Evaluation of Various \textit{Campylobacter}-Specific Quantitative PCR (qPCR) Assays for Detection and Enumeration of \textit{Campylobacteraceae} in Irrigation Water and Wastewater via a Miniaturized Most-Probable-Number–qPCR Assay

Graham S. Banting$^{a,b}$, Shannon Braithwaite,$^{a,b}$ Candis Scott,$^a$ Jinyong Kim,$^a$ Byeonghwa Jeon,$^b$ Nicholas Ashbolt,$^{a,b}$ Norma Ruecker,$^c$ Lisa Tymensen,$^d$ Jollin Charest,$^d$ Katarina Pintar,$^e$ Sylvia Checkley,$^{b,f}$ Norman F. Neumann$^{a,b}$

School of Public Health, University of Alberta, Edmonton, Alberta, Canada$^a$; Environmental Microbiology Program, Provincial Laboratory for Public Health, Edmonton, Alberta, Canada$^b$; Water Quality Services, Calgary, Alberta, Canada$^c$; Irrigation and Farm Water Division, Alberta Agriculture and Forestry, Lethbridge, Alberta, Canada$^d$; Infectious Disease Prevention and Control Branch, Public Health Agency of Canada, Ottawa, Ontario, Canada$^e$; Department of Ecosystem and Public Health, Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, Canada$^f$

**ABSTRACT**

\textit{Campylobacter} spp. are the leading cause of bacterial gastroenteritis worldwide, and water is increasingly seen as a risk factor in transmission. Here we describe a most-probable-number (MPN)–quantitative PCR (qPCR) assay in which water samples are centrifuged and aliquoted into microtiter plates and the bacteria are enumerated by qPCR. We observed that commonly used \textit{Campylobacter} molecular assays produced vastly different detection rates. In irrigation water samples, detection rates varied depending upon the PCR assay and culture method used, as follows: 0% by the de Boer Lv1-16S qPCR assay, 2.5% by the Van Dyke 16S and Jensen \textit{g}ly\textit{A} qPCR assays, and 75% by the Linton 16S endpoint PCR when cultured at 37°C. Primer/probe specificity was the major confounder, with \textit{Arco}bacter spp. routinely yielding false-positive results. The primers and PCR conditions described by Van Dyke et al. (M. I. Van Dyke, V. K. Morton, N. L. McLellan, and P. M. Huck, J Appl Microbiol 109:1053–1066, 2010, \url{http://dx.doi.org/10.1111/j.1365-2672.2010.04730.x}) proved to be the most stable and specific for \textit{Campylobacter} detection in water. \textit{Campylobacter} occurrence in irrigation water was found to be very low (<2 MPN/300 ml) when this \textit{Campylobacter}-specific qPCR was used, with the most commonly detected species being \textit{C. jejuni}, \textit{C. coli}, and \textit{C. lari}. \textit{Campylobacter} in raw sewage were present at ~10^7/100 ml, with incubation at 42°C required for reducing microbial growth competition from \textit{arcobacters}. Overall, when \textit{Campylobacter} prevalence and/or concentration in water is reported using molecular methods, considerable validation is recommended when adapting methods largely developed for clinical applications. Furthermore, combining MPN methods with molecular biology-based detection algorithms allows for the detection and quantification of \textit{Campylobacter} spp. in environmental samples and is potentially suited to quantitative microbial risk assessment for improved public health disease prevention related to food and water exposures.

**IMPORTANCE**

The results of this study demonstrate the importance of assay validation upon data interpretation of environmental monitoring for \textit{Campylobacter} when using molecular biology-based assays. Previous studies describing \textit{Campylobacter} prevalence in Canada utilized primers that we have determined to be nonspecific due to their cross-amplification of \textit{Arcobacter} spp. As such, \textit{Campylobacter} prevalence may have been vastly overestimated in other studies. Additionally, the development of a quantitative assay described in this study will allow accurate determination of \textit{Campylobacter} concentrations in environmental water samples, allowing more informed decisions to be made about water usage based on quantitative microbial risk assessment.

\textit{Campylobacter} spp. are Gram-negative, rod-shaped, motile bacteria of the class \textit{Epsilonproteobacteria} and the family \textit{Campylobacteraceae}, which contain the closely related genera \textit{Campylobacter}, \textit{Arcobacter}, and \textit{Helicobacter}. It was only in the 1990s that \textit{Arcobacter} and \textit{Campylobacter} were proposed to be separate genera due to the observation that \textit{Arcobacter} displays aerotolerance unlike \textit{Campylobacter}, which requires a microaerophilic atmosphere for culture (1). \textit{Campylobacter} and \textit{Arcobacter} are present in the gut of warm-blooded animals such as birds, cattle, and pigs (2–6). \textit{Campylobacter} is the leading cause of bacterial gastroenteritis in Canada, with an infection rate of 29/100,000 persons in 2013 (7). The predominant route of transmission is via contaminated foods (poultry in particular) (8), but with untreated water also being recognized as a potential source of infection. Campylobacteriosis rates in Canada have been shown to be elevated in comparison to those of control individuals if water consumption was from well water and the well was within 2 km of agricultural activity (9), and several campylobacteriosis outbreaks have been reported.
linked to water in Canada and Europe (10–13). Outbreaks are typically the result of either fecally contaminated surface runoff (manure) following rainfall or human sewage intrusion into source waters coupled with little or no treatment of drinking water supplies. High-intensity cattle, swine, or poultry farming combined with rainfall can result in fecal loading into groundwater and water of nearby waterways, which can subsequently be used for irrigation of crops. While there have been no confirmed campylobacteriosis outbreaks directly attributed to irrigation water, there have been multiple reports of gastroenteritis outbreaks associated with contaminated produce. Leafy and root vegetables have been associated with at least seven campylobacteriosis outbreaks between 1998 and 2008 in the United States (14). Additional studies have detected live Campylobacter spp. and closely related Arcobacter spp. on fresh produce (15, 16). Contamination of fresh produce with other enteric bacteria (Escherichia coli O157: H7) from water has led to gastroenteritis outbreaks (17), demonstrating the linkage between illness and consumption of contaminated vegetables. Direct and indirect costs due to gastroenteritis can be significant, e.g., >75 million euros/year in the Netherlands for campylobacteriosis alone in 2011 (18).

Understanding the influence of irrigation water quality on downstream risk, in terms of potential sources of contamination (i.e., wastewater or animal manure), is an important step in evaluating the public health risks posed by this organism in food and water. Evaluating the public health risks posed by this organism in food and water of nearby waterways, which can subsequently be used for irrigation of crops. While there have been no confirmed campylobacteriosis outbreaks directly attributed to irrigation water, there have been multiple reports of gastroenteritis outbreaks associated with contaminated produce. Leafy and root vegetables have been associated with at least seven campylobacteriosis outbreaks between 1998 and 2008 in the United States (14).

Bacterial strains and growth conditions. Campylobacter type strains were purchased from Cedarlane Laboratories (Burlington, Ontario, Canada) for use in the development of the MPN-qPCR assay and included the following strains: (i) Campylobacter jejuni ATCC 29428; (ii) Campylobacter coli ATCC 33559; (iii) Campylobacter lari ATCC 35221; (iv) Campylobacter fetus ATCC 27374; (v) Campylobacter hyointestinalis ATCC 35217; and (vi) Campylobacter upsaliensis ATCC 43954. Isolates were initially cultured on blood agar plates (BAP) (Dalynn Biologicals, Calgary, Alberta, Canada) at 37°C in a Mitsubishi AnaeroPak system (ThermoFisher Scientific, Ottawa, Ontario, Canada) with MicroAero Paks generating a microaerophilic environment. Human-derived Campylobacter isolates were obtained from the Alberta Provincial Laboratory for Public Health (ProvLab) and were cultured as described for the type strains.

Target cloning and limit-of-detection determination. Campylobacter PCR targets were amplified from genomic DNA from the type strains (listed above) and cloned into pCR2.1-TOPO (ThermoFisher Scientific, Ottawa, Ontario, Canada). (q)PCR was run on serial dilutions of 50,000 to 0.5 copies per reaction as described below. The 95% confidence limit of detection (LOD95) was determined by the method of Wilrich and Wilrich (29) for each target assayed (Table 1).

Sample preparation for irrigation water. Irrigation water samples from southern Alberta, collected during the 2014 and 2015 field seasons, were sampled in 1-liter sterile plastic jars and shipped overnight on ice to the ProvLab in Edmonton, Alberta, Canada. The following morning, 400 ml of each sample was spun at 10,000 × g in sterile Nalgene bottles in a Sorvall RC5C centrifuge at 20°C for 20 min (Fig. 1). For the 2014 samples, the resulting pellets were resuspended in Bolton broth (BB) (Oxoid CM0983; ThermoFisher, Nepean, Ontario, Canada) containing Bolton selective supplements (Oxoid R0183) and 25 mg/liter sulfamethoxazole (BBmx) (Sigma, Markham, Ontario, Canada) to a total volume of 4 ml. For the 2015 samples, sulfamethoxazole was omitted.

