Comparative quantification of *Campylobacter jejuni* from environmental samples using traditional and molecular biological techniques

Michael J. Rothrock Jr., Kimberly L. Cook, and Carl H. Bolster

Abstract: *Campylobacter jejuni* is one of the most common causes of gastroenteritis in the world. Given the potential risks to human, animal, and environmental health, the development and optimization of methods to quantify this important pathogen in environmental samples is essential. Two of the most commonly used methods for quantifying *C. jejuni* are selective plate counting and quantitative real-time PCR (qPCR). Unfortunately, little comparative research has been performed to evaluate the accuracy of these methods for quantification of *C. jejuni* in aqueous and solid matrices. In this study, the limit of detection and the level of resolution obtained using these 2 methods was evaluated for *C. jejuni* and compared with that of the common indicator organism *Escherichia coli*. The use of selective plate count media for quantification of *C. jejuni* resulted in a 0.7–1.2 log underestimation of cell concentrations, compared with qPCR in both water and column leachate samples, whereas *E. coli* concentrations were found to be similar with either technique. For *C. jejuni,* only the qPCR assay accurately measured 2-fold changes in cell concentrations in water samples, whereas concentrations of *E. coli* were accurately measured regardless of method. Based on these data, qPCR assays were found to be more accurate than selective plate counts for quantification of *C. jejuni* from environmental samples.

Key words: Campylobacter jejuni, quantitative real-time PCR, selective plate counts, *Escherichia coli*.

Résumé : *Campylobacter jejuni* est une des causes les plus fréquentes de gastroentérite dans le monde. Compte tenu du risque potentiel qu'elle constitue pour la santé humaine, animale et environnementale, le développement et l'optimisation des méthodes de quantification de cet important pathogène dans des échantillons environnementaux sont essentiels. Deux des méthodes les plus couramment utilisées pour quantifier *C. jejuni* sont la numération sélective sur plaque et la PCR quantitative en temps réel (qPCR). Malheureusement, il n'y a que peu de recherche comparative qui a été réalisée pour évaluer l'exactitude de ces méthodes pour quantifier *C. jejuni* dans des matrices aqueuses et solides. Dans cette étude, la limite de détection et le niveau de résolution obtenus par ces deux méthodes ont été évalués pour *C. jejuni* et comparés à ceux d'un organisme indicateur commun, *Escherichia coli*. L'utilisation de milieux de numération sélective sur plaque pour quantifier *C. jejuni* a résulté en une sous-estimation des concentrations cellulaires d'un facteur de 0,7–1,2 log, comparativement à la qPCR d'échantillons d'eau ou de lixiviat de colonne, alors que les concentrations de *E. coli* étaient estimées similaires en utilisant l'une ou l'autre technique. Pour *C. jejuni*, seule la qPCR a mesuré exactement des changements d'un facteur deux des concentrations cellulaires d'échantillons d'eau, alors que les concentrations d'*E. coli* étaient exactement mesurées quelles que soient les méthodes. Sur la base de ces résultats, les essais en qPCR se sont avérés plus fiables que les numérations sélectives sur plaque pour quantifier *C. jejuni* présent dans des échantillons environnementaux.

Mots-clés : Campylobacter jejuni, PCR quantitative en temps réel, numération sélective sur plaque, *Escherichia coli*.

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Introduction

Campylobacter jejuni is an obligately microaerophilic enteric pathogen that is a leading cause of gastroenteritis worldwide (Mead et al. 1999). *Campylobacter jejuni* is extremely sensitive to environmental parameters (i.e., light and desiccation) (Jones 2001), and it was originally shown to be unable to multiply outside of a host (Korhonen and Martikainen 1991; Wallace et al. 1993). It is therefore assumed to have low survivability in the environment for extended periods. Recent studies, however, have shown that *C. jejuni* is more persistent than originally thought, with the organism being isolated from environments such as river water, groundwater, sewage effluent, lagoon waters, and bedding on farm sites (Hanninen et al. 2003; Murinda et al. 2004).

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Considering the potential human, animal, and environmental health risks, the development and use of accurate methods to quantify *C. jejuni* from environmental samples is essential. The most common techniques for detection of campylobacters involve most probable number determinations or culturing following selective enrichment for several days (Nogva et al. 2000). Rather than simple presence/absence methodologies, quantitative techniques are essential to evaluate the environmental incidence of campylobacters (Hutchison et al. 2004; Stanley and Jones 2003). The lack of culturability of *Campylobacter* spp. on agar cannot be accurately equated with nonviability considering their ability to enter the viable but nonculturable (VBNC) state and persist at low concentrations for long periods (Rollins and Colwell 1986; Stern et al. 2001). To circumvent these issues, molecular-based methodologies that specifically detect and quantify targeted microbial populations are being utilized. Several quantitative real-time PCR (qPCR) assays have been developed for detection of *C. jejuni* (Inglis and Kalischuk 2004; Nogva et al. 2000; Yang et al. 2003). Many of these assays permit sensitive, specific detection of the organism, but the research has not been done to determine the resolution or limit for detection of the organism in environmental samples (i.e., water and soils).

Current environmental quality-monitoring procedures, either culture- or molecular-based, require the use of indicator organisms such as total coliforms, fecal coliforms, or enterococci (Hanninen et al. 2003). *Escherichia coli* is the most commonly used of the specific indicators of fecal contamination. The suitability of *E. coli* as an indicator organism for certain pathogens has been a point of debate, given that it has been shown to respond to environmental conditions quite differently than some targeted pathogens (Baggi et al. 2001; Cook and Bolster 2007; Ferguson et al. 1996; Harwood et al. 2005; Lemarchand and Lebaron 2003), including *C. jejuni*.

