Culture-based indicators of fecal contamination and molecular microbial indicators rarely correlate with *Campylobacter* spp. in recreational waters

Kristen N. Hellein, Cynthia Battie, Eric Tauchman, Deanna Lund, Omar A. Oyarzabal and Joe Eugene Lepo

ABSTRACT

Campylobacter spp. are the leading cause of gastroenteritis worldwide. Most human infections result from contaminated food; however, infections are also caused by recreational waterway contamination. *Campylobacter* culture is technically challenging and enumeration by culture-based methods is onerous. Thus, we employed qPCR to quantify *Campylobacter* spp. in fresh- and marine-water samples, raw sewage and animal feces. Multiplex PCR determined whether *Campylobacter jejuni* or *C. coli*, most commonly associated with human disease, were present in qPCR-positive samples. Campylobacters were detected in raw sewage, and in feces of all avian and mammalian species tested. *Campylobacter*-positive concentrations ranged from 68 to 2.3×10^6 cells per 500 mL. Although *C. jejuni* and *C. coli* were rare in waterways, they were prevalent in sewage and feces. *Campylobacter*-specific qPCR screening of environmental waters did not correlate with the regulatory EPA method 1600 (*Enterococcus* culture), nor with culture-independent, molecular-based microbial source tracking indicators, such as human polyomavirus, human *Bacteroidales* and *Methanobrevibacter smithii*. Our results suggest that neither the standard EPA method nor the newly proposed culture-independent methods are appropriate surrogates for *Campylobacter* contamination in water. Thus, assays for specific pathogens may be necessary to protect human health, especially in waters that are contaminated with sewage and animal feces.

Key words | *Campylobacter*, fecal indicator, microbial source tracking, molecular indicator, recreational waters

Kristen N. Hellein
Eric Tauchman
Joe Eugene Lepo (corresponding author)
Center for Environmental Diagnostics and
 Bioremediation,
University of West Florida,
11000 University Parkway,
Pensacola, FL 32514,
USA
E-mail: *jlepo@uwf.edu*

Cynthia Battie
Deanna Lund
Department of Public Health,
University of North Florida,
1 UNF Dr,
Jacksonville, FL 32250,
USA

Omar A. Oyarzabal
Department of Biological Sciences,
Alabama State University,
Montgomery, AL 36101,
USA

INTRODUCTION

To prevent waterborne illnesses in the USA, the Environmental Protection Agency (EPA) developed water quality methodology employing culture-based assays to determine levels of enterococci and fecal coliforms as surrogate indicators of waterborne pathogens (US Environmental Protection Agency 2002, 2003). As these methods are slow, labor intensive and not specific for human pathogens, real-time quantitative polymerase chain reaction (qPCR) detection of host-specific viruses and bacteria using molecular markers has been proposed as an alternative method to determine the risk to human health of exposure to potable and recreational waters (Guy *et al.* 2003; Santo Domingo *et al.* 2003; He & Jiang 2005; Layton *et al.* 2006; Stoeckel & Harwood 2007; US Environmental Protection Agency 2009).

Campylobacter spp. are Gram-negative, microaerobic, motile rods with a single flagellum. Two species, *C. jejuni* and *C. coli*, account for nearly 95% of campylobacteriosis cases and are a leading cause of bacterial gastroenteritis in the USA (Mead *et al.* 1999; Scallan *et al.* 2011), Canada (Public Health Agency of Canada 2010) and European countries (European Food Safety Authority 2006). Importantly, Guillain-Barré syndrome is thought to be a sequela

of campylobacteriosis. *Campylobacter* spp. have been implicated in several large outbreaks, such as the outbreak of *C. jejuni* in Walkerton, Ontario, Canada (Clark *et al.* 2003).

Prevention of campylobacteriosis is problematic because it is sporadic and difficult to predict. *C. jejuni* has a low infectious dose of approximately 500 cells (Robinson 1981), and the average incubation period for this disease is 3 to 5 days, which is longer than that of other bacterial gastrointestinal infections (Porter & Reid 1980; Evans *et al.* 1996; Skirrow & Blaser 2000). Moreover, many cases go unreported. The transmission of this pathogen is complicated with multiple reservoirs and routes of transmission (Bi *et al.* 2008). Sporadic infections are often the result of ingesting or handling raw or undercooked food, such as milk or poultry (Kilonzo-Nthenge *et al.* 2008), while outbreaks have been attributed primarily to the consumption of contaminated drinking water (Hänninen *et al.* 2003; Schuster *et al.* 2005; Martin *et al.* 2006). Indeed, in Canada, campylobacteriosis was the second most commonly reported illness attributable to potable water during 1974–2001 (Schuster *et al.* 2005).

Campylobacter spp. have been detected in a variety of aquatic environments, especially those that receive sewage effluent/contamination and agricultural runoff. Abulreesh *et al.* (2006) hypothesized that *Campylobacter* spp. are not indigenous to aquatic environments because of their growth requirements, and thus their presence is indicative of recent fecal contamination. *Campylobacter* spp. may persist in the environment by entering a viable but non-culturable (VBNC) state, although it is not clear if these states retain infectivity (Nogva *et al.* 2000; Daczkowska-Konon & Brzosteck-Nowakowska 2001). In summer, high concentrations of campylobacters were reported in a southeastern USA mixed-use watershed probably as the result of fecal contamination from both human and domestic animals, corresponding to the high rates of campylobacteriosis seen in summer (Vereen *et al.* 2007). *Campylobacter* spp. were frequently present in surface waters that received effluent from a wastewater treatment plant (WWTP), especially those that process both human and poultry waste and in areas receiving agricultural runoff (Vereen *et al.* 2007). While wastewater outflow drives a significant portion of *Campylobacter* contamination in the mixed-use watershed, the presence of these bacteria in

larger watersheds and upstream watersheds is more likely related to rainfall and runoff (Vereen *et al.* 2007).

Campylobacter spp. are present in many types of farm animal feces such as cattle, pigs and sheep (Sáenz *et al.* 2000; Uaboi-Egbenni *et al.* 2008; Zweifel *et al.* 2008; Hakkinen & Hänninen 2009; Horrocks *et al.* 2009); in wild animal and bird feces (Abulreesh *et al.* 2006; Schets *et al.* 2008; Uaboi-Egbenni *et al.* 2008; Zweifel *et al.* 2008); and in the feces of companion animals (Horrocks *et al.* 2009; Koene *et al.* 2009). Ducks and geese in the USA (Fallacara *et al.* 2001) and other countries (Aydin *et al.* 2001), and gulls of Lake Michigan beaches (Kinzelman *et al.* 2008) are well-known carriers of *Campylobacter* spp. Feces from wild birds contribute to *Campylobacter* infection in preschool children (Waldenström *et al.* 2006; Walters *et al.* 2007; French *et al.* 2009). Additionally, Pitkänen *et al.* (2008) reported that rainwater runoff in Finland flushes large amounts of wild bird fecal material into drinking water, causing *Campylobacter* infections. Thus, animal fecal contamination is a probable source of environmental cases of campylobacteriosis.