Sample preparation for wastewater. Sewage samples were provided by the City of Calgary from the Pine Creek wastewater treatment plant, a system that serves a mostly residential portion of the city. Samples were collected from the raw, post-girt-screened influent and shipped on ice by courier to the ProvLab in Edmonton within 24 h of collection. Samples were processed as described for irrigation water, with the following slight modifications: 100 ml of wastewater was diluted to 400 ml with sterile buffered water prior to centrifugation. Resuspension of the resulting pellet was done either in Bolton broth with selective supplement (BB) medium or BB supplemented with rifampin (10 mg/liter; Sigma, Markham, Ontario, Canada) and polymyxin B (5,000 IU/liter; Sigma, Markham, Ontario, Canada) (BBRP).

Matrix spikes. In order to validate assay recoveries, matrix spikes were performed on a random irrigation water sample from the sampling date.
| Assay and primer | Oligonucleotide sequence (5'-3') | Targeted organism | Product size (bp) | Primer/probe concentration (nM) | Annealing temperature (°C) | LOD95 copies (L/U) |
|-----------------|---------------------------------|-------------------|------------------|-------------------------------|---------------------------|---------------------|
| Linton 16S PCR  | Linton 16S F GGATGACACTTTTCGGAGC | Campylobacter spp. | 816              | 300/200                       | 55                        | 6.25 (1.5/17.1)     |
|                 |                                 |                   |                  |                               |                           |                     |
| de Boer LV1-16S| de Boer LV1 F CTGGAMGCAGCAACGCC | Campylobacter spp. | 107              | 300/100                       | 60                        | 3.2 (1.5/6.94)      |
|                 |                                 |                   |                  |                               |                           |                     |
| Van Dyke 16S qPCR | Van Dyke 16S F CTGCTTAACACAAGTTGAGTAGG | Campylobacter spp. | 287              | 300/100                       | 60                        | 4.3 (2.3/8.1)       |
|                 |                                 |                   |                  |                               |                           |                     |
| Jensen glyA qPCR | Jensen glyA F CCGTCTTGGAGCTTATCTTTTGCAGACA | Campylobacter coli | 80               | 300/100                       | 60                        | 2.1 (0.5/8.4)       |
|                 |                                 |                   |                  |                               |                           |                     |
| Jensen glyA cj qPCR | Jensen glyA cj F CAAATAAAGTTAGAGGTAGAATGT | Campylobacter jejuni | 135              | 300/100                       | 60                        | 2.1 (0.5/8.4)       |
|                 |                                 |                   |                  |                               |                           |                     |
| Jensen glyA cj qPCR | Jensen glyA cj F CAAATAAAGTTAGAGGTAGAATGT | Campylobacter lari | 96               | 300/100                       | 60                        | 2.1 (0.5/8.4)       |
|                 |                                 |                   |                  |                               |                           |                     |
| Yamazaki multiplex PCR | | Campylobacter coli | 502              | 200/—                       | 58                        | 6.25 (2.3/17.1)     |
|                 |                                 |                   |                  |                               |                           |                     |
under interrogation. *C. jejuni* ATCC 29428 was cultured as described above, scraped from the BAP agar into 10 ml of phosphate-buffered saline, and enumerated by plating serial dilutions back to BAP. One-milliliter-volume dilutions in the \(10^{-4}\) to \(10^{-6}\) range of these pure cultures were spiked into a random water sample prior to centrifugation and processed as described for the other samples. Spikes ranged from \(10^2\) to \(10^5\) CFU per sample. The number of *C. jejuni* bacteria spiked in each sample varied by week due to the fact that the bacteria in the spiked sample were...
enumerated concomitantly with those in the cultured stock in order to ensure accurate quantitation. This was due to our concern that the viability of *Campylobacter* could be affected in stock cultures when they were stored at 4°C for 48 h while plate quantifications were performed.

**Miniaturized MPN-qPCR assay for irrigation water.** The miniaturized three-tube MPN assay was based on methods described by Chenu et al. (28) with minor modifications. The resuspended pellet was split into three 1-ml aliquots and added to an initial deep-well (1-ml, 96-well) MPN plate (Greiner BioOne), followed by serial dilution to 10⁻³ for (for irrigation water) in BB (2015 samples) or BBsxm (2014 samples) media. The plates were covered with loose-fitting hard-shell lids and placed in AnaeroPack jars (Mitsubishi) with AnaeroPack-MicroAero (Mitsubishi) and incubated for 42 to 44 h at 37°C (2014 samples) or 42°C (2015 samples). Initially, incubation at 37°C was chosen based on the findings of Khan et al. (20), who observed that incubation at 37°C led to an increased overall recovery and a greater diversity of *Campylobacter* spp. from water. After incubation, the plates were opened and diluted 1:3 into a second MPN plate (0.2 ml, 96 wells; Greiner BioOne) containing BBsxm plus 150 μg/ml of the metabolic indicator triphenyltetrazolium chloride (TTC; Sigma, Markham, Ontario, Canada) (BBsxmT). Unlike the method of Chenu et al. (28), agar was not added to this medium and it was left as a liquid broth. This second plate was incubated as for the first plate but only for 18 to 20 h at 37°C. In the 2015 samples, the second plate was eliminated completely and the incubation temperature was changed to 42°C (based on results using wastewater as a matrix), in order to compare the relative sensitivities of a single-step enrichment assay for screening samples.

**Miniaturized MPN-qPCR assay for wastewater.** Due to the fact that very few irrigation water samples were contaminated with *Campylobacter* in Alberta, we used wastewater as a matrix to further optimize detection. However, because of the high microbial content of wastewater (and, in particular, *Arocobacter* spp.), we examined the effects of increasing the stringency of the culture conditions in some experiments by raising the incubation temperature to 42°C and adding two additional antibiotics (rifampin and polymyxin B). MPN cultures were grown at 37°C and 42°C in either BB or BBRP medium. Additionally, MPN cultures were diluted to 10⁻¹ to more accurately enumerate high-prevalence contaminating species (*Arocobacter* spp.). Only a single enrichment step was performed; time, temperature, and atmosphere conditions for this primary enrichment step were the same as for irrigation water samples.

**(q)PCR.** After incubation in the primary or secondary MPN plates, cultures were diluted 1:10 in H₂O and heated at 95°C for 10 min to lyse the cells. The initial screening for *Campylobacter* presence was performed on an Applied Biosystems TaqMan 7500 fast real-time PCR system using the qPCR primer/probe conditions described by de Boer et al. (30) (here referred to as the de Boer Lv1-16S qPCR assay) (Table 1) using 5 μL of the diluted, boiled cultures as the template. Subsequently, screening was performed with additional *Campylobacter* (q)PCR assays to compare the specificities of the primers for the detection of *Campylobacter*. These included the PCR primers and conditions described by Van Dyke et al. (19) (Van Dyke 16S qPCR), Jensen et al. (31) (Jensen glyA qPCR), and Linton et al. (32) (Linton 16S endpoint PCR), as outlined in Table 1. All qPCR assays were fast cycled using 1× TaqMan fast advanced master mix (ThermoFisher Scientific, Ottawa, Ontario, Canada) and 200 μg/ml bovine serum albumin (BSA; Sigma, Markham, Ontario, Canada) with the following cycling conditions: 50°C for 2 min, followed by 95°C for 20 s and then 40 cycles of 95°C for 3 s and 60°C for 30 s. The de Boer Lv1-16S and Van Dyke 16S assays were duplexed with an internal amplification control (IAC) assay (Table 1) in which 100 copies of IAC plasmid (33) was spiked into each reaction mixture to determine if PCR inhibition was occurring. Samples were deemed inhibited if cycle threshold (Cₚ) values of the IAC assay were shifted by ≥3. High-performance liquid chromatography-purified primers and probes were purchased from ThermoFisher Scientific. Primer/probe concentrations for each assay are shown in Table 1.

The Linton 16S endpoint PCR was also performed on all 2014 irrigation water MPN cultures. These reactions were amplified using Maxima hot start master mix (ThermoFisher Scientific, Ottawa, Ontario, Canada) containing 200 μg/ml BSA using the following cycling conditions: 95°C for 4 min, followed by 35 cycles of 95°C for 20 s, 55°C for 30 s, and 72°C for 1 min. Reactions were run on 2% agarose gels and photographed on an ImageQuant LAS4000 imager (GE Biosciences, Mississauga, Ontario, Canada).

MPN cultures were also screened with an Arcobacter butzleri-specific qPCR assay targeting the heat shock protein 60 gene (hsp60), as described by de Boer et al. (30) (Table 1). This was undertaken to resolve the specificity of the *Campylobacter* assays as a result of significant Arcobacter growth in the MPN cultures that confounded *Campylobacter* identification. Cycling conditions were as described for the *Campylobacter* qPCR assays described above.