Given that both culture-based (plate counting on selective media) and molecular-based (qPCR) methods are commonly used for the detection of *C. jejuni* and *E. coli*, the goal of this study was to evaluate the efficacy of these techniques for accurately quantifying (in terms of resolution and detection limits) these 2 organisms in environmental samples.

Materials and methods

Cultures

Campylobacter jejuni ATCC 49943 was grown and maintained on campylobacter-selective agar CM0689 (CSA) (Oxoid, Sparks, Maryland) supplemented with 5% horse blood (Hemostat, Dixon, California) and modified Preston campylobacter-selective supplement SR0204 (Oxoid), which contains polymixin B, rifampicin, trimethoprim, and amphotericin B. *Campylobacter jejuni* was incubated at 37 °C in anaerobic jars (BBL Gaspack System; Sparks, Maryland). Microaerophilic conditions were generated with the Campy-Pak Plus microaerophilic system (BD, Sparks, Maryland). An environmental *E. coli* strain was isolated from dairy manure from a pasture on the Western Kentucky University Agricultural Farm (Bowling Green, Kentucky). *Escherichia coli* was grown and maintained on BBL Eosin Methylene

Blue (EMB) agar (BD) and incubated aerobically at 37 °C. Stock cultures of each organism were maintained at –80 °C in liquid broth (trypticase soy broth (TSB) for *C. jejuni* and Luria broth (LB) for *E. coli*), supplemented with 10% glycerol.

Recovery of *C. jejuni* or *E. coli* from water samples

To achieve starting inocula of ~1×10^9 cells·mL^{-1}, duplicate 250 mL aliquots of TSB were inoculated with a single *C. jejuni* colony (from a CSA plate) and incubated microaerophilically under static conditions in an airtight jar for 48 h at 37 °C. For *E. coli*, a single colony (from an EMB plate) was used to inoculate 50 mL of LB, which was incubated aerobically with shaking (150 r·min^{-1}) for 24 h at 37 °C. After the appropriate incubation period, cells were centrifuged ($9000g$ for 10 min at 4 °C), and the supernatant was removed. Cell pellets were washed in 25 mL of sterile deionized H$_2$O (DI-H$_2$O), and, for *C. jejuni*, the pellets from each 250 mL culture were combined in a total of 50 mL of sterile DI-H$_2$O. Three cycles of centrifugation ($9000g$ for 10 min at 4 °C) and resuspension in DI-H$_2$O (25 mL total) were used to completely wash and rinse the cells prior to serial dilution. After 2 successive 10-fold dilutions of the inocula in sterile DI-H$_2$O, the cells were serially diluted using alternative 2-fold and 5-fold dilutions down to a final dilution of 1×10^{-7}. All dilutions were performed in triplicate.

For plate counts, 100 µL of each dilution was spread plated on the appropriate selective media (CSA for *C. jejuni* and EMB for *E. coli*) and incubated at 37 °C using the appropriate culture conditions (see earlier text). Standard plate-counting techniques were used to determine the cell concentrations for both organisms. For qPCR assays, inoculated water was vacuum filtered through 0.2 µm GTTP Isopore membrane filters (Millipore Corp., Billerica, Massachusetts). The volume of sample filtered was dependent on the approximate concentrations: 1 mL ($\geq 1 \times 10^8$ cells·mL^{-1}), 10 mL (1×10^7 to 1×10^5 cells·mL^{-1}), or 100 mL ($<1 \times 10^5$ cells·mL^{-1}). Filters were placed into sterile 1.7 mL tubes and stored at –20 °C until DNA extraction.

Recovery of *C. jejuni* or *E. coli* from column leachate

The transport of *C. jejuni* and *E. coli* through columns packed with acid-washed sand was measured following experimental protocols described in detail elsewhere (Bolster et al. 2006). Briefly, acid-washed, autoclaved sand, ranging in size from 250 to 350 µm, was wet-packed into 4.8-cm diameter Chromaflex chromatography columns (Kontes Glass Co., Vineland, New Jersey) filled with 10 mmol·L^{-1} KCl electrolyte solution. After packing was completed, the columns were operated in a downward direction using a peristaltic pump, and approximately 10 pore volumes of the electrolyte solution were passed through each column to equilibrate the sand pack. During the transport experiments, the columns were operated in a downward direction using a peristaltic pump to achieve a Darcian flow velocity of ~6.5 cm·h^{-1}. An approximate 100 mL volume pulse of electrolyte solution containing both *E. coli* and *C. jejuni* was injected at the top of each column followed by bacteria-free electrolyte solution. Effluent was collected every 11 min using a Spectra/Chrom CF-1 fraction collector (Spectrum Chromatography, Houston, Texas). Concentrations of *E. coli* and *C. jejuni* were determined by plate counts and qPCR.