The validity of conventional fecal indicators and molecular MST (microbial source tracking) indicators as sentinels for the presence of specific human pathogens, particularly *C. jejuni* and *C. coli*, in water sources is controversial. In terms of conventional fecal indicators, elevated counts of enterococci (Bi *et al.* 2008) and fecal coliforms (Savill *et al.* 2001; Vereen *et al.* 2007) correlated with *Campylobacter* spp. in an aquatic environment. In contrast, several researchers found that *Campylobacter* contamination was not predicted by culture-based indicator methods (e.g. enterococci, fecal coliforms) that are similar to United States EPA-approved methods and/or molecular MST indicators that rely on molecular host-specific markers (Stoeckel & Harwood 2007; USEPA 2009). Walters *et al.* (2007) reported that PCR detection of human-specific but not ruminant-specific *Bacteroidales* predicted the presence of viable campylobacters in river water. The positive correlation between a generic fecal marker for *Bacteroidales* and the presence of *E. coli* O157:H7, *Campylobacter* and *Salmonella* in river water, suggests that campylobacteriosis is not always zoonotic but might be transmitted via human fecal waste. The conflicting results described above necessitate further investigations of the validity of using EPA-approved methods or

molecular MST indicators to alert public health agencies of the possible threat of *Campylobacter* contamination of environmental waters.

Although not necessarily pathogens, certain microorganisms that seem to be exclusive to human sources, such as human polyomavirus, human *Bacteroidales*, and *Methanobrevibacter smithii*, have been proposed indicators of human pollution (Harwood *et al.* 2009). Molecular detection of these indicators through MST has been invaluable in the attribution of human fecal contaminants to water systems. However, any additional indicator of water quality based on molecular methods used for detection should correlate well with the presence of human pathogens, such as *Campylobacter* spp., in potable and recreational waters. The present work investigated the correlation of a traditional indicator organism (enterococcus) measured by EPA Method 1600 and molecular MST indicators (human polyomavirus, general *Bacteroidales*, human-specific *Bacteroidales* and *M. smithii*), measured by PCR, with the presence of *Campylobacter* spp. in fresh and marine water environments of Northwest Florida. Given that *Campylobacter* spp. are difficult to culture, we used qPCR targeting a 16S rRNA gene to detect bacterial presence, and multiplex PCR to identify *C. jejuni* and *C. coli*. *Campylobacter* spp. were identified in both fresh and marine water sites with *C. jejuni* and *C. coli* present in both water types, though not in all *Campylobacter* qPCR positive samples. No correlations were found between the presence of campylobacters and either the conventional EPA-approved enterococcus methods or the molecular MST indicators. Thus, *Campylobacter* is not easily predicted by indicator microorganisms and instead may need to be identified directly to assess the risk of campylobacteriosis from exposure to recreational waterways.

METHODS

Collection and processing of environmental water and raw sewage samples

Grab samples from 32 local fresh and marine water sites (Table 1) were collected in Pensacola, Florida, and surrounding areas from June 2008 to September 2009

(Figure 1). One liter of surface water was collected in a sterile 1-L Nalgene™ bottle (rinsed twice with the environmental water prior to collection). Salinity data were collected on site. Samples were transported to the laboratory for processing within 6 h of collection. We counted enterococci by the EPA Method 1600 (US Environmental Protection Agency 2002), and filtered the sample for DNA extraction. For PCR and qPCR assays, samples (500 mL) were concentrated by membrane filtration using 0.45 μm pore size, 47 mm diameter nitrocellulose filters (Fisher Scientific, Pittsburg, PA). If a sample was too turbid to filter 500 mL, filtering time was limited to 15 min. Samples processed for human polyomavirus were subject to an extra step before filtration. These samples were acid adjusted to pH 3.5 using 20% hydrochloric acid prior to filtration. Using sterile, DNA-free forceps, the filters were folded and placed into a PowerBead tube from the PowerSoil™ DNA kit (MO BIO Laboratories, Inc., Carlsbad, CA) and either used directly for DNA extraction or frozen at $-20\,^{\circ}C$ to be processed later. Negative controls (500 mL sterile water) were processed in parallel with the collected samples starting with filtration.

Raw sewage samples were obtained from wastewater treatment plants (WWTPs) and lift stations as described in Table 2 from 19 visits to three lift stations over a sampling period of 14 months (July 2008–September 2009). At the WWTPs, 1-L grab samples were captured by filling sterile Nalgene™ screw-cap bottles with raw sewage from the waste stream. For sampling at lift stations, a 250 mL sterile Nalgene™ snap-cap collection container was attached to a fishing line and lowered via a fishing pole into the raw sewage. Samples were immediately transferred back to the laboratory and 10 mL of each sample was concentrated by membrane filtration. Filters were processed as described above to extract DNA. The extracted DNA was used for PCR assays of the molecular MST indicators, multiplex PCR assays to identify pathogenic *Campylobacter* spp. and qPCR assay to quantify *Campylobacter* spp. Raw sewage from WWTPs rather than effluent was tested for the presence of *Campylobacter* spp. and molecular MST indicators to ensure that we could detect these organisms in human fecal material.

To compare conventional methods with the methods described above, enterococcus counts were also performed on the collected environmental water samples

Table 1 | *Campylobacter* presence in environmental waters

Sampling location	*Campylobacter* cells/500 mL[a]	Species[b]	Molecular MST indicators[c]	*Enterococcus* (CFU/dL)[d]
Escambia River	195	*C. coli* & *C. jejuni*	None	TNTC (poor)
Williams Creek	68	*C. coli*	None	11 (good)
UWF Duck Pond	213	*Campylobacter* spp.	None	0 (good)
Thompson's Bayou	122	*Campylobacter* spp.	None	11 (good)
Lakeview Park	2.3×10^6	*Campylobacter* spp.	None	37 (moderate)
Hwy 399 Bridge 1	366	*C. coli*	*Bacteroidales*	242 (poor)
Hwy 399 Bridge 2 (upstream of drain)	362	*Campylobacter* spp.	*Bacteroidales*, human-specific *Bacteroidales*	19 (good)
Hwy 399 Bridge 2 (downstream of drain)	176	*Campylobacter* spp.	*Bacteroidales*, human-specific *Bacteroidales*	40 (moderate)
Quietwater Beach	1,200	*Campylobacter* spp.	None	10 (good)
Quietwater Beach (8 June 09)	285	*Campylobacter* spp.	None	0.6 (good)
Quietwater Beach (15 June 09)	2,100	*Campylobacter* spp.	None	0.2 (good)
Quietwater Beach (15 June 09)	2,030	*Campylobacter* spp.	None	0.2 (good)
Quietwater Beach (15 June 09)	898	*Campylobacter* spp.	None	0 (good)
East Bay	232	*C. coli*	*Bacteroidales*	6.3 (good)

[a]The number of *Campylobacter* cells in a 500 mL grab sample from the given sampling locations as determined by qPCR.
[b]*Campylobacter* spp. detected by qPCR.
[c]Samples were also tested for the molecular MST indicators, HPyV, total and human-specific *Bacteroidales*, and *M. smithii*.
[d]Samples were also tested for *Enterococcus* using EPA Method 1600 and the number of enterococci CFU/dL are shown. The Florida Department of Health has set the following guidelines for water quality based on enterococcus counts. Water is considered 'good' if the EPA Method 1600 detects 0–35 enterococci per dL of marine water. Water is considered a 'moderate' threat if 36–104 enterococci are detected per dL of marine water. Water is considered to be of 'poor' quality if greater than 105 enterococci are detected per dL of marine water.

and raw sewage samples. From the 1 L of collected water, both 10-mL and 50-mL aliquots were filtered in duplicate through another set of 0.45 μm pore size, 47 mm diameter nitrocellulose filters. From the 1 L of WWTP sewage and the 250 mL of lift station sewage, a 10^{-3} and a 10^{-4} dilution of sewage was prepared in phosphate buffered saline (PBS) and a 10 mL and a 50 mL aliquot of these dilutions were filtered in duplicate through another set of 0.45 μm pore size, 47 mm diameter nitrocellulose filters. These filters were immediately transferred to BBL™ mEI Agar plates (BD, Franklin Lakes, NJ) and allowed to incubate overnight at 41 °C. The resulting blue colonies were counted after 24 ± 2 h according to EPA Method 1,600. Results were reported as colony forming units (CFU)/dL.