**Species confirmation.** MPN wells that showed exponential amplification by the *Campylobacter* genus-specific qPCR were selected for secondary screening for confirmation of the occurrence of *Campylobacter* spp. by using the methods of Yamazaki-Matsune et al. (34) (here referred to as the Yamazaki multiplex PCR; Table 1). As the Yamazaki multiplex PCR assay did not always identify a putative *Campylobacter* species, the Linton 16S PCR amplicons were also sequenced by Sanger method sequencing (Macrogen, Seoul, South Korea), and the resulting DNA sequences were subjected to Basic Local Alignment Search Tool (BLAST) analysis to confirm identity. qPCR-positive wells were plated on Bolton agar, and putative *Campylobacter* colonies were enriched in Bolton broth overnight at 37°C, followed by PCR (described below) to confirm identity.

For samples from the 2015 irrigation season, *Campylobacter*-positive samples and isolates were also screened by the Jensen glyA qPCR (three assays) (Table 1) and the Khan ITS multiplex PCR (35) (Table 1) to identify *Campylobacter* bacteria to the species level. The Jensen glyA assays for *C. jejuni* and *C. lari* were performed together (i.e., duplex), and the *C. coli* assay was processed by itself (i.e., simplex). Cycling conditions were the same as for the other assays described above. The Khan ITS assay was run with Qiagen multiplex PCR master mix (Qiagen, Toronto, Ontario, Canada) with the addition of 200 μg/ml BSA per the manufacturer’s instructions. Reactions were cycled using the following program: 95°C for 15 min, followed by 35 cycles of 95°C for 30 s, 46°C for 30 s, and 72°C for 60 s. Reaction mixtures were run on 2.25% agarose gels and photographed as described above. *Campylobacter* isolates from human feces and wastewater were tested with the same PCR panel described above.

**MPN enumeration.** The bacteria in the MPN cultures were enumerated based on either the TTC metabolic indicator change after selective enrichment as described by Chenu et al. (28) and/or the *Campylobacter* qPCR results. Wells were deemed positive by qPCR if they displayed exponential amplification with a Cₚ value of <35 and with no inhibition detected in IAC controls. Standard three-tube MPN tables were followed to determine an MPN/300 ml (irrigation water) or an MPN/100 ml (wastewater).

**RESULTS**

*Campylobacter* MPN-qPCR method validation using matrix spikes. In order to validate the performance of the MPN-qPCR assay, *C. jejuni* ATCC 29428 was used as a matrix spike in auto-claved irrigation water on each of the 16 sampling dates in the 2014 and 2015 irrigation field seasons. The initial spikes prior to the centrifugation step ranged from ~10⁰ to 10⁵ CFU in each sample. In 15 of 16 cases, the matrix spike was observed by qPCR positivity (Van Dyke 165 assay) in the MPN cultured wells (Table 2). Accounting for serial dilution in the MPN plates, PCR positivity was observed across the spiked cultures, with an inoculum equivalency between 1 and 500 CFU being detected by the method after culture enrichment. Matrix spike recoveries were calculated based on the calculated MPN of the spike (based on qPCR positivity of the individual wells) versus the plate-counted inoculum and ranged from 0.5 to 71% in the 37°C culture method and 9 to
TABLE 2 Matrix spike recoveries of C. jejuni in irrigation water using an MPN-qPCR assay

| Sample | No. of C. jejuni CFU/spike<sup>a</sup> | MPN detection limit (no. of C. jejuni CFU) | Spike recovery of C. jejuni (%) | A. butzleri MPN in spike sample<sup>a</sup> |
|---|---|---|---|---|
| 1<sup>b</sup> | 1.1 × 10<sup>2</sup> | 8.0 × 10<sup>0</sup> | 11.3 | 0 |
| 2<sup>b</sup> | 3.9 × 10<sup>2</sup> | 1.0 × 10<sup>0</sup> | 71.2 | 0 |
| 3<sup>b</sup> | 5.9 × 10<sup>2</sup> | 4.4 × 10<sup>2</sup> | >0.5 | 9.3 |
| 4<sup>b</sup> | 4.0 × 10<sup>3</sup> | 3.0 × 10<sup>3</sup> | 0.7 | 46 |
| 5<sup>b</sup> | 5.8 × 10<sup>4</sup> | 4.4 × 10<sup>1</sup> | >14.6 | 0.4 |
| 6<sup>b</sup> | 2.4 × 10<sup>5</sup> | 2.0 × 10<sup>0</sup> | 11 | >2,400 |
| 7<sup>b</sup> | 3.8 × 10<sup>5</sup> | 2.9 × 10<sup>2</sup> | 0.1 | 111 |
| 8<sup>b</sup> | 1.2 × 10<sup>6</sup> | 9.0 × 10<sup>0</sup> | 10.1 | 46 |
| 9<sup>b</sup> | 2.6 × 10<sup>6</sup> | 0.8 × 10<sup>0</sup> | 15.4 | 0 |
| 10<sup>b</sup> | ND<sup>c</sup> | DNQ<sup>d</sup> | DNQ | 0 |
| 11<sup>b</sup> | 1.0 × 10<sup>7</sup> | 2.5 × 10<sup>0</sup> | 9.3 | 2.3 |
| 12<sup>b</sup> | ND<sup>c</sup> | ND | ND | 0 |
| 13<sup>b</sup> | 2.6 × 10<sup>8</sup> | 0.6 × 10<sup>0</sup> | 177 | 0.4 |
| 14<sup>b</sup> | 2.8 × 10<sup>8</sup> | 0.7 × 10<sup>0</sup> | 33.2 | 0.9 |
| 15<sup>b</sup> | 2.8 × 10<sup>8</sup> | 0.7 × 10<sup>0</sup> | 164.3 | 0 |
| 16<sup>b</sup> | 1.4 × 10<sup>9</sup> | 0.4 × 10<sup>0</sup> | 171.4 | 0 |

<sup>a</sup> Spiked into 400 ml of irrigation water prior to centrifugation step.
<sup>b</sup> Double-enrichment MPN assay at 37°C (2014 samples).
<sup>c</sup> Single-enrichment MPN assay at 42°C (2015 samples).
<sup>d</sup> ND, indeterminate. Could not enumerate cells before spike due to swarming on plate.
<sup>e</sup> DNQ, detected by qPCR, but enumeration not possible due to swarming on plate.
<sup>f</sup> ND, not detected. No cells went into spike.
<sup>g</sup> Determined by the de Boer hsp60 qPCR

171% in the 42°C culture method (assay development described below). Matrix spike recoveries were consistently higher in the 42°C method, and this was attributed to the reduction in competition from Arcobacter and other bacteria (expanded upon in “Campylobacter detection in wastewater” below). The A. butzleri MPN in each spike sample was generally lower in the 2015 samples cultured at 42°C than in the 2014 samples cultured at 37°C (Table 2), supporting this hypothesis. These matrix spike results confirm the sensitivity of both the centrifugation and culture/PCR portions of the assay, with as little as 1 CFU of C. jejuni detectable by the method after culture enrichment (Table 2).

**Campylobacter occurrence in irrigation water in 2014.** The initial development of the MPN-qPCR assay was performed using 80 irrigation water samples from southern Alberta between June and September 2014. For these samples, a double-enrichment MPN assay at 37°C was performed with the metabolic indicator TTC in the second enrichment (Fig. 1A and B), as described by Chenu et al. (28). Initial qPCR screening with the Campylobacter genus-specific de Boer Lv1-16S qPCR assay (Table 1) failed to yield any samples showing exponential qPCR amplification (Table 3), yet 89% of the samples displayed a color change in the medium, indicating bacterial growth in these wells. In these metabolism-positive samples (i.e., TTC positive), an average MPN of 94.2 ± 178/300 ml was observed, which is an underestimation of occurrence, since four samples were not included in the calculation as they were outside the dynamic range of the MPN assay (>2,400 MPN/300 ml). Based on reports of high levels of Campylobacter detected in other Canadian studies, we rescreened the MPN cultures using the Linton 16S endpoint assay as described by Khan et al. (20, 21) (Table 1). The Linton 16S assay yielded a 75% positivity rate across MPN wells, with an average MPN value of 55.7 ± 184/300 ml (Table 3), with one sample excluded from the calculation for having an MPN value of >2,400/300 ml. To confirm sequence identity and specificity of the assay, 65 of the Linton 16S endpoint PCR amplicons (originating from 60 water samples) were sequenced and subjected to BLAST analysis. DNA sequence analysis revealed that 60 of 65 amplicons were actually Arcobacter butzleri, while 3 of 65 were confirmed to be Arcobacter cryaerophilus. Only 2 of 65 were confirmed as C. lari, suggesting that the primer set of Linton et al. (32) was not specific to the Campylobacter genus. These results made us question the Campylobacter PCR assay specificities used by other researchers when examining the occurrence of Campylobacter in water. As such, all MPN cultures were rescreened using five additional PCR assays (see below).

