

Detection of potentially pathogenic *Arcobacter* spp. in Bangkok canals and the Chao Phraya River

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ABSTRACT

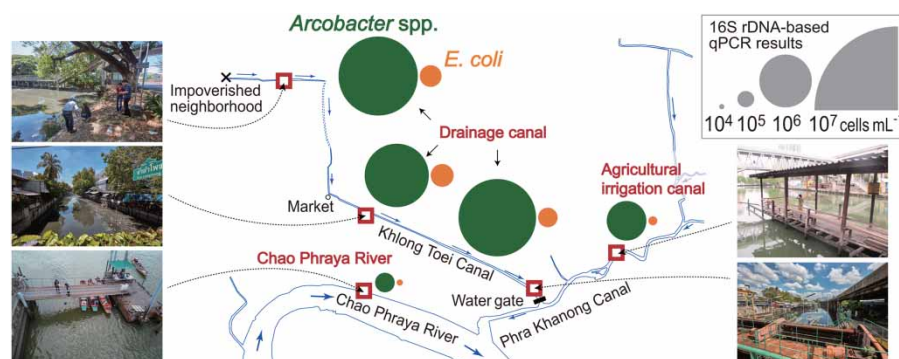
The management of pathogenic bacteria in waterways is a public health issue. Here, we investigated the concentrations of potentially pathogenic bacteria, *Arcobacter* spp. and *Campylobacter* spp., and *Escherichia coli*, by quantifying species-specific genes in surface water samples from canals and the Chao Phraya River from June 2017 to June 2018 in Bangkok, Thailand. We assessed the relationship between the specific bacterial concentrations, water quality, and seasonal changes. *Arcobacter* spp. were detected at high density in all samples and showed seasonal fluctuations according to analyses based on 16S rDNA and the invasion gene *ciaB*. High levels of 16S rDNA and *dut* gene of *E. coli* were detected in the polluted drainage canals. A high correlation was observed between *E. coli* and chemical and biochemical oxygen demand (COD and BOD), suggesting that untreated domestic wastewater was the source of the *E. coli*. In contrast, *Arcobacter* spp. were detected with high density even in water samples with relatively low COD, suggesting that *Arcobacter* spp. are more likely than *E. coli* to survive in the water environment. The analysis of 16S rDNA and *ciaB* gene sequence analyses indicated that the *Arcobacter* spp. isolated from the drainage canals were *A. butzleri* and *A. cryaerophilus*.

Key words: *Arcobacter*, *Campylobacter*, *Escherichia coli*, public health, surface water pollution

HIGHLIGHTS

- We conducted a survey of water quality and analyzed the concentrations of *E. coli*, *Arcobacter* spp., and *Campylobacter* spp. in canals in Bangkok.
- Potentially pathogenic *Arcobacter* spp. were detected in particularly high levels in the heavily polluted canals.
- *Arcobacter* spp. are likely to survive in the environment for longer periods than *E. coli*.

GRAPHICAL ABSTRACT



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INTRODUCTION

Arcobacter spp. have, in recent years, received increasing interest as emerging enteropathogens and potential zoonotic agents (Rasmussen *et al.* 2013). *Arcobacter butzleri*, *Arcobacter cryaerophilus*, and *Arcobacter skirrowii*, in particular, have been associated with human and animal diseases (Doudah *et al.* 2012). *A. butzleri* (Lau *et al.* 2002) and *A. cryaerophilus* (Hsueh *et al.* 1997) have been detected in patients with bacteremia. Taylor *et al.* (1991) reported that *A. cryaerophilus* was detected in a child with diarrhea in Thailand, and Morita *et al.* (2004) isolated *A. butzleri* from a drainage canal in Bangkok. Both *A. butzleri* and *A. cryaerophilus* have been detected in high concentrations in domestic sewage (Kirs *et al.* 2017; Cui *et al.* 2019). The surface water quality in Bangkok has deteriorated due to the population becoming more concentrated and the delay in the introduction of wastewater treatment facilities (Tabucanon 2006). Therefore, since the waterways in Bangkok may be widely contaminated with *Arcobacter* spp., it is important to investigate the concentrations of *Arcobacter* spp. in canals and rivers close to areas of human habitation in Thailand.