Collection and processing of animal fecal samples

Several grams of fresh fecal material from individual animals (Table 3) were collected using sterile swabs, at separate times and locations from the collection of the water and raw sewage samples, and placed into sterile microcentrifuge tubes. Samples were transported on ice to the laboratory for immediate processing or for storage at −80 °C until used in the *Campylobacter* qPCR. Approximately 0.25–0.3 g of fecal material of the original sample was placed into a MoBio Powersoil™ bead microcentrifuge tube for DNA extraction. In order to ensure that the extraction kits were free of contaminated reagents, reagent blanks that included all extraction kit reagents but no fecal material were included in each batch preparation. *Enterococcus* counts were not performed on collected animal fecal samples.

PCR detection of the molecular MST indicators

Water, sewage and animal fecal samples were tested for the presence of the following agents: human polyomavirus
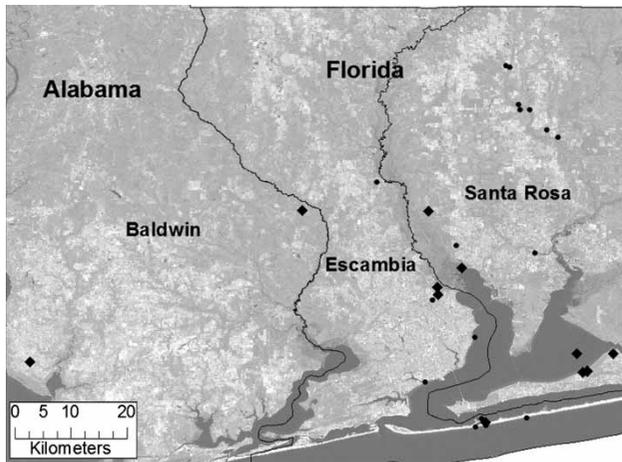
Figure 1 | Sampling sites within and near Northwest Florida. Grab samples from regional fresh and marine waters were collected over a period of 14 months (July 2008 to September 2009) in the vicinity of Pensacola, Florida, spanning three counties (Baldwin in Alabama, Escambia and Santa Rosa in Florida). The samples were analyzed by qPCR for Campylobacter spp. and by conventional (endpoint) PCR for other molecular microbial source tracking markers. Enterococcus numbers were determined by the regulatory US EPA Method 1600 (US Environmental Protection Agency 2002). Sampling sites represented by a solid diamond (◆) were qPCR-positive for Campylobacter spp.; additional data for Campylobacter-positive sites are summarized in Table 1. Sampling sites represented by a solid circle (•) were qPCR-negative for Campylobacter spp.

(HPyV), general Bacteroidales, human-specific Bacteroidales, and M. smithii using the PCR methods published by Harwood et al. (2009), with the filtered samples processed as described above. The HPyV PCR assay targets the conserved T antigen region of the genome; the general and human-specific Bacteroidales assays both target the 16S rRNA gene; and the M. smithii assay targets the nifH gene.

Detection of Campylobacter by qPCR

A Taqman-based qPCR assay was used for the detection of thermotolerant Campylobacter spp. using the primers and probe, CampF2, CampR2 and CampP2 (Table 4). Each reaction mixture consisted of 2.5 mM magnesium chloride, 200 μM dNTPs (deoxyribonucleotide triphosphate), 200 nM forward and reverse primers, 8 nM probe and 0.05 U/μL Taq polymerase (Roche Applied Science, Indianapolis, IN) in 1× PCR buffer; 2 μL of template DNA was added to 23 μL of prepared master-mix for a final volume of 25 μL per reaction. A Corbett Rotor-Gene™ 3000 (Qiagen, Valencia, CA) was used for all reactions with

Table 2 | Campylobacter presence in raw sewage

Lift station[a]	Sampling date	Estimated Campylobacter cells/500 mL[b]	Species[c]	Molecular MST indicators[d]	Enterococcus (CFU/dL)[e]
89	3 Nov 08	3.5×10^3	C. jejuni	All	3.0×10^7
89	18 May 08	4.8×10^3	Campylobacter spp.	All	TNTC
89	28 July 08	5.4×10^3	Campylobacter spp.	All	4.4×10^6
76	24 July 08	7.1×10^2	Campylobacter spp.	All	1.2×10^7
89	18 Aug 08	2.9×10^3	C. jejuni/C. coli	All	TNTC
89	19 Aug 08	7.6×10^2	Campylobacter spp.	All	2.7×10^6
54	16 Oct 08	6.8×10^2	C. jejuni	All	5.0×10^6
89	17 Oct 08	2.5×10^3	C. jejuni	All	3.7×10^6
89	11 Feb 09	3.1×10^7	Campylobacter spp.	All	1.1×10^5
89	30 July 08	2.6×10^3	Campylobacter spp.	All	2.2×10^6
54	26 Aug 08	1.5×10^4	C. coli	All	1.6×10^5
54	7 Oct 08	4.2×10^4	Campylobacter spp.	All	8.2×10^5
54	14 Oct 08	1.0×10^4	Campylobacter spp.	All	1.7×10^5
89	15 Oct 08	6.1×10^3	Campylobacter spp.	All	8.5×10^4
89	9 Feb 09	3.9×10^3	Campylobacter spp.	All	1.1×10^5

[a]Lift stations in Northwest Florida are given a designated number. This number describes the location of the lift station.
[b]The number of Campylobacter cells in a 500 mL grab sample from the given sampling locations as determined by qPCR.
[c]Campylobacter spp. detected by qPCR.
[d]Samples were also tested for the molecular MST indicators, HPyV, total and human-specific Bacteroidales and M. smithii.
[e]Samples were also tested for Enterococcus using EPA Method 1600 and the number of enterococci CFU/dL are shown.

Table 3 | Presence of *Campylobacter* spp. in mammal and bird feces

Animal	Date sampled	Number of samples[a]	Number positive for *Campylobacter*[b]	Pathogenic species[c]	Molecular MST indicators[d]
Cat	Aug 2008	18	9	*C. coli* (2)	None
Dog	Aug 2008	25	19	*C. coli* (1) *C. jejuni* (3)	None
Cow	21 Aug 2008	25	23	*C. coli* (1) *C. jejuni* (17)	None
Seagull	27 Apr 2009, 1 May 2009	18	6	*C. coli* (4)	None
Chicken	25 Oct 2008	25	18	*C. coli* (4)	None
Duck	14 Oct 2008, 22 Apr 2009	25	16	*C. coli* (3) *C. jejuni* (11)	None
Geese	9 Mar 2009	12	2	None detected	None
Pigs	20 Dec 2005	25	16	*C. coli* (3) *C. jejuni* (2)	*M. smithii* (4)

[a]Number of fecal samples tested for thermophilic *Campylobacter* spp. by qPCR.
[b]Number of fecal samples that tested positive for thermophilic *Campylobacter* spp.
[c]Number (in parenthesis) of *C. coli* and *C. jejuni* that was present, if any, in those samples that tested positive for thermophilic *Campylobacter* spp.
[d]Samples were also tested for the molecular MST indicators, HPyV, total- and human-specific *Bacteroidales* and *M. smithii*.