**Campylobacter PCR assay sensitivity/specificty comparisons.** Due to the lack of concordance between the de Boer Lv1-16S qPCR, Linton 16S endpoint PCR, and metabolic TTC assays, we rescreened all 2014 irrigation samples from MPN cultures by using the Van Dyke 16S qPCR, the Jensen glyA assays (C. coli/C. jejuni/C. lari), and an A. butzleri-specific qPCR assay (hsp60) (Table 1). The Van Dyke 16S qPCR and Jensen glyA qPCR assays both identified the two samples previously classified as C. lari through sequencing of the Linton 16S PCR amplicons, both of which were missed by the de Boer Lv1-16S qPCR assay (Table 3). The Van Dyke 16S qPCR and Jensen glyA qPCR assays did not identify any additional Campylobacter-positive samples not identified by Linton 16S ampiclon sequencing, demonstrating their strong specificity to the Campylobacter genus (and not Arcobacter).

During the initial PCR screen with the de Boer Lv1-16S assay, nonexponential amplification was often observed (Fig. 2) in a portion of the irrigation water samples, which we originally assumed to be nonspecific. However, this nonspecific amplification often coincided with the presence of A. butzleri in the sample, as determined by the hsp60 amplification assay results (Fig. 2B to E). In some cases, low-level nonspecific amplification was observed in the de Boer Lv1-16S assay in the absence of a positive A. butzleri hsp60 assay result (Fig. 2A). In these cases, sequencing of the Linton 16S ampiclon determined that A. cryaerophilus was present.
FIG 2  Five MPN cultures amplified by various qPCR assays for *Campylobacter* (de Boer Lv1-16S, circles; Van Dyke 16S, triangles) and *Arcobacter butzleri* (hsp60, squares). The five wells were determined to contain *Arcobacter cryaerophilus* (A), *A. butzleri* and *C. lari* (B and C), *A. butzleri* (D), and *A. butzleri* with a *C. jejuni* spike (E). (F) The same samples were run with the Yamazaki multiplex PCR (containing the Linton 16S assay amplicon), with individual lanes 1 to 5 corresponding to panels A to E. Lane 6, Yamazaki multiplex positive control; lane 7, no-template control; lane 8, 100-bp ladder. The Van Dyke 16S assay was able to amplify *Campylobacter* in the presence of *Arcobacter*, while the de Boer Lv1-16S assay did not, due to a cross-reaction with *Arcobacter*. The Yamazaki multiplex PCR was unable to identify the two samples containing *C. lari* (B and C) but could identify *C. jejuni* from a matrix spike sample (E). \( \Delta R_n \), normalized fluorescence minus the background fluorescence, where normalized fluorescence refers to the ratio of the probe fluorescence to the fluorescence of the passive reference dye (ROX).
suggesting that the Linton 16S endpoint PCR assay cross-reacted with the 16S gene of both *A. butzleri* and *A. cryaerophilus* while the *Arcobacter* *hs*p60 assay did not recognize *A. cryaerophilus*. Interestingly, in situations where both *Arcobacter* and *Campylobacter* were observed growing in the same well, not all assays were able to detect *Campylobacter* against this *Arcobacter* background. This may be due to the fact that based on MPN values, *Arcobacter* spp. were numerically superior (2 to 3 log<sub>10</sub>) to *Campylobacter* spp., and that PCR bias may occur, especially for assays that display some weak cross-amplification between the two genera. For MPN wells in which *A. butzleri* and *C. lari* were present (Fig. 2B and C), or for which *A. butzleri* and *C. jejuni* (Fig. 2E) were present, the *hs*p60 assay was positive for *A. butzleri* and the Van Dyke 16S assay was positive for *Campylobacter*, but the de Boer LV1-16S assay displayed nonexponential amplification curves indicative of *Arcobacter*. This result suggests that PCR bias may occur in the de Boer LV1-16S assay and therefore it may not be useful for detection of *Campylobacter* from samples in which *Arcobacter* may be present.

Overall, *A. butzleri* was detected in 79% of samples and on a well-to-well basis closely mimicked the Linton 16S assay results described above (Table 3), suggesting that *A. butzleri* was the major contaminating bacterial species in the MPN cultures and present in irrigation water at an MPN of 55.7 ± 184/300 ml (based on the Linton 16S assay) to 96.8 ± 257/300 ml (based on the *hs*p60 assay). The MPN associated with the Linton 16S assay is slightly lower than those from assays using *hs*p60 and TTC, likely due to the fact that amplification of *Arcobacter* by the Linton primers is a nonspecific reaction with low PCR efficiency. These MPNs are all likely an underestimate of the true occurrence of *Arcobacter*, since two samples were excluded from the calculation because the MPN exceeded the upper limit of detection of 2,400/300 ml. When these same samples were amplified by the Yamazaki multiplex PCR, the 16S band (same as that for the Linton 16S assay) was positive for all samples, albeit with differing amplification efficiencies (Fig. 2F). The Yamazaki multiplex PCR was unable to detect the two samples containing *C. lari* (Fig. 2F, wells 2 and 3) but could detect the *C. jejuni* from the matrix spike (Fig. 2F, well 5). Based on the results from all PCR assays tested, only the Van Dyke 16S qPCR-positive samples were confirmed to be *Campylobacter* positive (Fig. 3A), suggesting that the Van Dyke 16S assay was the most specific and accurate assay for use in screening MPN cultures for the presence of *Campylobacter*, despite the fact that the LOD<sub>95</sub> for this assay was at least an order of magnitude higher than that of the de Boer LV1-16S assay (Table 1). All subsequent samples (2015 irrigation samples and wastewater) were screened using the Van Dyke 16S assay.

**MPN enumeration.** For determination of the MPN of *Campylobacter* spp. growing in the enrichment broth, we compared two methods: PCR positivity in the culture well and color change in the metabolic indicator, TTC, as described by Chenu et al. (28). TTC color change resulted in the identification of 89% of the 2014 irrigation water samples as positive for metabolism in Bolton selective enrichment broth when cultured at 37°C, with an average MPN value of 94.2 ± 178/300 ml (Table 3). The MPN values based on TTC ranged from 0 to >2,400/300 ml, the limit of our assay for irrigation water. When PCR positivity was used to determine MPN values, the results differed based on the PCR assay used (see above). The Linton 16S assay resulted in a positivity rate of 75% with an average MPN of 55.7 ± 184/300 ml (range, 0 to >2,400), whereas both the Van Dyke 16S and Jensen *gly*A assays resulted in a positivity rate of 2.5% with an average MPN of <1/300 ml (Table 3). When the same cultures were enumerated for *Arcobacter butzleri* (*hs*p60 assay), a positivity rate of 79% was observed, with an average MPN of 96.8 ± 257/300 ml (range, 0 to >2,400). The majority of wells positive for metabolism (TTC) were also positive for *A. butzleri* by the *hs*p60 assay, suggesting that this species was the major nontarget microbe growing in the Bolton broth culture medium and metabolizing the TTC.

**Campylobacter detection in wastewater.** Due to the relatively low prevalence and concentration of *Campylobacter* spp. in irrigation water samples in Alberta, we adapted the *Campylobacter* MPN-qPCR for use with raw sewage in order to further optimize the MPN-qPCR assay. However, due to the high bacterial content in this water matrix, the input volume of the assay was reduced to 100 ml and a 42°C incubation temperature was utilized to suppress growth of competing microbiota. Sulfamethoxazole was also
removed from the Bolton broth, since preliminary testing determined that it provided no reduction in growth from competing bacteria (data not shown), likely due to this drug being commonly found in wastewater effluent (36). We tested two temperature conditions (37°C versus 42°C) in combination with two antibiotic combinations (Bolton broth with selective supplement [BB] versus BB plus rifampin and polymyxin B [BBRP]) in a single-enrichment MPN-qPCR (Fig. 1B) for Campylobacter. Each of the four culture combinations were tested with three Campylobacter (q)PCR assays (de Boer Lv1-16S qPCR, Van Dyke 16S qPCR, Lin ton 16S endpoint PCR and one A. butzleri qPCR assay (hsp60)).

Wastewater proved a very challenging matrix for the recovery of Campylobacter growth in the MPN assay. Frequently, Campylobacter could not be detected in the undiluted wastewater MPN samples, an outcome attributed to the intense microbial growth competition in wastewater (i.e., Arcobacter) and not PCR inhibition (based on IAC reactions). Arcobacter was present in wastewater at concentrations up to 4 \( \log_{10} \) higher than Campylobacter (Table 4), suggesting that it (and potentially other bacterial species) simply outcompetes the Campylobacter spp. for available resources when seeded at high concentrations.