In the second set of column experiments, the transport behavior of *E. coli* and *C. jejuni* through a silty-loam soil (Table 1) was investigated. PVC columns (6-in. diameter, 1 in = 25.4 mm) were packed with air-dried soil sieved through a 2-mm screen to a height of 20 cm. The bottom of the PVC pipe was fitted with an endcap with a fitting placed in the center to allow collection of the effluent. Cheese cloth was placed between the endcap and the soil column to keep the soil from exiting the column during the experiment. Once in place, the end cap was glued to the column. Prior to the transport experiments, the columns were saturated by placing the soil-filled columns into a bucket filled with distilled water. This allowed the water to infiltrate upwards through the column to enhance saturation. Once the top of the soil had become saturated with water, the columns were removed from the bucket and the water was allowed to drain for 48 h so that the soil saturation level would be near field capacity. The water draining from the column during this time was collected and sampled to obtain background concentrations of *E. coli* and *C. jejuni*. Once the column had reached field capacity, poultry litter inoculated with *C. jejuni* was applied to the surface of the column at a rate of 4.5 tons·acre^{-1} (1 ton = 0.907 184 74 Mg; 1 acre = 0.404 685 6 ha); the poultry litter was not incorporated into the soil. After poultry litter application, distilled water was applied to the top of the column for 2 h at an intensity of 0.5 in.·h^{-1}. The leachate following water application was collected every 2 h until the water stopped draining. *Escherichia coli* and *C. jejuni* concentrations were determined by plate counts and qPCR. Subsequent applications of water occurred 8 and 21 days later. The physiochemical properties of the solid matrices can be seen in Table 1.

Recovery of *C. jejuni* or *E. coli* from soil samples

Campylobacter jejuni and *E. coli* inocula (at starting concentrations of ~1 × 10^9 cells·mL^{-1}) were prepared as described for the water samples. Inocula were diluted 10-fold in sterile DI-H$_2$O (to a final dilution of 1 × 10^{-7}), and 5 mL of each dilution (including initial) or sterile DI-H$_2$O (uninoculated control) was added to 15 g of nonsterile topsoil. Samples were mixed thoroughly in a Whirl-Pak bag (Nasco, Fort Atkinson, Wisconsin) to homogenize. Triplicate 0.3 g samples were taken and stored at –20 °C until DNA extraction.

DNA extraction and qPCR analyses

DNA was extracted from the environmental sample using the FastDNA Spin kit for soils (MP Biomedical, Solon, Ohio), following the manufacturer's instructions. For the water–leachate samples, the filter was aseptically transferred from the filtration apparatus to the initial extraction tube provided with the extraction kit, while for the soil samples, 0.3 g of soil was extracted. Dilutions (1:10) of all extracted DNA samples were used as the template for the qPCR assays.

The qPCR assays were run on the DNA Engine Opticon 2 (MJ Research, Inc., Waltham, Massachusetts). The primers were obtained from Sigma Genosys (St. Louis, Missouri), and the dual-labeled Black Hole Quencher probes were prepared by Biosearch Technologies, Inc. (Novato, California) (Table 2). Assays were carried out in QIAGEN HotStart *Taq* Master Mix (QIAGEN, Valencia, California) in a total volume of 25 µL. For *Campylobacter jejuni* qPCR quantifi-

cation, the real-time PCR method developed by Nogva et al. (2000) was used. The amplification mixture contained 3.5 mmol·L^{-1} MgCl$_2$, 300 nmol·L^{-1} of each primer, 200 nmol·L^{-1} of probe, and 10–100 ng of sample DNA or dilutions of plasmid PCR 2.1 vector (Invitrogen, Carlsbad, California) carrying the 86 bp insert sequence as a standard (from 1 × 10^1 to 1 × 10^8 copies). For *E. coli* qPCR quantification, the method of Frahm and Obst (2003) was used to target the *uidA* gene of *E. coli*. The amplification mixture contained 3.5 mmol·L^{-1} MgCl$_2$, 600 nmol·L^{-1} of each primer, 200 nmol·L^{-1} of probe, and 10–100 ng of sample DNA or dilutions of plasmid pCR 2.1 vector (Invitrogen) carrying the 82 bp insert sequence as a standard (from 1 × 10^1 to 1 × 10^8 copies).

For both assays, the published PCR protocols were used, with the addition of an initial step of 95 °C for 15 min to activate the HotStart *Taq* polymerase enzyme. For all reactions, the baseline values were set as the lowest fluorescence signal measured in the well over all cycles. The baseline was subtracted from all values and the threshold was set to 1 times the standard deviation. Assays were performed using duplicates of each extracted sample, and all PCR runs included standards and control reactions without template.

Physiochemical analysis of the solid matrices

Moisture was determined by drying the matrices at 65 °C overnight and comparing the mass before and after drying. pH was determined using a combination electrode (Fisher Scientific, Hampton, New Hampshire) at a 5:1 deionized water – solid ratio. Conductivity was determined using an Orion conductivity meter model 126 (Thermo Scientific, Waltham, Massachusetts). Total N and total C were determined by combustion (Watson et al. 2003) using a Vario-Max CN analyzer (Elementar Americas, Inc., Mt. Laurel, New Jersey). The NH$_4$–N content was determined after a 1:60 solid to 2 mol·L^{-1} KCl extraction (Peters et al. 2003) followed by flow injection analysis using the Quickchem FIA+ (method No. 12-107-06-2-A; Lachat Instruments, Milwaukee, Wisconsin). The NO$_3$–N content was also assessed after this KCl extraction using Quickchem FIA+ (method No. 12-107-04-1-B; Lachat Instruments). Total P was determined using inductively coupled plasma – optical emission spectroscopy (ICP–OES) analysis after HNO$_3$ and HCl microwave digestion (Walter et al. 1997). Microwave digestion was performed in a Mars 5 Microwave (CEM Corp., Matthews, North Carolina). The procedure consisted of mixing 0.5 g of solid with 9 mL of HNO$_3$ and 3 mL of HCl in a Teflon microwave digestion vessel. This mixture was allowed to predigest for 45 min at room temperature and was then placed in the microwave. A 6.5 min ramp time was used to achieve a digestion temperature of 175 °C, which was held for 12 min. Samples were allowed to cool to room temperature and were then filtered through a Whatman 42 filter before ICP–OES analysis.