Table 4 | Primers used for detection of *Campylobacter* spp. by qPCR and multiplex-PCR

Primer name	Primer sequence	Species amplified	Reference
CampF2	5′-CAC GTG CTA CAA TGG CAT AT-3′	*Campylobacter* spp.	Lund *et al.* (2004)
CampR2	5′-GGC TTC ATG CTC TCG AGT T-3′	*Campylobacter* spp.	Lund *et al.* (2004)
CampP2	5′-FAM CAG AGA ACA ATC CGA ACT GGG ACA BHQ1-3′	*Campylobacter* spp.	Lund *et al.* (2004)
16S-F	5′-GGA GGC AGC AGT AGG GAA TA-3′	*Campylobacter* spp.	Cloak & Fratamico (2002)
16S-R	5′-TGA CGG GCG GTG AGT ACA AG-3′	*Campylobacter* spp.	Cloak & Fratamico (2002)
CC18F	5′-GGT ATG ATT TCT ACA AAG CGA G-3′	*C. coli*	Cloak & Fratamico (2002)
CC519R	5′-ATA AAA GAC TAT CGT CGC GTG-3′	*C. coli*	Cloak & Fratamico (2002)
HipO-F	5′-GAC TTC GTG CAG ATA TGG ATG CTT-3′	*C. jejuni*	Cloak & Fratamico (2002)
HipO-R	5′-GCT ATA ACT ATC CGA AGA AGC CAT CA-3′	*C. jejuni*	Cloak & Fratamico (2002)

the following program: 94 °C for 10 min followed by 40 cycles of 15 s at 94 °C and 60 s at 58 °C. Each reaction was performed in duplicate. Fluorescence signals were measured every cycle at the end of the annealing step. The resulting data were analyzed using Corbett's Rotor-Gene™ software. A standard curve was constructed relating the number of bacterial cells to the cycle threshold (C_T) of qPCR for known concentrations of bacteria determined by culture (CFU). Standard curves were prepared in fresh water, marine water and buffered water and the standard curve that was run with each set of samples had the corresponding water matrix. Water matrices were tested for the presence of *Campylobacter* spp. Only matrices negative for *Campylobacter* spp.

were used in the preparation of standard curves. Samples that amplified before a C_T value of 40 and before both the negative and no template controls started showing non-specific amplification were considered positive, corresponding to approximately 50 cells –5×10^8 cells/500 mL.

Laboratory culture of *Campylobacter* spp.

C. jejuni and *C. coli* were grown at 37 °C in a microaerobic environment on tryptic soy agar (TSA) supplemented with 5% defibrinated sheep's blood (TSAB) for 72 h. A microaerobic environment was created by placing a MicroAero pack (Mitsubishi Gas Chemical America, Inc.,

New York, USA) directly into a 2.5 L sealable jar. Upon exposure to air, the pack rapidly removes oxygen to produce a final atmosphere of approximately 6–12% O_2 and 5–8% CO_2. Cells were harvested by scraping into PBS and cells were diluted from 10^{-1} to 10^{-6}; 100 µL of each dilution was plated on TSA and allowed to grow for 72 h in microaerobiosis. After 72 h, colonies were counted and used to calculate the number of cells present in each dilution. Immediately after dilution, 1 mL of each dilution was spiked into 500 mL of three different water matrices: phosphate-buffered water, and recently collected marine water and fresh water. These dilutions were filtered and the filters processed as described above.

Detection of *Campylobacter* by multiplex PCR

Table 4 shows the primers used in a multiplex PCR adapted from Oyarzabal *et al.* (2005) to determine if *C. jejuni* and/or *C. coli* were present in *Campylobacter*-positive samples. Each reaction mixture consisted of 3.0 mM magnesium chloride, 200 µM dNTPs, 200 nM each primer (Table 4), and 0.05 U/µL *Taq* polymerase (Roche Applied Science) in 1× PCR buffer; 2 µL of template DNA was added to 23 µL of prepared master-mix for a final volume of 25 µL per reaction. Reactions were performed in duplicate. A Corbett Palm Cycler (Qiagen) was used for all reactions with the following program: 94 °C for 10 min followed by 30 cycles of 60 s at 94 °C, 60 s at 50 °C, and 60 s at 72 °C. A final extension was performed for 5 min at 72 °C and finally samples were held at 4 °C until subjected to agarose gel electrophoresis. PCR products were visualized on a 1% agarose gel with 0.05 µg/mL ethidium bromide using standard techniques.

Statistical analysis

Statistical analyses were performed using GraphPad InStat[TM] software (GraphPad Software, Inc., La Jolla, CA, USA). A probability of <0.05 was considered significant. Regression analyses elucidated correlations between *Campylobacter* and enterococcus concentrations. C_T values (dependent variable) from *Campylobacter* qPCR and *Enterococcus* counts (independent variable) in the same sample were used for these analyses. Correlation analysis also compared the C_T values from *Campylobacter* qPCR and the

presence/absence of molecular MST indicators. Lastly, we analyzed a contingency table using Fisher's exact test to determine if the Florida Department of Health guidelines for water quality based on enterococcus counts could predict the presence of *Campylobacter*. For this analysis, water samples were considered a potential health threat if there were ≥36 enterococci per dL of marine water, in accordance with the Florida Department of Health standards, which are based on bacterial water-quality criteria articulated by the US Environmental Protection Agency (1986, 2003). Samples were considered positive for *Campylobacter* based upon qPCR results.

RESULTS AND DISCUSSION

Detection of the molecular MST indicators, enterococci and *Campylobacter* spp. in environmental water

To determine whether surface waters in Northwest Florida could pose a human health threat through the presence of *Campylobacter* spp. and whether conventional indicators or potential molecular MST indicators could correctly identify surface waters contaminated with *Campylobacter* spp., a variety of fresh and marine water sources were sampled over a period of 14 months (July 2008–September 2009). Comparison of qPCR results for the fresh and marine water samples versus the PBS samples indicated that the more complex matrices did not inhibit the qPCR reaction. In the case of fresh water, *Campylobacter* spp. were detected at 38% (8 of 21) of the sampling sites. *Campylobacter* cell numbers ranged from approximately 70 to 2.3×10^6 cells/500 mL. *Campylobacter jejuni* was present at one site and *C. coli* at three (Table 1). The indicators, HPyV, *Bacteroidales* and *M. smithii*, were not detected in any of these water samples with the exception of those from the Highway 399 site. Total *Bacteroidales* was found at all sampling locations at Hwy 399, even those that were negative for *Campylobacter* (data not shown). Human-specific *Bacteroidales* was also detected in *Campylobacter*-positive samples taken both upstream and downstream of a storm drain on this road. Enterococci were present in all but one sample that was positive for *Campylobacter*

spp. Most importantly, enterococci were detected in samples positive for *C. jejuni* and *C. coli* but were also detected in a greater number of samples that were negative for *Campylobacter* spp., indicating a lack of specificity for the presence of this pathogen.