The addition of rifampin and polymyxin B to Bolton broth increased the suppression of background microbiota in wastewater, with a concomitant reduction in \( C_T \) values in the Campylobacter (q)PCR assays (Table 4), suggesting a reduction in growth competition from Arcobacter and other bacteria and an increase in the overall growth of Campylobacter spp. in these cultures. A. butzleri levels were reduced by \( \sim 2 \log_{10} \) in cultures containing the two additional antibiotics (Table 4). The lowering of \( C_T \) values in the Campylobacter assays in BBRP medium, however, came at a cost of lower MPN values (\( \sim 40\% \) lower in BBRP at 42°C than in BB at 42°C). While the Campylobacter MPNs were not substantially different between the three different qPCR assays tested, the \( C_T \) values for the de Boer Lv1-16S assay were significantly lower (6 to 15 \( C_T \) units lower). This was attributed to nonspecific amplification, with the high level of Arcobacter bacteria present affecting overall fluorescence in the qPCR.

Culturing at 42°C had the benefit of reducing the overall A. butzleri levels by \( \sim 1 \log_{10} \) and concomitantly raising the \( C_T \) values (19.0 for BB at 37°C versus 35.3 for BB at 42°C), suggesting that A. butzleri grew poorly in BB at 42°C (Table 4). In BBRP medium, however, another phenomenon was observed. While A. butzleri MPN values were 1 to 2 \( \log_{10} \) lower in BBRP than in BB at 37°C, they were the same in BBRP at 37°C and 42°C (Table 4). This suggests the presence of multiple strains of A. butzleri, with one or more of these strains being inherently resistant to all six antimicrobials present in the BBRP medium and tolerant to 42°C, albeit with a lower growth rate.

The average Campylobacter MPNs were not significantly different between the BB and BBRP cultures at 42°C, while culture at 37°C allowed too much background growth for Campylobacter to survive, as evidenced by the low MPN values observed (Table 4). Based on the growth characteristics of Campylobacter and Arcobacter under the four culture conditions tested, we achieved the best compromise for encouraging Campylobacter growth and suppressing Arcobacter by culture in standard Bolton broth with selective supplements at 42°C. This methodology allowed for optimal recovery of thermotolerant Campylobacter spp. in a complex wastewater matrix and was subsequently applied to irrigation water samples for the 2015 field season.

### Campylobacter detection in irrigation water in 2015

Following the optimization of the MPN-qPCR assay for irrigation and wastewater samples, an additional set of irrigation water samples (n = 74) was tested in 2015. Based on optimization from wastewater samples, a single-enrichment MPN assay at 42°C followed by qPCR (Van Dyke 16S qPCR) was used for the detection of Campylobacter. Matrix spikes were again performed and showed that the assay was very sensitive for recovery of C. jejuni in an irrigation water matrix (Table 2), with as little as 2.6 CFU/300 ml being recovered. A total of six irrigation water samples were identified as containing Campylobacter (Fig. 3; Table 5) in 2015, with one sample containing two different species. Similar to the findings in 2014, the Campylobacter MPN was exceptionally low (1 to 2 Campylobacter/300 ml), depending upon the PCR assay used (Table 5). A. butzleri was again present in a large number of samples (54%), but the MPN was much lower than that in 2014 (13.5 versus 210/300 ml), likely due to the elevated incubation temperature of 42°C, which was not optimal for Arcobacter.

### Table 4: Enumeration of Campylobacter and Arcobacter bacteria in raw sewage (2 trials) as detected by an MPN-qPCR single-enrichment assay with four culture conditions and five different PCR assays

| Bacterium and PCR assay | Trial | BB, 37°C | BB, 42°C | BBRP, 37°C | BBRP, 42°C |
|-------------------------|-------|----------|----------|------------|------------|
|                         |       | MPN/100 ml Avg \( C_T \) | MPN/100 ml Avg \( C_T \) | MPN/100 ml Avg \( C_T \) | MPN/100 ml Avg \( C_T \) |
| Campylobacter            |       |          |          |            |            |
| de Boer Lv1-16S qPCR    | 1     | 0        | NA*      | 4.3 \( \times 10^2 \) | 2.4 \( \times 10^4 \) |
| Van Dyke 16S qPCR       | 1     | 3.6 \( \times 10^0 \) | 42.9     | 6.2 \( \times 10^6 \) | 1.9 \( \times 10^4 \) |
| Jensen glyA qPCR        | 1     | 3.0 \( \times 10^0 \) | 37.1     | 9.2 \( \times 10^4 \) | 3.0 \( \times 10^4 \) |
| Linton 16S endpoint PCR | 1     | 0        | 4.6 \( \times 10^2 \) | 9.3 \( \times 10^4 \) | 1.5 \( \times 10^5 \) |
| de Boer Lv1-16S qPCR    | 2     | 2.0 \( \times 10^1 \) | 4.6 \( \times 10^2 \) | 9.3 \( \times 10^4 \) | 7.9 \( \times 10^3 \) |
| Van Dyke 16S qPCR       | 2     | 3.0 \( \times 10^2 \) | 4.3 \( \times 10^2 \) | 4.3 \( \times 10^2 \) | 3.0 \( \times 10^3 \) |
| Jensen glyA qPCR        | 2     | 1.5 \( \times 10^0 \) | 4.6 \( \times 10^2 \) | 9.6 \( \times 10^2 \) | 1.1 \( \times 10^3 \) |
| Linton 16S endpoint PCR | 2     | 2.4 \( \times 10^0 \) | 7.5 \( \times 10^4 \) | 3.5 \( \times 10^3 \) | 2.4 \( \times 10^3 \) |
| Arcobacter hsp60 qPCR   |       | 1        | 19.6     | 2.4 \( \times 10^6 \) | 2.1 \( \times 10^4 \) |
|                         |       | 2        | 9.3 \( \times 10^3 \) | 35.5 \( \times 10^3 \) | 35.8 \( \times 10^3 \) |

* BB, Bolton broth; BBRP, Bolton broth plus rifampin and polymyxin B.

\( \dagger \) NA, not applicable.
The Van Dyke 16S assay-positive MPN wells and isolates from BB agar were subsequently tested by four other PCR assays to determine their performance in detecting Campylobacter and in identifying it to the species level (Jensen Campylobacter not C. jejuni, Campylobacter Bacterium and assay DNA amplified from isolate.

IW, irrigation water; WW, wastewater; PI, patient isolate.

/a/H11001/H11001
/H11001/H11001
/H11001/H11001
/H11001/H11001
/H11001/H11001
/H11001/H11001
/H11001/H11001
/H11001/H11002/H11002/H11002/H11001
/H11001/H11002/H11002/H11002/H11001
/H11001/H11001
/H11001/H11001

TABLE 5 Frequency and enumeration of Campylobacter and Arcobacter bacteria in irrigation water from 2015 as detected by a single-enrichment MPN-(q)PCR enrichment assay at 42°C (n = 74)

| Bacterium and assay | No. (% of bacteria detected) | MPN (avg ± SD)/300 ml |
|---------------------|------------------------------|-----------------------|
| Campylobacter       |                              |                       |
| Van Dyke 16S qPCR   | 6 (8.1)                      | 1.0 ± 1.4             |
| Jensen glyA qPCR    | 3 (4.1)                      | 1.9 ± 2.1             |
| Arcobacter hsp60 qPCR | 40 (54.1)                   | 13.5 ± 38.6           |

TABLE 6 Campylobacter PCR assay performance on Campylobacter enrichment cultures and isolates from irrigation water, wastewater, or human stool

| Sample | Van Dyke 16S qPCR | Jensen glyA qPCR | Yamazaki multiplex PCR | Khan ITS multiplex PCR | Linton 16S endpoint PCR | DNA sequence confirmation (16S gene) by Linton 16S endpoint PCR |
|--------|------------------|------------------|------------------------|------------------------|------------------------|---------------------------------------------------------------|
| IW-1   | +                | + (C. jejuni)    | + (C. jejuni)          | + (C. jejuni)          | +                     | C. jejuni/C. coli                                            |
| IW-2   | +                | + (C. lari)      | -                      | + (C. lari)            | +                     | C. lari                                                       |
| IW-3   | +                | -                | -                      | -                      | +                     | C. jejuni/C. coli                                            |
| IW-4   | +                | -                | -                      | + (C. coli)            | +                     | C. jejuni/C. coli                                            |
| IW-5   | +                | -                | -                      | -                      | +                     | C. jejuni/C. coli                                            |
| IW-6   | +                | + (C. lari)      | -                      | + (IND)                | +                     | C. jejuni/C. coli + C. lari                                  |
| WW-1   | +                | + (C. coli)      | + (C. coli)            | + (C. coli)            | +                     | C. jejuni/C. coli                                            |
| WW-2   | +                | + (C. coli)      | + (C. coli)            | + (C. coli)            | +                     | C. jejuni/C. coli                                            |
| WW-3   | +                | + (C. coli)      | + (C. coli)            | + (C. coli)            | +                     | C. jejuni/C. coli                                            |
| WW-4   | +                | + (C. jejuni)    | + (C. jeuni)           | + (IND)                | +                     | C. jejuni/C. coli                                            |
| WW-5   | +                | + (C. jejuni)    | + (C. jejuni)          | + (IND)                | +                     | C. jejuni/C. coli                                            |
| WW-6   | +                | + (C. jejuni)    | + (C. jejuni)          | + (IND)                | +                     | C. jejuni/C. coli                                            |
| WW-7   | +                | + (C. coli)      | + (C. coli)            | + (C. coli)            | +                     | C. jejuni/C. coli                                            |
| PI-1   | +                | + (C. coli)      | + (C. coli)            | + (C. coli)            | +                     | C. jejuni/C. coli                                            |
| PI-2   | +                | + (C. jejuni)    | + (C. jejuni)          | + (C. jejuni + C. lari)| +                     | C. jejuni/C. coli                                            |
| PI-3   | +                | + (C. jejuni)    | + (jejunii)            | + (C. jejuni + C. lari)| +                     | C. jejuni/C. coli                                            |
| PI-4   | +                | + (C. jejuni)    | + (jejunii)            | + (C. jejuni + C. lari)| +                     | C. jejuni/C. coli                                            |