Statistical analyses

The results from triplicate samples (n = 3) were used for each treatment, at each sampling point. All statistically significant differences were at the $p \leq 0.05$ level. Paired *t* tests and linear regression analyses were performed using Graph-Pad Prism version 5.01 (GraphPad Software, Inc., Lo Jolla,

Table 1. Physiochemical characteristics of the solid matrices used in this study.

Characteristic	Matrix	
	Soil[a]	Poultry litter[b]
Total N (mg·kg^{-1})	1.63 (SD 0.26)$\times 10^{3b}$	2.80 (SD 0.14)$\times 10^4$
NH$_4$-N (mg·kg^{-1})	1.66 (SD 0.03)$\times 10^{1b}$	2.61 (SD 0.09)$\times 10^3$
NO$_3$-N (mg·kg^{-1})	2.62 (SD 0.10)$\times 10^{1b}$	9.91 (SD 0.62)$\times 10^1$
Total C (mg·kg^{-1})	1.85 (SD 0.05)$\times 10^{4b}$	3.00 (SD 0.16)$\times 10^5$
Total P (mg·kg^{-1})	4.49 (SD 0.04)$\times 10^{2b}$	1.85 (SD 0.42)$\times 10^4$
Moisture (% mass)	2.46 (SD 0.42)^b	23.32 (SD 0.38)
pH	6.57 (SD 0.19)^b	8.81 (SD 0.03)
Conductivity (mS)	0.11 (SD 0.002)^b	12.77 (SD 0.29)
Sand (%)	7.56	NA
Clay (%)	22.38	NA
Silt (%)	70.06	NA
Texture and class	Silty-loam	NA

Note: NA, not available.

[a]Used for the soil column leachate study and the soil study.

[b]Values represent mean (±SD) for triplicate (n = 3) samples.

Table 2. Primers and probes used for quantitative PCR in this study.

Oligo name	Target	Sequence (5′–3′)	Insert size (bp)	Reference
Nogva F	*Campylobacter jejuni*	CTGAATTTGATACCTTAAGTGCAGC	86	Nogva et al. (2000)
Nogva R	*C.jejuni*	AGGCACGCCTAAACCTATAGCT		
NogCampBHQ	*C.jejuni*	TCTCCTTGCTCATCTTTAGGATAAATTCTTTCACA		
UidA784 F	*Escherichia coli*	GTGTGATATCTACCCGCTTCGC	82	Frahm and Obst (2003)
UidA866 R	*E. coli*	AGAACGGTTTGTGGTTAATCAGGA		
UidA807FAM	*E. coli*	TCGGCATCCGGTCAGTGGCAGT		

California). Paired t tests were determined using a two-tailed approach. For linear regression analysis, concentration data was log$_{10}$-transformed, and the resulting slopes and y intercepts were compared. Data were also statistically analyzed by analysis of variance (proc ANOVA) for multiple comparisons among means with SAS version 9.2 (SAS Institute Inc., Cary, North Carolina).

Results and discussion

Limit of detection and level of discrimination (resolution) for *C. jejuni* in water samples

To evaluate the limit for detection of *C. jejuni* in water, cell concentrations from 2.7 ± 0.4 × 10^9 cells·mL^{-1} were initially diluted 100-fold, and then serially diluted to extinction using alternating 2- and 5-fold dilutions. The detection limit (calculated from the lowest dilution that gave a positive result) in water samples using either plate counts or qPCR was similar (6.0 ± 1.9 × 10^1 and 5.8 ± 2.6 × 10^1 cells·mL^{-1}, respectively). These values are in agreement with those previously determined for *C. jejuni* from environmental water samples using culture-based (2.0 × 10^1 cells·mL^{-1}) (Cook and Bolster 2007) and PCR-based (0.3 × 10^1 to 1.5 × 10^1 cells·mL^{-1}) detection methods (Waage et al. 1999). The calculated concentrations from the same dilution tube, however, were significantly different (p values ranging from 0.001 to 0.025) between the 2 methods, with CSA plate counts averaging 0.7 log lower than the concentration determined by qPCR (Table 3). There was a strong linear relationship between the expected (based on in-

itial inoculum) and observed *C. jejuni* concentrations (Fig. 1A) for both CSA plate count (r^2 = 0.961) and qPCR (r^2 = 0.987) assays. One-way ANOVA analyses demonstrated that there was a statistically significant (p = 0.0453) difference between the qPCR and CSA plate-counting methods. Regression analysis showed no significant difference between the slopes for the CSA plate counts and qPCR methods (p = 0.5690), but the y intercepts were significantly different (p < 0.0001), indicating that the CSA plate-count numbers consistently underestimated the actual cell concentration across the entire dilution range. The qPCR method provided greater resolution between cell concentrations in successive 2- or 5-fold dilutions. When compared with cell concentrations in the preceding dilution, qPCR values were significantly different in 83% of cases, whereas CSA plate counts were significantly different for only 33% of successive dilutions (Table 3).