Campylobacter spp. were detected in water samples from 2 of the 11 marine water sites (18.2%). East Bay samples were positive for *Campylobacter* spp. at a single sampling time and Quietwater Beach samples were positive on three different sampling days (Table 1). Both of these sites had experienced several days of intermittently heavy rain prior to the sampling event. As a result, the salinity at Quietwater Beach was diluted to 10.1 ppt (typically 13–17 ppt), and that of East Bay was 8.1 ppt (typically 13 ppt).

Linear regression analysis showed no correlation between the estimated *Campylobacter* cell number in a given sample and the enterococcus counts for that sample ($p = 0.79$). When looking at fresh water sites and marine water sites separately, there was still no correlation ($p = 0.70$ and $p = 0.11$, respectively). Additionally, the Florida Department of Health guidelines for the determination of surface waters that pose a health risk would not have predicted the presence of *Campylobacter*, as the analysis of the contingency table by Fisher's exact test did not indicate an association between waters containing ≥ 36 enterococci per dL and the presence of *Campylobacter* (two-sided p value $= 0.08$). In addition, the estimated number of *Campylobacter* cells in a sample did not correlate with the presence of any of the molecular MST indicators ($p = 0.38$).

Detection of molecular MST indicators and *Campylobacter* spp. in raw sewage

To determine if raw sewage could be a source of *Campylobacter* contamination in surface waters in Northwest Florida, sampling was conducted during 19 visits to three lift stations over a sampling period of 14 months (July 2008–September 2009). *Campylobacter* spp. were detected in samples from 15 visits but only two samples were positive for *C. coli* and four samples were positive for *C. jejuni* (Table 2). *Campylobacter* spp. were present in raw sewage obtained from all three Northwest Florida WWTPs at an estimated range of 1.4×10^3–5.9×10^3 *Campylobacter* cells/500 mL. *Campylobacter jejuni* was

present at the Milton, FL, WWTP. *Campylobacter jejuni* and *C. coli* were present at both the Main Street (Pensacola, FL, USA) WWTP and the Tiger Point (Gulf Breeze, FL, USA) WWTP. The molecular MST indicators were present in all samples. Enterococcus counts ranged from 1.1×10^6 CFU/dL to TNTC (too numerous to count).

Detection of the molecular MST indicators and *Campylobacter* spp. in animal fecal samples

To help establish potential reservoirs for contamination of recreational water, fecal samples from 173 animals, including cats, dogs, cows, seagulls, chickens, ducks, geese and pigs, were tested for *Campylobacter* spp. using qPCR (Table 3). These fecal samples tested negative for the molecular MST indicators with the exception of pigs in which four samples tested positive for *M. smithii*. In contrast to the molecular MST indicators, *Campylobacter* spp. were detected in all animal groups. *Campylobacter* spp. were detected in 50% of the feline samples, 64% of porcine and 76% of the canine fecal samples, but neither *C. jejuni* nor *C. coli* were present. *Campylobacter* spp. were detected in 92% of the bovine fecal samples with the majority of samples containing *C. jejuni*. On the other hand, *C. coli* was present in the majority of positive seagull and chicken fecal samples, both of which had high levels of *Campylobacter* spp. contamination. Duck feces were highly contaminated with *Campylobacter* spp. and *C. jejuni* was prevalent in a high number of samples. Geese fecal samples were less prone to *Campylobacter* spp. contamination than other birds and *C. jejuni* and *C. coli* were not detected in these samples.

Campylobacter spp. detected by qPCR were prevalent in many recreational freshwater sites in Northwest Florida, but were only present in recreational marine water sites after heavy rains. Not all sites had detectable *C. jejuni* and *C. coli*, but those sites positive for these two species had concentrations that could pose a potential health risk (~500 cells) (Black *et al.* 1988; Medema *et al.* 1996; Forsythe 2002). Additionally, our results indicate that sources of *Campylobacter* spp. may be human wastewater and/or wild animal feces. In all water matrices, the presence of *Campylobacter* spp. was not predicted by the conventional EPA method (enterococci counts), nor by the molecular MST indicators.

In Florida, the 2007 campylobacteriosis rate was approximately 5.42 cases per 100,000 (Florida Department of Health 2007). In 2005, campylobacteriosis was the ninth most reported gastrointestinal illness in Escambia County, Florida (Escambia County Health Department 2005). The majority of campylobacteriosis cases are reported in the spring and summer months (Schuster *et al.* 2005; Vereen *et al.* 2007), consistent with increased precipitation and recreational activity with weather variables including temperature and rainfall potentially influencing the transmission of this disease (Bi *et al.* 2008). Prüss (1998) reported an increased incidence of gastrointestinal symptoms in swimmers vs. non-swimmers in several countries worldwide. Most likely, rates of campylobacteriosis are much higher than reported because gastroenteric diseases are typically under-reported as they often are self-limiting and treatment is not sought. Moreover, the etiologic agent of most cases of gastroenteritis treated by medical professionals is not determined. Source determination is further complicated by the long incubation period of campylobacter infections (Porter & Reid 1980; Evans *et al.* 1996; Skirrow & Blaser 2000).

Although sporadic infections are often the result of ingesting or handling raw or undercooked food such as milk or poultry (Kilonzo-Nthenge *et al.* 2008), more widespread outbreaks are attributed to contaminated drinking or recreational water (Prüss 1998; Bolton *et al.* 1999; Hänninen *et al.* 2003; Schuster *et al.* 2005; Martin *et al.* 2006). We observed the presence of *Campylobacter* spp. in several local fresh and marine waters, most of which are typically used for recreational aquatic activities. The presence of *Campylobacter* in Northwest Florida fresh waterways is not surprising. Around the world, *Campylobacter* is frequently isolated from fresh water sources such as lakes, river waters, ground waters and surface waters (Lund 1996; Daczkowska-Kozon & Brzosteck-Nowakowska 2001; Savill *et al.* 2001; Hörman *et al.* 2004; Walters *et al.* 2007), and our overall results coincide with the different reports that suggested that aquatic environments are regularly contaminated with *Campylobacter* spp. (Koenraad *et al.* 1997; Desmond *et al.* 2008). In agreement with the present study, *Campylobacter* is not as frequently detected in marine water environments as in fresh water (Alonso & Alonso 1993), although it has been shown to be a common contaminant of shellfish (Wilson & Moore 1996).

Many sources of *Campylobacter* in surface waters have been proposed, including sewage runoff, effluent discharge, agricultural runoff and animal feces (Vereen *et al.* 2007; Pitkänen *et al.* 2008; Schets *et al.* 2008). In a study in the southern USA, the highest concentrations of campylobacters were detected directly downstream from a wastewater treatment plant that processed both human and poultry slaughterhouse waste (Vereen *et al.* 2007). However, Vereen *et al.* (2007) hypothesized that while wastewater drives a significant portion of *Campylobacter* loading in the watershed, its presence was more likely related to rainfall, runoff and wildlife fecal contamination. Our results demonstrated that *Campylobacter* were frequently isolated from raw sewage samples taken from both local area lift stations and waste water treatment facilities and that *Campylobacter* was present in all eight types of animal fecal material tested from both mammalian and avian sources. The presence of *Campylobacter* in wild bird fecal material is in agreement with many other studies (Broman *et al.* 2002; Wahlström *et al.* 2003; Abulreesh *et al.* 2006; Schets *et al.* 2008). *Campylobacter* has also been found in the gastrointestinal tract of domesticated animals, including livestock and pets, and many wild animals (Wahlström *et al.* 2003).