/a IW, irrigation water; WW, wastewater; PI, patient isolate. 
/b, detected; -, not detected; IND, indeterminate. 
/c C. jejuni and C. coli cannot be distinguished from each other based on sequencing by the Linton 16S amplicon, whereas all other species of Campylobacter can be. The designation of C. jejuni/C. coli in this column reflects this. 
/d DNA amplified from MPN culture well. 
/e DNA amplified from isolate.
origin but not of environmental origin (Table 6). There was, however, a lack of concordance with the results of the Khan ITS assay (Table 6). In particular, the Khan ITS assay often produced an indeterminate result due to abnormal band sizes or multiple bands (data not shown).

The results from comparing Campylobacter spp. isolated from different sources suggest that some environmental C. jejuni/C. coli isolates (samples IW-3 to IW-6) may be distinct from human isolates or type strains, with animals being the likely source. We were able to identify them as C. jejuni/C. coli by analysis of their 16S rRNA gene sequences. This observation suggests that current PCR methods for the identification of C. jejuni/C. coli from surface waters, which use PCR assays developed with patient isolates and/or type strains, are likely inadequate. The more inclusive and specific assay described by Van Dyke and colleagues (19) was the preferred detection/screening method, followed by 16S rRNA gene sequencing using a larger amplicon for better species resolution.

**DISCUSSION**

Campylobacter is an enteric bacterium that can cause serious gastrointestinal illness in humans and more serious sequelae in a small percentage of cases. It is found in the gut of warm-blooded vertebrates such as birds, cattle, and pigs (reviewed in reference 37). Campylobacter is commonly found on food products due to fecal cross-contamination during slaughter. The identification of Campylobacter in water, however, is an indication of fecal contamination as Campylobacter is not thought to grow in water under aerobic conditions. Instead, it adapts to living in water, however, is an indication of fecal contamination during slaughter. The identification of Campylobacter in water may come from human sewage discharge, overland runoff of feces from domestic farm animals, runoff from manure applied to fields, or direct deposition of feces from aquatic birds or mammals. The Campylobacter spp. from these sources are representative of the fecal input, as determined by multilocus sequence typing (39–41).

Several studies in Canada have reported high frequencies of detection of Campylobacter from surface waters (19, 21, 41–43), while others have reported detection of Campylobacter at levels up to 10^5 MPN/100 ml near a wastewater discharge location (20). Ingestion of contaminated water can lead to outbreaks of campylobacteriosis, as the infectious dose of Campylobacter is not thought to grow in water due to its aerotolerance and specific growth needs. Instead, it adapts to living in water, however, is an indication of fecal contamination as Campylobacter is not thought to grow in water due to its aerotolerance and specific growth needs. Instead, it adapts to living in water, however, is an indication of fecal contamination as Campylobacter is not thought to grow in water due to its aerotolerance and specific growth needs.

Campylobacter detection contributing to Campylobacter deposition in water may come from human sewage discharge, overland runoff of feces from domestic farm animals, runoff from manure applied to fields, or direct deposition of feces from aquatic birds or mammals. The Campylobacter spp. from these sources are representative of the fecal input, as determined by multilocus sequence typing (39–41).

Several studies in Canada have reported high frequencies of detection of Campylobacter from surface waters (19, 21, 41–43), while others have reported detection of Campylobacter at levels up to 10^5 MPN/100 ml near a wastewater discharge location (20). Ingestion of contaminated water can lead to outbreaks of campylobacteriosis, as the infectious dose of Campylobacter is not thought to grow in water due to its aerotolerance and specific growth needs. Instead, it adapts to living in water, however, is an indication of fecal contamination as Campylobacter is not thought to grow in water due to its aerotolerance and specific growth needs.

Campylobacter is commonly found on food products due to fecal cross-contamination during slaughter. The identification of Campylobacter in water, however, is an indication of fecal contamination as Campylobacter is not thought to grow in water under aerobic conditions. Instead, it adapts to living in water, however, is an indication of fecal contamination during slaughter. The identification of Campylobacter in water may come from human sewage discharge, overland runoff of feces from domestic farm animals, runoff from manure applied to fields, or direct deposition of feces from aquatic birds or mammals. The Campylobacter spp. from these sources are representative of the fecal input, as determined by multilocus sequence typing (39–41).

Using qPCR to score the MPN reduced the overall time and cost required to complete the assay, yet care must be taken to ensure the specificity of the PCR assays. Our testing of a variety of Campylobacter PCR assays demonstrated how paramount this decision is in data interpretation. The only assay that was fully inclusive of Campylobacter and exclusive of Arcobacter was the 16S rRNA gene assay developed by Van Dyke and colleagues (19), even though the assay itself had a higher LOD50 than other Campylobacter assays tested. The other molecular assays tested (i) cross-reacted with Arcobacter (Linton 16S and de Boer Lv1–16S assays), (ii) were unable to detect Campylobacter in a background of Arcobacter (de Boer Lv1–16S assay), or (iii) missed a certain percentage of Campylobacter (Yamazaki multiplex, Khan ITS, and Jensen glyA assays) in the environment. PCR inclusivity is particularly important, as the Campylobacter spp. we isolated from irrigation water appeared to be distinct from the human and wastewater isolates that we tested. C. lari is a genetically diverse species, and the inability of the Yamazaki multiplex PCR to detect a subgroup of this species has been previously reported (34). The inability of...
C. jejuni- or C. coli-specific PCR assays (i.e., Khan ITS and Jensen gtvα assays) to detect environmental isolates has not to our knowledge been reported before. In order to accurately quantify *Campylobacter* spp. in irrigation water and to use this information for microbial risk assessments, the PCR assays must be as inclusive as possible of all *Campylobacter* spp. but exclusive of nontarget organisms (i.e., *Arcobacter* spp.), noting, however, that various *Campylobacter* species/strains from animals are less likely to be infectious in humans (46). Several reports of *Campylobacter* prevalence/concentration in water have relied on the Linton 16S primer set for some of their interpretations (20, 21, 43). We urge caution in the interpretation of the aforementioned results due to possible conflation with *Arcobacter* spp. in these studies. We suggest that the recommended procedure for *Campylobacter* detection in surface water should encompass the genus-specific 16S gene detection methods of Van Dyke et al. (19), followed by species identification of isolates by 16S gene sequencing and/or another genomic method such as comparative genomic fingerprinting, multilocus sequence typing (MLST), or flaA typing (methods reviewed in reference 47) for the resolution of *C. coli* and *C. jejuni*.

Limitations of culture-based assays to detect *Campylobacter* in surface waters include the findings that *Campylobacter* enters into a dormant or VNBC state in aquatic environments and if stored for a prolonged period at 4°C (48). Once in a VNBC state, recovery in a rich medium is often not possible (48). Hence, only relatively “fresh” campylobacters will be detected by culture-based methods. This, in combination with transportation time of samples to the laboratory, likely leads to an underestimation of the true viable *Campylobacter* numbers at the time of sampling. The use of propidium monoazide (PMA)-PCR has been reported for the direct detection by PCR of live (including VNBC) versus dead *Campylobacter* (49), but caution must be used, as uptake of this DNA intercalating dye that inhibits PCR has been shown to be inconsistent, affecting its efficacy (50, 51). Additionally, primer/probe selection is key to the accurate interpretation of any PMA assay due to the potential for nonspecific amplification of *Arcobacter* spp.

*Arcobacter* is an emerging pathogen, with several studies reporting it to be the fourth most common bacterial pathogen present (up to 1.3%) in human diarrheic stools (52–54). Previous reports have shown *Arcobacter butzleri* to be present at high levels in wastewater (24, 55, 56), and our results confirm *A. butzleri* levels to be >10^5 MPN/100 ml in raw wastewater in Alberta, Canada (J. Kim, G. Banting, B. Jeon, N. Ashbolt, and N. Neumann, unpublished data). *Arcobacter* has also been reported in fresh-vegetable processing plants (16) and fresh shellfish (57), both of which can be impacted by wastewater discharge. Based on our study of irrigation water in Alberta, Canada, *Arcobacter butzleri* was found at levels 2 to 3 log_10 higher than *Campylobacter* spp. This observation, along with known environmental tolerance of *Arcobacter* (23), suggests that *Arcobacter* may represent a greater threat to human health than *Campylobacter* in the context of irrigation water. Hence, we feel that the detection and enumeration of *Arcobacter* levels in irrigation water (and other surface water) warrant further study. The described assay can easily be modified to quantify *Arcobacter* bacteria by lowering the incubation temperature to 30°C, a more optimal growth temperature for this organism (58).