The cause of the underestimation of *C. jejuni* concentrations in environmental samples using selective media is uncertain, although 2 important factors should be considered. First, the use of selective media (i.e., CSA) typically results in lower concentrations of *C. jejuni* than does general media (i.e., TSA blood) (Colles et al. 2003), owing in part to the inability of this media to recover injured forms of *C. jejuni* (Buswell et al. 1998). Our lab has found that plate counts using TSA blood agar were on average 2 times greater than CSA plate counts (data not shown), but *C. jejuni* concentrations were still significantly underestimated compared with those from qPCR. It should also be noted that it would be impossible to use an enriched medium to isolate *C. jejuni*

Table 3. Determination of the level of resolution for quantification of *Campylobacter jejuni* or *Escherichia coli* in water samples by quantitative PCR and plate counts.

Species	Expected fold reduction	qPCR			Plate counts		
		Mean concn.[a]	Measured fold reduction	p	Mean concn.	Measured fold reduction	p[a]
Campylobacter jejuni		3.6 (SD 0.6)$\times 10^7$			7.3 (SD 0.7)$\times 10^6$		
	10	3.9 (SD 0.1)$\times 10^6$	9.3	0.000 05	1.2 (SD 0.3)$\times 10^6$	6.1	*0.172 94*
	2	1.5 (SD 0.4)$\times 10^6$	2.5	0.004 84	3.6 (SD 0.1)$\times 10^5$	3.4	0.017 20
	5	2.0 (SD 0.5)$\times 10^5$	7.5	0.000 47	4.6 (SD 1.2)$\times 10^4$	7.7	*0.171 12*
	2	5.6 (SD 0.1)$\times 10^4$	3.6	0.002 47	1.9 (SD 0.1)$\times 10^4$	2.5	*0.085 73*
	5	9.3 (SD 2.7)$\times 10^3$	6.0	0.012 76	2.3 (SD 0.1)$\times 10^3$	8.1	0.001 69
	2	4.3 (SD 0.4)$\times 10^3$	2.2	*0.063 92*	1.1 (SD 0.6)$\times 10^3$	2.2	*0.125 67*
	5	4.0 (SD 1.1)$\times 10^2$	10.8	0.000 12	1.2 (SD 2.0)$\times 10^2$	8.9	0.029 48
	2	1.9 (SD 0.7)$\times 10^2$	2.1	0.031 71	6.0 (SD 1.9)$\times 10^1$	2.0	0.017 54
	5	1.2 (SD 0.2)$\times 10^2$	1.5	*0.161 66*	ND	NA	NA
	2	5.8 (SD 2.6)$\times 10^1$	2.1	0.003 81	ND	NA	NA
	5	BD	NA	NA	ND	NA	NA
Escherichia coli		3.9 (SD 0.5)$\times 10^8$			2.6 (SD 0.3)$\times 10^8$		
	10	3.1 (SD 0.5)$\times 10^7$	12.6	0.000 58	2.7 (SD 0.1)$\times 10^7$	9.6	0.005 11
	2	1.5 (SD 0.5)$\times 10^7$	2.1	0.009 15	1.3 (SD 0.1)$\times 10^7$	2.1	0.007 42
	5	2.3 (SD 0.3)$\times 10^6$	6.5	0.002 00	2.2 (SD 0.2)$\times 10^6$	5.9	0.007 94
	2	9.1 (SD 2.4)$\times 10^5$	2.5	0.000 50	1.3 (SD 0.1)$\times 10^6$	1.7	0.020 45
	5	2.0 (SD 0.3)$\times 10^5$	4.5	0.000 26	2.3 (SD 0.1)$\times 10^5$	5.7	0.000 10
	2	7.3 (SD 3.4)$\times 10^4$	2.8	0.012 17	1.1 (SD 0.2)$\times 10^5$	2.1	*0.091 01*
	5	1.5 (SD 0.8)$\times 10^4$	4.9	0.003 98	2.6 (SD 0.3)$\times 10^4$	4.2	*0.116 14*
	2	1.3 (SD 0.5)$\times 10^3$	11.4	0.007 15	1.5 (SD 0.3)$\times 10^4$	1.7	*0.088 20*
	5	5.9 (SD 0.9)$\times 10^2$	2.2	0.010 80	2.4 (SD 0.2)$\times 10^3$	6.3	0.018 44
	2	3.6 (SD 1.3)$\times 10^2$	1.6	0.015 13	1.6 (SD ±0.1)$\times 10^3$	1.5	*0.166 15*
	5	7.4 (SD 1.3)$\times 10^1$	4.9	*0.059 22*	4.3 (SD 0.4)$\times 10^2$	3.7	0.021 26

Note: Bold italicized values represent nonsignificant differences in cell concentrations ($p > 0.05$). ND, not detected; NA, not available; BD, below detection: $<1 \times 10^1$ copies per PCR reaction.

[a]Mean of triplicate samples ($n = 3$).

from mixed-community environmental samples because of its slow-growing nature and more stringent culturing conditions. While selective enrichment of *C. jejuni* from environmental samples prior to plating has been used, Sails et al. (2003) have shown that the genome-equivalent counts of *C. jejuni* from poultry carcasses based on their designed qPCR assay were greater than the viable cell counts using selective media after selective enrichment in over 80% of their samples, with the selective enrichment counts being ~1.3 log lower than the qPCR counts. The authors indicated that the selective nature of the enrichment and plating inhibited the recovery of injured or suboptimal *C. jejuni* cells, thus leading to the underestimation of cell concentrations.