The detection of *Arcobacter* spp. has been shown to be related to the detection of fecal contamination indicators such as fecal coliforms, *Escherichia coli*, and intestinal enterococci in environmental waters (Collado *et al.* 2008). Lee *et al.* (2012) measured the levels of the 23S rRNA gene of *Arcobacter* spp., the 16S rRNA gene of *Bacteroides-Prevotella* (HuBac; human-specific fecal marker), and the *udiA* gene of *E. coli* (general fecal marker), and found that the density of *Arcobacter* spp. was significantly correlated with both fecal markers. However, *Arcobacter* spp. have been frequently isolated from the natural world (Wesley & Miller 2010), and it is not clear whether the changes of *Arcobacter* spp. in the environment, particularly in areas of human habitation, can be adequately evaluated with fecal markers. Furthermore, in Bangkok, where the dry season and the rainy season are separated, the concentration of *Arcobacter* spp. is likely to differ depending on the season.

Miller *et al.* (2007) analyzed the complete genome sequence of *A. butzleri* RM4018 and reported that it encodes virulence-associated genes *cadF*, *ciaB*, *cj1349*, *mviN*, *pldA*, and *tlyA*. Doudah *et al.* (2012) reported that virulence gene *ciaB* was present in all three of the allegedly pathogenic strains of *Arcobacter* that they tested. In addition, Levican *et al.* (2013) studied the adhesion and invasion of *Arcobacter* spp. by using Caco-2 cells and also showed that *ciaB* was present in all invasive strains. Therefore, *ciaB* is expected to be useful for quantitative analysis of potentially pathogenic strains of *Arcobacter*.

Here, we analyzed the concentrations of potentially pathogenic *Arcobacter* spp. and *Campylobacter* spp. by targeting specific genes in the surface waters (canal water and river water) of Bangkok, Thailand to assess the hygiene risks due to water pollution. To gather basic information for quality management of surface water, we investigated the relationship between the concentrations of these bacteria and water quality standard items [e.g., pH, dissolved oxygen (DO), biochemical oxygen demand (BOD), chemical oxygen demand (COD), oxidation–reduction potential (ORP), NH₄-N, NO₃-N, and *E. coli* colony forming units (CFU)]. The water samples tested were collected over a 13-month period at points with various levels of pollution in cooperation with the Department of Drainage and Sewerage (DDS) of the Bangkok metropolitan administration (BMA).

MATERIALS AND METHODS

Sampling

All water sampling was done in Bangkok. Sampling was performed at Site 1 (272), Site 2 (303), Site 3 (611), Site 4 (612), and Site 5 (Chao Phraya River); the numbers in parentheses are the sampling point number according to the BMA Water Control System Office. Site 1 was located near the confluence of an agricultural irrigation canal (Phra Khanong canal) from the Bang Pakong River and the Chao Phraya River. Sites 2, 3, and 4 were located on drainage canals. Drainage water flows from Site 2 to Site 4 and Site 3. Site 2 is on a minor drainage canal near an impoverished neighborhood, and Site 4 is near Bangkok's largest market (Khlong Toei Market) and an impoverished neighborhood. The Khlong Toei drainage canal merges with the irrigation canal at Site 3 and flows into the Chao Phraya River. Site 3 is inside the water gate. The Khlong Toei drainage canal is stagnant, because its flow is controlled by a sluice gate downstream of Site 3, whereas the irrigation canal with Site 1 flows freely into the Chao Phraya River. Site 5 is located slightly downstream of Klong Toei Nok Temple on the Chao Phraya River in central Bangkok (Figure 1).

Surface water from the sampling points on the drainage canals and irrigation canal (Sites 1–4) were collected monthly from June 2017 to June 2018, and water samples from Site 5 were collected from August 2017 to June 2018.

Samples for the measurement of suspended solids (SS), total nitrogen (TN), and CFU of *E. coli* were brought back to the laboratory on ice; the procedure for *E. coli* measurement was started within 5 h of sampling.