Our findings suggest that the reporting of *Campylobacter* levels in water is highly dependent upon the methods used and that great care must be used to ensure that *Arcobacter* is not being misidentified as *Campylobacter*. As a result of our comprehensive evaluation of both culture and molecular biology-based detection of *Campylobacter* spp. in water, we report that the prevalence of *Campylobacter* in irrigation water in Alberta is extremely low (2.5% in 2014 [2 of 80 samples] and 8% in 2015 [6 of 74 samples]), and even in cases where it is found in the water samples, the concentration of the bacteria is also low (<2 MPN/300 ml). In this context, *Arcobacter* spp. may represent a greater threat to human health than *Campylobacter* spp. from contact with irrigation water. The miniaturized MPN-qPCR assay described in this paper for estimating the occurrence of *Campylobacter* and *Arcobacter* in irrigation water and wastewater discharges should provide valuable input for the quantitative microbial risk assessment of water for which human contact or contaminated food consumption is likely.

ACKNOWLEDGMENT

We thank Linda Chui from the Provincial Laboratory for Public Health for providing the *Campylobacter* patient isolates used in this study.

FUNDING INFORMATION

Funding for this work was made possible by grants provided from Alberta Innovates—Energy and Environment Solutions, Alberta Agriculture and Forestry, and the Public Health Agency of Canada.

REFERENCES

1. Vandamme P, Vancanneyt M, But P, Mels L, Hoste B, Dewettinck D, Vlaes L, van den Borre G, Higgins R, Hommez J. 1992. Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter butzleri* comb. nov. and *Arcobacter skirrowii* sp. nov., an aerotolerant bacterium isolated from veterinary specimens. Int J Syst Bacteriol 42:344–356. http://dx.doi.org/10.1099/00207713-42-3-344.

2. Guévermont E, Lamoureux E, Loubier CB, Villeneuve S, Dubuc J. 2014. Detection and characterization of *Campylobacter* spp. from 40 dairy cattle herds in Quebec, Canada. Foodborne Pathog Dis 11:388–394. http://dx.doi.org/10.1089/fpd.2013.1706.

3. Boes J, Neresting L, Nielsen EM, Kransker S, Eneoe C, Wachmann HC, Baggesen DL. 2005. Prevalence and diversity of *Campylobacter jejuni* in pig herds on farms with and without cattle or poultry. J Food Prot 68:722–727.

4. van Driessche E, Hovf K, van Hoof J, De Zutter L, Vandamme P. 2003. Isolation of *Arcobacter* species from animal feces. FEMS Microbiol Lett 229:243–248. http://dx.doi.org/10.1016/S0378-1079(03)00840-1.

5. Wesley IV, Wells SJ, Harmon KM, Green A, Schroder-Tucker L, Glover M, Siddique I. 2000. Fecal shedding of *Campylobacter* and *Arcobacter* spp. in dairy cattle. Appl Environ Microbiol 66:1994–2000. http://dx.doi.org/10.1128/AEM.66.5.1994-2000.2000.

6. Atabay HI, Gorry JE, On SL. 1998. Diversity and prevalence of *Arcobacter* spp. in broiler chickens. J Appl Microbiol 84:1007–1016. http://dx.doi.org/10.1046/j.1365-2672.1998.00437.x.

7. Public Health Agency of Canada. Foodnet Canada 2013 short report. http://www.phac-aspc.gc.ca/foodnetcanada/report-rapport-2013-eng.pdf.

8. Donadogues AR, Piros SM, Halasa T, Hald T. 2012. Source attribution of human campylobacteriosis using a meta-analysis of case-control studies of sporadic infections. Epidemiol Infect 140:970–981. http://dx.doi.org/10.1017/S0014482711002676.

9. Galanis E, Mak S, Otterstatter M, Taylor M, Zubel M, Takaro TK, Kuo M, Michel P. 2014. The association between campylobacteriosis, agriculture and drinking water: a case-case study in a region of British Columbia, Canada, 2005-2009. Epidemiol Infect 142:2075–2084. http://dx.doi.org/10.1017/S001448271400123X.

10. Clark CG, Price L, Ahmed R, Woodward DL, Melito PL, Rodgers FG, Jamieson F, Ciebin B, Li A, Ellis A. 2003. Characterization of waterborne outbreak-associated *Campylobacter jejuni*, Walkerton, Ontario. Emerging Infect Dis 9:1232–1241. http://dx.doi.org/10.3201/eid910.020584.

11. Hänninen M-L, Haajanen H, Pummi T, Werumenden K, Katila M-L,
Sarkkinen H, Miettinen I, Rautelin H. 2003. Detection and typing of Campylobacter jejuni and Campylobacter coli and analysis of indicator organisms in three waterborne outbreaks in Finland. Appl Environ Microbiol 69:1391–1396. http://dx.doi.org/10.1128/AEM.69.3.1391-1396.2003.

Schuster CJ, Ellis AG, Robertson WJ, Charron DF, Aramini JJ, Marshall BJ, Medeiros DT, 2005. Infectious disease outbreaks related to drinking water in Canada, 1974-2001. Can J Public Health 96:254–258.

Guzman-Herrador B, Carlander A, Ethelberg S, Freiesleben de Blasio B, Khan IUH, Gannon V, Loughborough A, Jokinen CC, Kent R, Koning W, Lapen DR, Midwinter A, Holland B, Collins-Emerson J, Pattison R, Koning K, Bartelt E, Sommerfeld C, Hildebrandt G. 2006. Comparison and characterization and description of Arcobacter faecis sp. nov., isolated from a human waste disposal site. Syst Appl Microbiol 29:405–414. http://dx.doi.org/10.1016/j.syapm.2015.12.002.

Diergaardt SM, Venter SN, Spreech A, Theron J, Brozel VS. 2004. The occurrence of campylobacters in water sources in South Africa. Water Res 38:2589–2595. http://dx.doi.org/10.1016/j.watres.2004.03.004.

Khan IUH, Vann V, Loughborough A, Jonkens C, Kent R, Koning W, Lapen DR, Medeiros D, Miller J, Neumann NF, Phillips R, Scherer H, Topp E, van Bochove E, Edge TA. 2009. A methods comparison for the isolation and detection of thermophilic Campylobacter sp. in raw retail chicken legs. Int J Food Microbiol 108:115–119. http://dx.doi.org/10.1016/j.ijfoodmicro.2005.08.031.

Chen JW, Pavic A, Cox JM. 2013. A novel miniaturized most probable number method for the enumeration of Campylobacter spp. from poultry-associated matrices. J Microbiol Methods 93:12–19. http://dx.doi.org/10.1016/j.mimet.2013.01.013.

Wilrich C, Wilrich P-T. 2009. Estimation of the POD function and the LOD of a qualitative microbiological measurement method. J AOAC Int 92:1763–1772.

de Boer RF, Ott A, Gülen P, van Zanten E, van Belkum A, Koostia-Smidt AMD. 2013. Detection of Campylobacter species and Arcobacter butzleri in stool samples by use of real-time multiplex PCR. J Clin Microbiol 51:253–259. http://dx.doi.org/10.1128/JCM.01716-12.

Jensen AN, Andersen MT, Dalsgaard A, Baggesen DL, Nielsen EM. 2005. Development of real-time PCR and hybridization methods for detection and identification of thermophilic Campylobacter spp. in pig faecal samples. J Appl Microbiol 99:292–300. http://dx.doi.org/10.1111/j.1365-2672.2005.02616.x.

Linton D, Owen RJ, Stanley J. 1996. Rapid identification by PCR of the genus Campylobacter and of five Campylobacter species enteropathogenic for man and animals. Res Microbiol 147:707–718. http://dx.doi.org/10.1016/S0924-2539(96)80103-3.

Van Dyke MI, Morton VK, McLellan NL, Huck PM. 2014. Occurrence and genetic diversity of Arcobacter spp. in a spinach-processing plant and evaluation of two Arcobacter-specific quantitative PCR assays. Syst Appl Microbiol 36:235–243. http://dx.doi.org/10.1016/j.syapm.2013.02.003.

Mangen M-JJ, Bouwknecht M, Friisen I, Haagsma JA, Kortbeek LM, Tariq I, Wilson M, van Pelt W, Havelaar AH. 2015. Cost-of-illness and disease burden of food-related pathogens in the Netherlands, 2011. Int J Food Microbiol 164:84–93. http://dx.doi.org/10.1016/j.ijfoodmicro.2014.11.022.

Van Dyke MI, Morton VK, McLellan NL, Huck PM. 2010. The occurrence of Campylobacter in river water and waterfowl within a watershed in southern Ontario, Canada. J Appl Microbiol 109:1053–1066. http://dx.doi.org/10.1111/j.1365-2672.2010.04730.x.