Second, the ability of *C. jejuni* cells to enter a VBNC state could explain the significant difference between plate count and qPCR concentrations. *Campylobacter jejuni* cells can transform into a coccoid, suspended-state form during environmental stress (Park 2002; Rollins and Colwell 1986). There is an ongoing debate about whether these VBNC *C. jejuni* cells can be resuscitated from this state upon entry into a suitable animal host (Jones et al. 1991; Murphy et al. 2006; Pearson et al. 1993) or if VBNC cells are nonrecoverable degenerate forms of the organism (Hazeleger et al. 1998; Medema et al. 1992; Ziprin and Harvey 2004). The ability for *C. jejuni* to become infective following entering the VBNC state appears to be strain- and host-specific (Jones et al. 1991; Medema et al. 1992; van de Giessen et al. 1996), therefore their presence in environmental samples should be considered potentially infective, and these cells must be accounted for by the detection system

being used. Considering that the qPCR assay was both sensitive and specific regardless of the physiological state of the *C. jejuni* cells, and selective plate counts underestimate *C. jejuni* concentrations compared with qPCR, the use of the qPCR assay is recommended for detecting and quantifying *C. jejuni* from environmental samples.

Alternatively, qPCR has the potential to amplify DNA from dead, but intact, cells, thus artificially increasing the measured concentrations. This limitation of molecular techniques for microbial quantification from environmental samples has been demonstrated (Nocker and Camper 2006). It has been previously shown in groundwater samples that plate counts consistently underestimated respiring cells counts for *C. jejuni* but not *E. coli* (Cook and Bolster 2007). Given these results, and considering that the underestimation using plate counts occurred only for *C. jejuni* and not *E. coli* (Fig. 1 and Table 3), even though both were grown under optimal growth conditions for each organism, the effect of the amplification of DNA from dead cells was considered minimal.

Recovery of *C. jejuni* from leachate and soil samples

When sterile, uncharged sand was used as the column matrix, *C. jejuni* concentrations in the leachate were highest between 130 and 230 min for both the CSA plate count and the qPCR assays, with individual leachate fractions ranging from 1.6×10^6 to 3.2×10^6 cells·mL^{-1} to 2.0×10^7 to 8.7×10^7 cells·mL^{-1}, respectively (Fig. 2A). As was observed in the water samples, the detection method (qPCR or CSA plate counts) significantly affected the measured con-
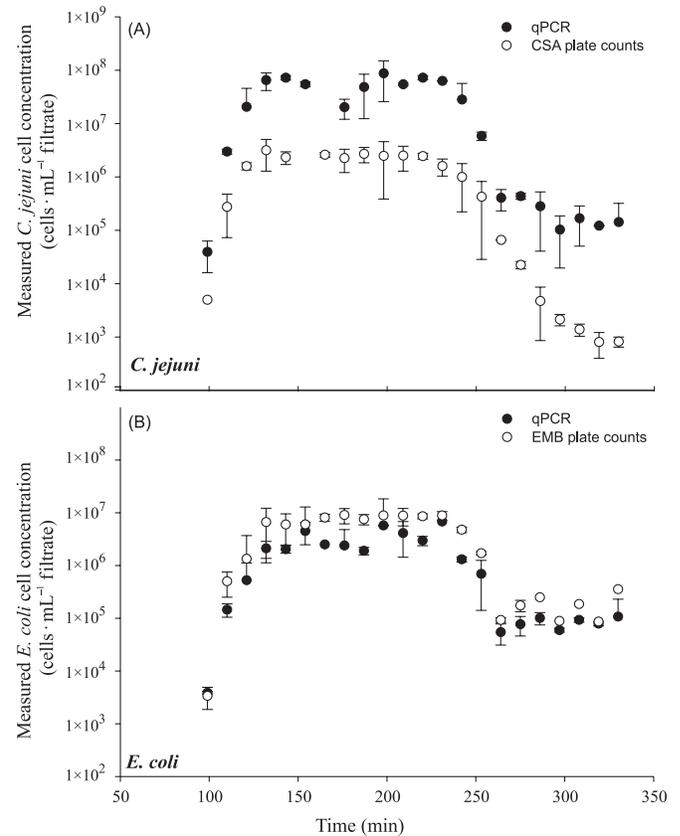
Fig. 1. The relationship between expected and measured cell concentrations for serial dilutions in water as determined by selective plate count (○) or quantitative PCR (●) assays in water. (A) Serial dilutions of *Campylobacter jejuni*. Campylobacter-selective agar (CSA) plate counts: $\log(y) = 1.14 \log(x) - 1.79$, $r^2 = 0.961$; quantitative PCR (qPCR): $\log(y) = 1.13 \log(x) - 0.988$, $r^2 = 0.987$. (B) Serial dilutions of *Escherichia coli*. Eosin Methylene Blue (EMB) plate counts: $\log(y) = 0.985 \log(x) - 0.01$, $r^2 = 0.999$; qPCR: $\log(y) = 1.14 \log(x) - 1.17$, $r^2 = 0.994$. Error bars indicate the standard deviation of triplicate samples.