The present research explored possible correlations between *Campylobacter* detection in local waterways and the presence of the conventional indicator organism, enterococcus. EPA method 1600 is the typical method for determining fecal contamination of a waterway. One limitation of this method is that the presence of enterococci does not indicate a source of human fecal pollution, as these organisms are found in the intestinal tract of all warm-blooded mammals. In addition, *Campylobacter* spp., in particular *C. jejuni* and *C. coli*, are also present in the fecal material from warm-blooded mammals. Therefore, we were unable to correlate the presence of *Campylobacter* spp. to enterococcus counts in Northwest Florida local waterways. Contamination of waterways with *Campylobacter* spp. is a complex process that can result from avian and mammalian fecal contamination and/or wastewater contamination (Skelly & Weinstein 2003), and the correlation between fecal indicator bacteria and *C. jejuni* and *C. coli* is inconclusive in the literature. Several researchers have found that *Campylobacter* detection was poorly

correlated with traditional indicator organisms (Lund 1996; Waage *et al.* 1999; Savill *et al.* 2001; Hörman *et al.* 2004; Ahmed *et al.* 2008). When the waterborne campylobacters are prevalent, as is the case in mixed-use rural watersheds in the coastal plain of southern Georgia, USA (Vereen *et al.* 2007), and New Zealand's waterways (Desmond *et al.* 2008), a positive correlation between the presence of *Campylobacter* and fecal coliforms in an aquatic environment is apparent. It is important to emphasize that in New Zealand approximately 400 campylobacteriosis cases are notified annually per 100,000 population, which is the highest rate of notified campylobacteriosis in the developed world (Desmond *et al.* 2008).

Host-specific PCR assays of molecular MST indicators have been suggested as useful additional methods of detecting human fecal pollution in water and identifying sources of pollution (Harwood *et al.* 2009). However, host-specific assays must correctly identify health risk by predicting the presence of human pathogens. This predictive power would ideally also extend to pathogens from non-human sources, such as *Campylobacter*. We were unable to correlate *Campylobacter* presence in a waterway to any of the newly proposed human-specific molecular MST indicators, suggesting that they are not sufficient for predicting the presence of pathogens that are not human-specific. The correlation between waterborne pathogens and these newly proposed markers is inconclusive in the literature (Vereen *et al.* 2007; Walters *et al.* 2007). Walters *et al.* (2007) reported a positive relationship between PCR detection of a general fecal marker for *Bacteroidales* and the presence of *E. coli* O157:H7, *Campylobacter* and *Salmonella* in river water; moreover, they found a threefold increase in detection of *Campylobacter* spp. when human-specific *Bacteroidales* markers were present. Although *Campylobacter* spp. were detected in our local fresh and marine waterways, no waterborne outbreaks of campylobacteriosis were reported during our sampling time (Florida Department of Health 2009).

Whereas our results indicate that *Campylobacter* presence in water is not predicted by conventional EPA methods or molecular MST indicators, there are methodological limitations that need to be considered. In previous studies, C_T values of qPCR analyses were extrapolated to cell counts using most probable numbers methods (Rothrock *et al.* 2009). Our study estimated the number of *Campylobacter* cells in a given sample by comparison to a standard curve based on serial dilutions of campylobacters whose concentrations were determined by concurrent plate culturing. Moreover, possible PCR inhibitors in environmental samples could generate false negatives for both *Campylobacter* and MST markers. We tried to minimize these effects by preparing qPCR standard curves in environmental waters to more closely represent C_T values in the presence of inhibitors. We found few samples that were positive for *C. jejuni* and *C. coli*, especially within salt water samples, although the number positive at the species level was larger. A larger study similar to this one should be performed to validate results presented in this manuscript. As with any study using PCR to detect *Campylobacter* spp., one must consider the presence of DNA from VBNC cells. However, the contribution of VBNC states in the contamination cycle of *Campylobacter* spp. is considered insignificant (Koenraad *et al.* 1997).

A method of concentrating water samples, followed by treatment with propidium monoazide (PMA) has been gaining acceptance as a means of selectively amplifying DNA from cells in a sample with intact cell membranes (Nocker *et al.* 2006, 2007; Vesper *et al.* 2008; Bae & Wuertz 2009; Varma *et al.* 2009). Our further studies will involve the use of PMA to determine the percentage of the campylobacters detected by PCR in Northwest Florida waterways attributable to membrane-compromised cells, which will improve the risk estimation of campylobacteriosis from recreational waters.

CONCLUSIONS

This study suggests that contamination of subtropical recreational waterways with *Campylobacter* spp., including those that cause human disease, may be a source of sporadic acute gastrointestinal disease, and that both the standard EPA method and the newly proposed culture-independent methods may not be useful indicators for the presence of *Campylobacter* in recreational water. Thus, assays for the specific detection of these pathogens may be necessary when testing recreational waters, especially those contaminated with sewage and animal feces. Further research is

needed to determine the significance of environmental campylobacters, including *C. jejuni* and *C. coli*, in public health. For instance, studies could elucidate the infectivity of these environmental strains and any seasonal risks they may pose.

ACKNOWLEDGEMENTS

REFERENCES

Abulreesh, H. H., Paget, T. A. & Goulder, R. 2006 Campylobacter in waterfowl and aquatic environments: incidence and methods of detection. Environ. Sci. Technol. 40 (23), 7122–7131.

Ahmed, W., Huygens, F., Goonetilleke, A. & Gardner, T. 2008 Real-time PCR detection of pathogenic microorganisms in roof-harvested rainwater in Southeast Queensland, Australia. Appl. Environ. Microbiol. 74 (17), 5490–5496.

Alonso, J. L. & Alonso, M. A. 1993 Presence of Campylobacter in marine waters of Valencia, Spain. Water Res. 27 (10), 1559–1562.

Aydin, F., Atabay, H. I. & Akan, M. 2001 The isolation and characterization of Campylobacter jejuni subsp. jejuni from domestic geese (Anser anser). J. Appl. Microbiol. 90 (4), 637–642.

Bae, S. & Wuertz, S. 2009 Discrimination of viable and dead fecal Bacteroidales bacteria by quantitative PCR with propidium monoazide. Appl. Environ. Microbiol. 75 (9), 2940–2944.

Bi, P., Cameron, A. S., Zhang, Y. & Parton, K. A. 2008 Weather and notified Campylobacter infections in temperate and sub-tropical regions of Australia: an ecological study. J. Infect. 57 (4), 317–323.

Black, R. E., Levine, M. M., Clements, M. L., Hughes, T. P. & Blaser, M. J. 1988 Experimental Campylobacter jejuni infection in humans. J. Infect. Dis. 157 (3), 472–479.

Bolton, F. J., Surman, S. B., Martin, K., Wareing, D. R. A. & Humphrey, T. J. 1999 Presence of Campylobacter and Salmonella in sand from bathing beaches. Epidemiol. Infect. 122 (1), 7–13.

Broman, T., Palmgren, H., Bergstrom, S., Sellin, M., Waldenstrom, J., Danielsson-Tham, M. L. & Olsen, B. 2002 Campylobacter jejuni in black-headed gulls (Larus ridibundus): prevalence,

genotypes, and influence on C. jejuni epidemiology. J. Clin. Microbiol. 40 (12), 4594–4602.

Clark, C. G., Price, L., Ahmed, R., Woodward, D. L., Melito, P. L. & Rodgers, F. G. 2003 Characterization of waterborne outbreak-associated Campylobacter jejuni, Walkerton, Ontario. Emerg. Infect. Dis. 9, 1232–1241.

