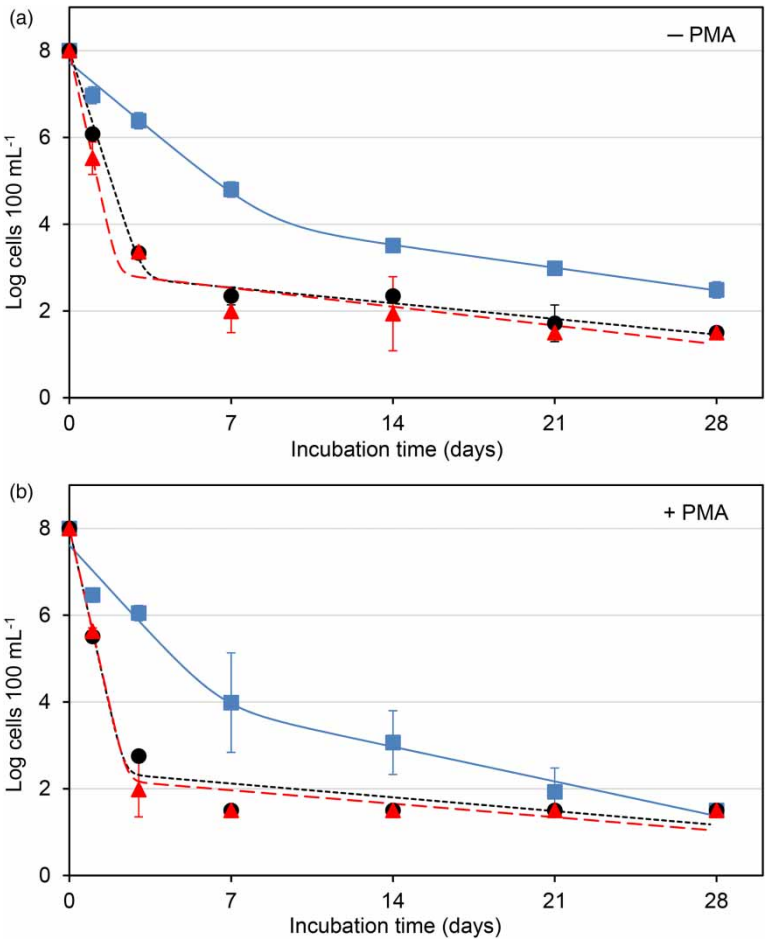

Figure 4 | Survival of *Arcobacter butzleri* in river water incubated at 5°C (■), 15°C (●), or 25°C (▲) and quantified by qPCR without (a) or with (b) PMA pretreatment. Error bars represent the 95% confidence interval of duplicate PCR measurements. Lines show the calculated model decay curves.

Table 2 | F-values determined by two-way ANOVA analysis to test the effect of temperature on pathogen persistence in river water

	Between 5 and 15°C		Between 15 and 25°	
Strain	**+ PMA**	**– PMA**	**+ PMA**	**– PMA**
Yersinia enterocolitica	5.62*	62.27*	13.15*	54.40*
Salmonella enterica	17.09*	33.21*	1.75	13.36*
Campylobacter jejuni	245.42*	230.44	23.82*	23.40*
Arcobacter butzleri	115.86*	1166.40*	0.85	24.76*

The $F_{critical}$ value at $\alpha = 0.05$ is 4.75, and values that show a significant difference are marked (*).

(i.e. Grand River water) can contain lytic enzymes and nucleases (Bazelyan & Ayzatullin 1979), and at higher temperatures the bacterial cell wall may be more fragile while interacting with extracellular enzymes (Baatout *et al.* 2005). This extracellular enzymatic activity could also be increased at high temperatures (Deming & Baross 2002) and may be the reason for faster cell and DNA degradation. These additive effects and higher microflora activity may be the reason why total (live + dead) cell reduction was more affected by high temperatures. Therefore, measuring viable cells is important in order to avoid overestimating the effect of temperature on cell reduction.

CONCLUSIONS

Molecular methods are more commonly used to assess pathogens in water, as they can provide specific and accurate detection in natural environmental samples. Using a

qPCR assay to compare pathogen persistence in sterile and non-sterile river water, this study showed that the presence of background autochthonous microbiota highly accelerated the reduction of inoculated strains of two bacterial pathogens (*Y. enterocolitica* and *S. enterica*). As well, cells in sterile water showed different inactivation patterns and were less affected by temperature compared with non-sterile water, therefore showing the importance of conducting studies in natural (untreated) samples. Bacterial persistence (both live and total) for all four strains tested (*Y. enterocolitica*, *S. enterica*, *C. jejuni* and *A. butzleri*) was significantly enhanced at lower temperatures in natural (non-sterile) river water, especially during the first week. Therefore, seasonal trends in pathogen occurrence in river water would be expected to occur, with higher survival at colder temperatures. This is supported by previous data on pathogen occurrence in the Grand River watershed using qPCR analysis, where higher *Campylobacter* concentrations were detected at colder water temperatures (Van Dyke *et al.* 2010), and *Yersinia* were detected more frequently at colder water temperatures (Cheyne *et al.* 2010).

By using a long amplicon PMA-PCR assay that can accurately assess viable cells, information in this study provided a better understanding on the persistence of waterborne bacteria and the effects of temperature as an environmental stress. In non-sterile river water, all four strains tested showed a more rapid decline when measured using PMA-PCR compared with no PMA pretreatment, especially at low incubation temperatures. By using the approach taken in this study, a better understanding of how environmental conditions can affect the persistence of important enteric pathogens in the aquatic environment was obtained. Information on pathogen longevity in water following point or non-point source contamination can provide improved data for health risk assessments, water treatment and regulatory decision-making.

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