Fig. 2. Effluent cell concentrations of (A) *Campylobacter jejuni* and (B) *Escherichia coli* recovered from 10 cm columns containing acid-washed sand. Cell concentrations were determined by selective plate count (○) or quantitative PCR (●) assays. Error bars indicate the standard deviation of triplicate samples. qPCR, quantitative PCR; CSA, campylobacter-selective agar; EMB, Eosin Methylene Blue.
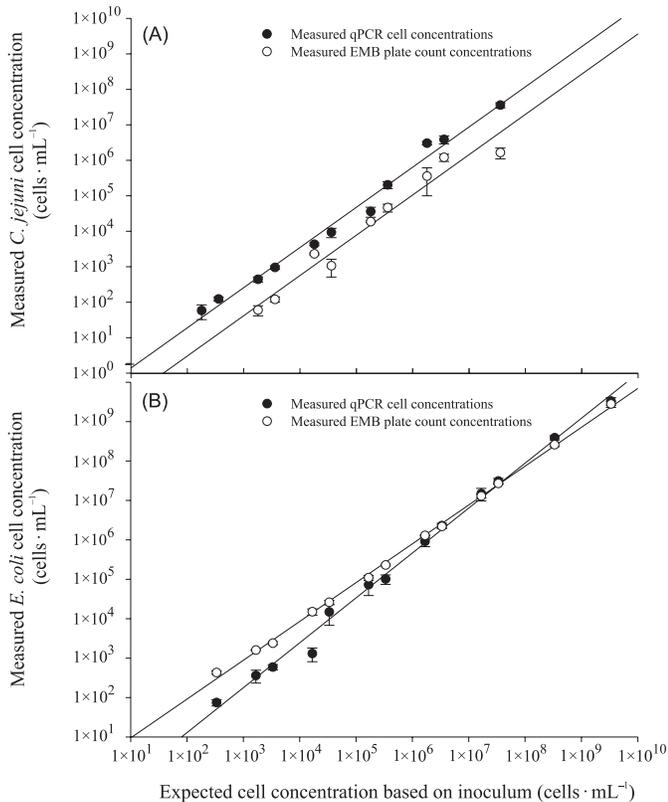
centration of *C. jejuni* from the sand-column leachate samples ($p < 0.001$), with CSA plate count concentrations averaging 1.2 log lower than the qPCR estimates. This discrepancy in concentrations was consistent for leachate samples collected for the first 4.5 h after addition to the sand column, after which time (Fig. 2A; $T \geq 280$ min) the CSA plate counts consistently estimated 2 log lower *C. jejuni* concentrations compared with qPCR. There was no significant difference between the initial inoculum concentration and the total concentration of *C. jejuni* that passed through the column according to either CSA plate counts ($p = 0.106$) or qPCR ($p = 0.198$), suggesting that *C. jejuni* is readily transported through an uncharged sand column.

The transport of *C. jejuni* through a poultry litter amended soil column following a simulated rainfall event of 2 h at an intensity of 0.5 in.·h^{-1} (Table 4) resulted in a >4.0 log reduction (column 1, 1.1×10^3 cells·mL^{-1}; column 2, 1.6×10^2 cells·mL^{-1}; Table 4) in cell concentrations compared with the concentration of *C. jejuni* in the amended soil prior to the rain event (7.1×10^7 cells·mL^{-1}; Table 4). This is in contrast to the transport though the sand column, in which there was no significant difference in the concentrations of *C. jejuni* in the initial or leached liquid. This result was not

unexpected, since the presence of the charged soil particles has been previously shown to inhibit the transport of both *C. jejuni* and *E. coli* through the soil matrix compared with an uncharged sand matrix (Bolster et al. 2001, 2006). *Campylobacter jejuni* was detected for 3–7 days following the rain event, albeit at significantly reduced concentrations (Table 4). After 1 week, *C. jejuni* was not detected by the qPCR assay (<1 × 10^1 copies per PCR reaction), even after additional rain simulations.

In terms of recovery from inoculated soil, *C. jejuni* could be detected in serial dilutions down to 1×10^6 cells·g^{-1}. There was a strong linear relationship ($r^2 = 0.998$) between the expected concentrations (relative to the initial inoculum concentration) and the qPCR-measured concentrations (Fig. 3), and these measured concentrations were not found to be significantly different ($p = 0.3211$) from the expected concentrations over this range. *Campylobacter jejuni* was not detected in the soil at concentrations <1 × 10^6 cells·g^{-1}. The reason for this higher limit of detection from soil is unknown and is currently under investigation.

Detection and recovery of *E. coli* from environmental samples

There was a strong linear relationship between the expected and measured concentrations of *E. coli* as determined

Table 4. Concentrations of *Campylobacter jejuni* and *Escherichia coli* in leachate from poultry litter amended soil columns by quantitative PCR.

| | Concn. (cells·mL^{-1} of filtrate)[a] | | | |
| | *C. jejuni* | | *E. coli* | |
Sample day[b]	Column 1	Column 2	Column 1	Column 2
0 (Treated litter before rain event)	7.1 (SD 3.6)×10^7	7.1 (SD 3.6)×10^7	1.0 (SD 0.1)×10	1.0 (SD 0.1)×10^6
1	1.1 (SD 0.3)×10^3	1.6 (SD 0.6)×10^2	BD1	BD1
2	1.4 (SD 0.7)×10^2	5.3 (SD 2.3)×10^1	BD1	BD1
3	1.5 (SD 0.4)×10^2	NA	BD1	BD1
7	NA	1.1(SD 0.3)×10^2	BD1	BD1
9[c]	BD2	BD2	BD1	BD1
30[c]	BD2	BD2	BD1	BD1

Note: BD1, below detection (<1 × 10^2 copies per PCR reaction); BD2, below detection (<10^1 copies per PCR reaction); NA, no filtrate recovered.

[a]Mean and SD of triplicate (*n* = 3) samples.

[b]Days after the first rainfall simulation event.

[c]Additional rain events occurred on days 8 and 21.