Khan IUH, Hill S, Nowak E, Edge TA. 2013. Effect of incubation temperature on the detection of thermophilic Campylobacter species from freshwater beaches, nearby wastewater effluents, and bird faecal droppings. Appl Environ Microbiol 79:7639–7645. http://dx.doi.org/10.1128/AEM.02324-13.

Khan IUH, Gannon V, Jokinen CC, Kent R, Koning W, Lapen DR, Medeiros D, Miller J, Neumann NF, Phillips R, Scherer H, Topp E, van Bochove E, Wilkes G, Edge TA. 2014. A national investigation of the prevalence and potentially thermophilic Campylobacter species in agricultural waterbodies in Canada. Water Res 61:243–252. http://dx.doi.org/10.1016/j.watres.2014.05.027.

Rice EW, Rodgers MR, Wesley IV, Johnson CH, Tanner SA. 2009. Isolation of Arcobacter butzleri from ground water. Lett Appl Microbiol 103:2561–2569. http://dx.doi.org/10.1111/j.1574-6968.2009.05111.x.

Jasinska EJ, Goss GG, Gillis PL, Van Der Kraak GJ, Matsumoto J, de Souza Machado AA, Giacomin M, Moon TW, Massarsky A, Gagné F, Servos MR, Wilson J, Sultana T, Metcalfe CD. 2015. Assessment of biomarkers for contaminants of emerging concern on aquatic organisms downstream of a municipal wastewater discharge. Sci Total Environ 530–531:140–153. http://dx.doi.org/10.1016/j.scitotenv.2015.03.080.

Ragimbeau C, Schneider F, Losch S, Even J, Mossipon J. 2008. Multilocus sequence typing, pulsed-field gel electrophoresis, and flu short variable region typing of clonal complexes of Campylobacter jejuni strains of human, bovine, and poultry origins in Luxembourg. Appl Environ Microbiol 74:7715–7722. http://dx.doi.org/10.1128/AEM.00865-08.

Bronowski C, James CE, Winstanley C. 2014. Role of environmental survival in transmission of Campylobacter jejuni. FEMS Microbiol Lett 356:18–19. http://dx.doi.org/10.1111/femsle.12488.

Jokinen CC, Scherer H, Mauro W, Taboada E, Isaac-Renton JL, Topp E, Edge TA, Thomas JF, Wilkins G, Vann V, Loughborough A, Jonkens C, Kent R, Koning W, Lapen DR, Medeiros D, Miller J, Neumann NF, Phillips R, Scherer H, Topp E, van Bochove E, Edge TA. 2009. The occurrence and sources of thermophilic Campylobacter jejuni strains of human, bovine, and poultry origins in Luxembourg. Appl Environ Microbiol 74:374–386. http://dx.doi.org/10.1128/AEM.01940-08.

French NP, Midwinter A, Holland B, Collins-Emerson J, Pattison R, Colles F, Carter P. 2009. Molecular epidemiology of Campylobacter jejuni isolates from wild-bird fecal material in children’s playgrounds. Appl Environ Microbiol 75:779–783. http://dx.doi.org/10.1128/AEM.01979-08.

Jokinen CC, Scherer H, Mauro W, Taboada E, Isaac-Renton JL, Topp E, Edge TA, Thomas JF, Vann V. 2010. The occurrence and sources of Campylobacter spp., Salmonella enterica and Escherichia coli O157:H7 in the Salmon River, British Columbia, Canada. J Water Health 8:374–86. http://dx.doi.org/10.2166/wh.2009.076.

Wilkes G, Edge TA, Thomas JF, Wilkins G, Jonkens C, Luytey E, Medeiros D, Neumann N, Rucker E, Topp E, Lapen DR. 2009. Seasonal relationships among indicator bacteria, pathogenic bacteria, Cryptosporidium oocysts, Giardia cysts, and hydrological indices for surface waters within an agricultural landscape. Water Res 43:2209–2223. http://dx.doi.org/10.1016/j.watres.2009.01.033.

Schmitz PJ, Pintar KDM, Fazil AM, Fleming CA, Lanthier M, La-prade N, Sunohara MD, Simhon A, Thomas JL, Topp E, Wilkes G, Lapen DR. 2013. Using Campylobacter spp. and Escherichia coli data and...
Bayesian microbial risk assessment to examine public health risks in agricultural watersheds under tile drainage management. Water Res 47:3255–3272. http://dx.doi.org/10.1016/j.watres.2013.02.002.

44. Robinson DA. 1981. Infective dose of Campylobacter jejuni in milk. BMJ (Clin Res ed) 282:1584. http://dx.doi.org/10.1136/bmj.282.6276.1584.

45. Miller WG, Parker CT, Rubenfield M, Mendz GL, Wösthen MMSM, Ussery DW, Stolz JF, Binnewies TT, Hallin PF, Wang G, Malek JA, Rogosin A, Stanker LH, Mandrell RE. 2007. The complete genome sequence and analysis of the epsilonproteobacterium Arcobacter butzleri. PLoS One 2:e1358. http://dx.doi.org/10.1371/journal.pone.0001358.

46. Lu J, Ryu H, Santo Domingo J, Griffith JF, Ashbolt N. 2011. Molecular detection of Campylobacter spp. in California Gull (Larus californicus) excreta. Appl Environ Microbiol 77:5034–5039. http://dx.doi.org/10.1128/AEM.00018-11.

47. Taboada EN, Clark CG, Sproston EL, Carrillo CD. 2013. Current methods for molecular typing of Campylobacter species. J Microbiol Methods 95:24–31. http://dx.doi.org/10.1016/j.mimet.2013.07.007.

48. Chaisowwong W, Kusumoto A, Hashimoto M, Harada T, Maklon K, Kawamoto K. 2012. Physiological characterization of Campylobacter jejuni under cold stress conditions: its potential for public threat. J Vet Med Sci 74:43–50. http://dx.doi.org/10.1292/jvms.11-0305.

49. Banihashemi A, Van Dyke MI, Huck PM. 2012. Long-amplicon propidium monoazide-PCR enumeration assay to detect viable Campylobacter and Salmonella. J Appl Microbiol 113:863–873. http://dx.doi.org/10.1111/j.1365-2672.2012.05382.x.

50. Pacholewicz E, Swart A, Lipman LJ, Wagenaar JA, Havelaar AH, Duim B. 2013. Propidium monoazide does not fully inhibit the detection of dead Campylobacter on broiler chicken carcasses by qPCR. J Microbiol Methods 95:32–38. http://dx.doi.org/10.1016/j.mimet.2013.06.003.

51. Seinige D, Krischek C, Klein G, Keihenber C. 2014. Comparative analysis and limitations of ethidium monoazide and propidium monoazide treatments for the differentiation of viable and nonviable Campylobacter cells. Appl Environ Microbiol 80:2186–2192. http://dx.doi.org/10.1128/AEM.03962-13.

52. Vandenberg O, Dediste A, Houf K, Ihekwe S, Souayah H, Cadranel S, Douat N, Zissis G, Butzler J-P, Vandamme P. 2004. Arcobacter species in humans. Emerg Infect Dis 10:1863–1867. http://dx.doi.org/10.3201/eid1010.040241.

53. Prouzet-Mauléon V, Labadi L, Bouges N, Ménard A, Mégraud F. 2006. Arcobacter butzleri: underestimated enteropathogen. Emerg Infect Dis 12:307–309. http://dx.doi.org/10.3201/eid1202.050570.

54. Van den Abeele A-M, Vogelaers D, Van Hende J, Houf K. 2014. Prevalence of Arcobacter species among humans, Belgium, 2008–2013. Emerg Infect Dis 20:1731–1734. http://dx.doi.org/10.3201/eid2010.140338.

55. Fisher JC, Levican A, Figueras MJ, McLellan SL. 2014. Population dynamics and ecology of Arcobacter in sewage. Front Microbiol 5:552.

56. Lu X, Zhang X-X, Wang Z, Huang K, Wang Y, Liang W, Tan Y, Liu B, Tang J. 2015. Bacterial pathogens and community composition in advanced sewage treatment systems revealed by metagenomics analysis based on high-throughput sequencing. PLoS One 10:e0125549. http://dx.doi.org/10.1371/journal.pone.0125549.

57. Levican A, Collado L, Yustes C, Aguilar C, Figueras MJ. 2014. Higher water temperature and incubation under aerobic and microaerobic conditions increase the recovery and diversity of Arcobacter spp. from shellfish. Appl Environ Microbiol 80:385–391. http://dx.doi.org/10.1128/AEM.03014-13.

58. Hilton CI, Mackey BM, Hargreaves AJ, Forsythe SJ. 2001. The recovery of Arcobacter butzleri NCTC 12481 from various temperature treatments. J Appl Microbiol 91:929–932. http://dx.doi.org/10.1046/j.1365-2672.2001.01457.x.