Cloak, O. M. & Fratamico, P. M. 2002 A multiplex PCR for the differentiation of Campylobacter jejuni and C. coli from a swine processing facility and characterization of isolates by PFGE and antibiotic resistance profiles. J. Food Protect. 65 (2), 266–273.

Daczkowska-Kozon, E. & Brzosteck-Nowakowska, J. 2001 Campylobacter spp. in waters of three main Western Pomerania water bodies. Int. J. Hyg. Environ. Health 203 (5–6), 435–443.

Desmond, T., McBride, G., Ball, A., Taylor, K. & Pyle, E. 2008 Large-scale freshwater microbiological study: rationale, results and risks. J. Water Health 6 (4), 443–460.

Escambia County Health Department 2005 Epidemiology Program 2005 Year-end Summary. Available from: http://www.escambiahealth.com/epi/pdfs/epi_yearly_report/ECHD_epi_report_2005.pdf (accessed 21 September 2009).

European Food Safety Authority 2006 Trends and Sources of Zoonoses, Zoonotic Agents and Antimicrobial Resistance in the European Union in 2004. European Food Safety Authority 96-16. Available from: www.efsa.europa.eu/en/scdocs/doc/310anr.pdf (accessed 23 June 2011).

Evans, M. R., Roberts, R. J., Ribeiro, C. D., Gardner, D. & Kemberley, D. 1996 A milk-borne Campylobacter outbreak following an educational farm visit. Epidemiol. Infect. 117, 457–462.

Fallacara, D. M., Monahan, C. M., Morishita, T. Y. & Wack, R. F. 2001 Fecal shedding and antimicrobial susceptibility of selected bacterial pathogens and a survey of intestinal parasites in free-living waterfowl. Avian Dis. 45, 128–135.

Florida Department of Health 2007 Summary of Selected Notifiable Diseases and Conditions: Campylobacteriosis. Available from: http://www.doh.state.fl.us/Disease_ctrl/epi/Morbidity_Report/2007/Disease/Campylobacteriosis.pdf (accessed 4 August 2009).

Florida Department of Health 2009 Epidemiological Updates – 2009. Available from: http://www.doh.state.fl.us/disease_ctrl/epi/Epi_Updates/2009_index.html (accessed 23 May 2011).

Forsythe, S. F. 2002 Risk analysis. In: The Microbiological Risk Assessment of Food (S. F. Forsythe, ed.). Wiley-Blackwell, Malden, MA, p. 96.

French, N., Midwinter, P. 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 (3), 779–783.

Guy, R. A., Payment, P., Krull, U. J. & Horgen, P. A. 2003 Real-time PCR for quantification of Giardia and Cryptosporidium in environmental water samples and sewage. Appl. Environ. Microbiol. 69 (9), 5178–5185.

Hakkinen, M. & Hänninen, M.-L. 2009 Shedding of Campylobacter spp. in Finnish cattle on dairy farms. J. Appl. Microbiol. 107 (3), 898–905.

Hänninen, M. J., Haajanen, H., Pummi, T., Wermundsen, 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 (3), 1391–1396.

Harwood, V. J., Brownell, M., Wang, S., Lepo, J. E., Ellender, R. D., Ajidahun, A., Hellein, K. N., Kennedy, E., Ye, W. & Flood, C. 2009 Validation and field testing of library-independent microbial source tracking methods in the Gulf of Mexico. Water Res. 43 (19), 4812–4819.

He, J.-W. & Jiang, S. 2005 Quantification of enterococci and human adenoviruses in environmental samples by real-time PCR. Appl. Environ. Microbiol. 71 (5), 2250–2255.

Hörman, A., Rimhanen-Finne, R., Maunula, L., von Bonsdorff, C. H., Torvela, N., Heikinheimo, A. & Hänninen, M. J. 2004 Campylobacter spp., Giardia spp., Cryptosporidium spp., noroviruses, and indicator organisms in surface water in southwestern Finland, 2000–2001. Appl. Environ. Microbiol. 70 (1), 87–95.

Horrocks, S. M., Anderson, R. C., Nisbet, D. J. & Ricke, S. C. 2009 Incidence and ecology of Campylobacter jejuni and coli in animals. Anaerobe 15 (1–2), 18–25.

Kilonzo-Nthenge, A., Nahashon, S. N., Chen, F. & Adefope, N. 2008 Prevalence and antimicrobial resistance of pathogenic bacteria in chicken and guinea fowl. Poultry Sci. 87 (8), 1841–1848.

Kinzelman, J., McLellan, S. L., Amick, A., Preedit, J., Scopel, C. O., Olapade, O., Gradus, S., Singh, A. & Sedmak, G. 2008 Identification of human enteric pathogens in gull feces at Southwestern Lake Michigan bathing beaches. Can. J. Microbiol. 54 (12), 1006–1015.

Koene, M. G. J., Houwers, D. J., Dijkstra, J. R., Duim, B. & Wagenaar, J. A. 2009 Strain variation within Campylobacter species in fecal samples from dogs and cats. Vet. Microbiol. 133 (1–2), 199–205.

Koenraad, P. M. F. J., Rombouts, F. M. & Notermans, S. H. W. 1997 Epidemiological aspects of thermophilic Campylobacter in water-related environments: a review. Water Environ. Res. 69, 52–63.

Layton, A., McKay, L., Williams, D., Garrett, V., Gentry, R. & Saylar, G. 2006 Development of Bacteroides 16S rRNA gene Taqman-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Appl. Environ. Microbiol. 72 (6), 4214–4224.

Lund, M., Nordentoft, S., Pedersen, K. & Madsen, M. 2004 Detection of Campylobacter spp. in chicken fecal samples by real-time PCR. J. Clin. Microbiol. 42 (11), 5125–5132.

Lund, V. 1996 Evaluation of E. coli as an indicator for the presence of Campylobacter jejuni and Yersinia enterocolitica in chlorinated and untreated oligotrophic lake water. Water Res. 30 (6), 1528–1534.

Martin, S., Penttinen, P., Ljungström, M., Allestam, G., Andersson, Y., Giesecke, J. & Hedin, G. 2006 A case-cohort study to investigate concomitant waterborne outbreaks of Campylobacter and gastroenteritis in Söderhamn, Sweden, 2002–3. J. Water Health 4, 417–424.

Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M. & Tauxe, R. V. 1999 Food-related illness and death in the United States. Emerg. Infect. Dis. 5 (5), 607–625.

Medema, G. J., Teunis, P. F., Havelaar, A. H. & Haas, C. N. 1996 Assessment of the dose–response relationship of Campylobacter jejuni. Int. J. Food Microbiol. 30 (1–2), 101–111.

Nocker, A., Cheung, C.-Y. & Camper, A. K. 2006 Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J. Microbiol. Meth. 67 (2), 310–320.

Nocker, A., Sossa-Fernandez, P., Burr, M. & Camper, A. K. 2007 Use of propidium monoazide for live/dead distinction in microbial ecology. Appl. Environ. Microbiol. 73 (16), 5111–5117.

Nogva, H. K., Bergh, A., Holck, A. & Rudi, K. 2000 Application of the 5′-nuclease PCR assay in evaluation and development of methods for quantitative detection of Campylobacter jejuni. Appl. Environ. Microbiol. 66 (9), 4029–4036.