Fig. 3. The relationship between the expected and measured *Campylobacter jejuni* concentrations after serial dilution in agricultural soil as determined by quantitative PCR (qPCR). Log(*y*) = 0.88 log(*x*) + 1.04, *r*^{2} = 0.998. *Campylobacter jejuni* was not detected after the lowest dilution on the regression line (final dilution was 1 × 10^{-6} times the dilution of the inocula). *Escherichia coli* exhibited a background level of ~1 × 10^5 cells·g^{-1}. Error bars indicate the standard deviation of triplicate samples.
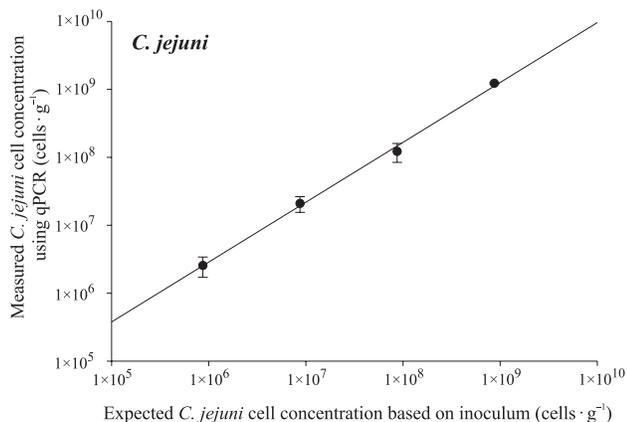
by both EMB plate counts (*r*^{2} = 0.999) and qPCR (*r*^{2} = 0.994) (Fig. 1B). Unlike plate counts for *C. jejuni*, the concentrations of *E. coli* based on EMB plate counts were virtually identical (0.06 log higher) to the concentrations based on qPCR (Table 3) from water samples. One-way ANOVA analyses showed that there was no statistical difference (*p* = 0.656) between these detection methods (qPCR and EMB plate counts) on measured *E. coli* concentrations. At cell densities <5 × 10^4 cells·mL^{-1}, the qPCR assay underestimated the *E. coli* concentrations in water samples, compared with EMB plate counts (Fig. 1B). The percent recovery of microorganisms after filtration, as determined by qPCR, has been shown to range from 20% to 65% (Fuhrman et al. 1988; Weinbauer et al. 2002; Wolffs et al. 2006). In this study, the recovery of *E. coli* by qPCR (compared with plate counts) averaged 26% at cell densities <5 × 10^4 cells·mL^{-1}, but it was nearly 100% at higher cell densities. The effect of

the reduced recovery of DNA from filtered cells would be more evident at lower concentrations, potentially resulting in the observed underestimation of *E. coli* concentrations. It is also possible that filtering only 100 mL of water was insufficient to accurately quantify *E. coli* at low concentrations. Volumes of up to 20 L have been shown to be required to accurately quantify low concentrations of enteric pathogens from some water sources (Jenkins et al. 2008). As was the case for *C. jejuni*, the qPCR assay targeting the *E. coli uidA* gene exhibited a higher level of discrimination between successive 2- or 5-fold dilutions (92% accuracy compared with 67% accuracy for plate counts) (Table 3).

Our results suggest that *E. coli* is not a suitable indicator for either the presence/absence or concentration of *C. jejuni* from leachate or soil samples. The transport behavior of *E. coli* (Fig. 2B) was similar to that of *C. jejuni* (Fig. 2A) in the sand-column studies, with the highest concentrations of *E. coli*, as measured by EMB plate counts (4.8 × 10^6 to 9.1 × 10^6 cells·mL^{-1}) and qPCR (1.3 × 10^6 to 6.8 × 10^6 cells·mL^{-1}), occurring in the leachate samples between 130 and 230 min. Unlike *C. jejuni*, however, ANOVA analysis showed that the method of detection did not significantly effect the measured *E. coli* concentrations (*p* = 0.3901). In the poultry litter amended soil-column experiment (Table 4), *E. coli* was never detected (<1 × 10^2 copies per PCR reaction) in the leachate samples, whereas *C. jejuni* concentrations were detectable up to 1 week after the initial rain event. These results from both column experiments indicate significant differences in transport characteristics between *C. jejuni* and *E. coli*, which have been previously demonstrated in our lab (Cook and Bolster 2007). The ability of *C. jejuni* to be present in environmental samples in the absence of common indicator organisms has been reported from environmental water samples collected from mountain catchments (Schaffter and Parriaux 2002).

In terms of recovery from soil, high background *E. coli* concentrations were found in the uninoculated controls (2 × 10^5 cells·g^{-1}), and, therefore, no less than 1 × 10^5 cells·g^{-1} could be detected using the *E. coli* qPCR assay. While this limit of detection may seem high, previous qPCR based studies on *E. coli* from fecal samples have shown a similar

detection limit $(5 \times 10^5$ cells·g^{-1}) (Rinttila et al. 2004). Meanwhile, *C. jejuni* was not detected in the uninoculated control, suggesting that background levels of *C. jejuni* will not artificially raise the limit of detection for the qPCR assay, as was seen with *E. coli*.

Conclusions

Considering the obvious risk of *C. jejuni* to human and environmental health (Mead et al. 1999), the paucity of research on the detection and fate of *C. jejuni* in the environment is surprising. While numerous culture- and molecular-based detection methods are being used, we believe our results show that qPCR assays more accurately quantify *C. jejuni* concentrations from environmental samples compared with culture-based techniques, and that qPCR assays can detect as low as 2-fold differences in *C. jejuni* concentrations among samples. As technologies improve and the limit for detection of organisms improves, traditional methods (culturing and selective enrichment) for evaluating the presence of pathogens should be re-evaluated.

Acknowledgements

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