Oyarzabal, O. A., Macklin, K. S., Barabaree, J. M. & Miller, R. S. 2005 Evaluation of agar plates for direct enumeration of Campylobacter spp. from poultry carcass rinses. Appl. Environ. Microbiol. 71 (6), 3351–3354.

Pitkänen, T., Miettinen, I. T., Nakari, U. M., Takkinen, J., Nieminen, K., Siitonen, A., Kuusi, M., Holopainen, A. & Hänninen, M. L. 2008 Faecal contamination of a municipal drinking water distribution system in association with Campylobacter jejuni infections. J. Water Health 6 (3), 365–376.

Porter, I. A. & Reid, T. M. S. 1980 A milk-borne outbreak of Campylobacter infection. J. Hyg. 84, 415–419.

Prüss, A. 1998 Review of epidemiological studies on health effects from exposure to recreational water. Int. J. Epidemiol. 27 (1), 1–9.

Public Health Agency of Canada 2010 C-EnterNet 2008 Annual Report, National Integrated Enteric Pathogen Surveillance Program, Public Health Agency of Canada. Available from: (http://www.phac-aspc.gc.ca/c-enternet/pubs/2008/index-eng.php (accessed 23 May 2011).

Robinson, D. A. 1981 Infective dose of Campylobacter jejuni in milk. Br. Med. J. 282, 1584.

Rothrock, M. J., Cook, K. L. & Bolster, C. H. 2009 Comparative quantification of Campylobacter jejuni from environmental samples using traditional and molecular biological techniques. Can. J. Microbiol. 55 (6), 633–641.

Sáenz, Y., Zarazaga, M., Lantero, M., Gastañares, J., Baquero, F. & Torres, C. 2000 Antibiotic resistance in Campylobacter strains isolated from animals, food, and humans in Spain in 1997–1998. Antimicrob. Agents Ch. 44 (2), 267–261.

Santo Domingo, J. W., Seifring, S. C. & Haugland, R. A. 2003 Real-time PCR method to detect Enterococcus faecalis in water. Biotechnol. Lett. 25 (3), 261–265.

Savill, M. G., Hudson, J. A., Ball, A., Klena, J. D., Scholes, P., Whyte, R. J., McCormick, R. E. & Jankovic, D. 2001

Enumeration of *Campylobacter* in New Zealand recreational and drinking waters. *J. Appl. Microbiol.* **91** (1), 38–46.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Jones, J. L. & Griffin, P. M. 2011 Foodborne illness acquired in the United States—major pathogens. *Emerg. Infect. Dis.* **17**, 7–15.

Schets, F. M., van Wijnen, J. H., Schijven, J. F., Schoon, H. & de Roda Husman, A. M. 2008 Monitoring of waterborne pathogens in surface waters in Amsterdam, The Netherlands, and the potential health risk associated with exposure to *Cryptosporidium* and *Giardia* in these waters. *Appl. Environ. Microbiol.* **74** (7), 2069–2078.

Schuster, C. J., Ellis, A. G., Robertson, W. J., Charron, D. F., Aramini, J. J., Marshall, B. J. & Medeiros, D. T. 2005 Infectious disease outbreaks related to drinking water in Canada, 1974–2001. *Can. J. Public Health* **96** (4), 254–258.

Skelly, C. & Weinstein, P. 2003 Pathogen survival trajectories: an eco-environmental approach to the modeling of human campylobacteriosis ecology. *Environ. Health Perspect.* **111** (1), 19–28.

Skirrow, M. B. & Blaser, M. J. 2000 Clinical aspects of Campylobacter infection. In: *Campylobacter*, 2nd edition. (M. J. Nachamkin & M. J. Blaser, eds.). American Society for Microbiology, Washington, DC, pp. 66–88.

Stoeckel, D.M. & Harwood, V.J. 2007 Performance, design, and analysis in microbial source tracking studies. *Appl. Environ. Microbiol.* **73** (8), 2405–2415.

Uaboi-Egbenni, P. O., Okolie, P. N., Adesanya, O. D., Omonigbehin, E. & Sobande, A. O. 2008 Epidemiological studies of the incidence of pathogenic *Campylobacter* spp. amongst animals in Lagos metropolis. *Afr. J. Biotechnol.* **7** (16), 2852–2856.

US Environmental Protection Agency 1986 *Ambient Water Quality for Bacteria*. EPA440/5-84-002, US Environmental Protection Agency, Office of Water, Regulations and Standards, Washington, DC.

US Environmental Protection Agency 2002 *Method 1600: Enterococci in water by membrane filter using membrane-enterococcus indoxyl-β-glucoside agar (mEI)*. EPA/821-R02-022, US Environmental Protection Agency, Office of Water, Washington, DC.

US Environmental Protection Agency 2003 *Bacterial water quality standards for recreational waters (freshwater and marine waters)*. EPA/823.R-03/008, US Environmental Protection Agency, Washington, DC.

US Environmental Protection Agency 2009 *Review of published studies to characterize relative risks from different sources of fecal contamination in recreational water*. EPA 822-R-09-001, US Environmental Protection Agency, Washington, DC.

Varma, M., Field, R., Stinson, M., Rukovets, B., Wymer, L. & Haugland, R. 2009 Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater. *Water Res.* **43** (19), 4790–4801.

Vereen Jr, E., Lowrance, R. R., Cole, D. J. & Lipp, E. K. 2007 Distribution and ecology of campylobacters in coastal plain streams (Georgia, United States of America). *Appl. Environ. Microbiol.* **73** (5), 1395–1403.

Vesper, S., McKinstry, C., Hartmann, C., Neace, M., Yoder, S. & Vesper, A. 2008 Quantifying fungal viability in air and water samples using quantitative PCR after treatment with propidium monoazide (PMA). *J. Microbiol. Meth.* **72** (2), 180–184.

Waage, A. S., Varund, T., Lund, V. & Kapperud, G. 1999 Detection of small numbers of *Campylobacter jejuni* and *Campylobacter coli* cells in environmental water, sewage, and food samples by a seminested PCR assay. *Appl. Environ. Microbiol.* **65** (4), 1636–1643.

Wahlström, H., Tysén, E., Olsson Engvall, E., Brändström, B., Vågsholm, I., Eriksson, E. & Mörner, T. 2003 Survey of *Campylobacter* species, VTEC O157, and *Salmonella* species in Swedish wildlife. *Vet. Rec.* **153** (3), 74–80.

Waldenström, J., On, S.L.W., Ottvall, R., Hasselquist, D. & Olsen, B. 2006 Species diversity of campylobacteria in a wild bird community in Sweden. *J. Appl. Microbiol.* **102** (2), 424–432.

Walters, S. P., Gannon, V. P. J. & Field, K. G. 2007 Detection of *Bacteroidales* fecal indicators and the zoonotic pathogens *E. coli* O157: H7, *Salmonella*, and *Campylobacter* in river water. *Environ. Sci. Technol.* **41** (6), 1856–1862.

Wilson, I. G. & Moore, J. E. 1996 Presence of *Salmonella* spp. and *Campylobacter* spp. in shellfish. *Epidemiol. Infect.* **116**, 147–153.

Zweifel, C., Scheu, K. D., Keel, M., Renggli, F. & Stephan, R. 2008 Occurrence and genotypes of *Campylobacter* in broiler flocks, other farm animals, and the environment during several rearing periods on selected poultry farms. *Int. J. Food Microbiol.* **125** (2), 182–187.