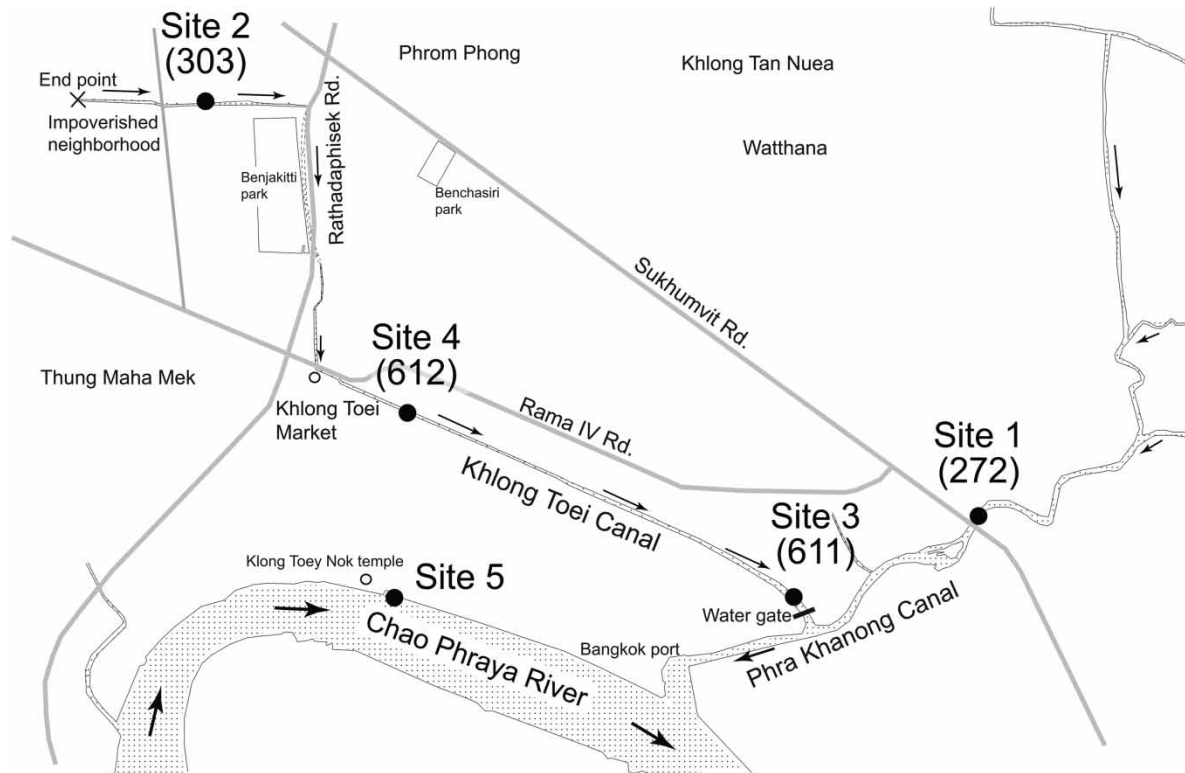


Figure 1 | Sampling points at the end of an agricultural irrigation canal (Site 1), at drainage canals (Sites 2, 3, and 4), and at the Chao Phraya River (Site 5), in central Bangkok. Arrows indicate the direction of water flow. The numbers in parentheses are the sampling points of the Water Control System Office of the Bangkok metropolitan administration. Rd, road.

Subsamples (40 mL) for DNA and nutrient analysis were fixed with ethanol (final ethanol concentration, 20%) to suppress biological activity and then stored at 4 °C until filtration. Samples for the isolation of *Arcobacter* spp. were taken at drainage canal Sites 2 and 4 on May 30, 2019. In addition, domestic wastewater (400 m³ day⁻¹; from a housing complex) flowing into the Bongai Sewage Treatment Plant (STP) in Bangkok was sampled on February 15 and June 6, 2019.

Water quality analyses

DO, pH, electrical conductivity, ORP, and temperature were measured on site using a DO meter (DO-31P; DKK-TOA Corporation, Tokyo, Japan), a pH meter (HM-30P; DKK-TOA Corporation), a conductivity tester (C65; Milwaukee Electronics Kft., Szeged, Hungary), an ORP tester (HI 98120; Hanna Instruments, Woonsocket, RI, USA), and a thermometer.

The SS concentrations were determined using a glass fiber filter with 0.4- μ m pore size (GB140; Advantec Co. Ltd, Tokyo, Japan). The BOD and TN measurements were conducted according to APHA Standard Methods. COD was determined by using a spectrophotometer (HACH DR2800; HACH Co., Loveland, CO, USA).

Twenty milliliters of each ethanol-fixed sample were filtered through a Whatman GF/F filter (Cytiva, Amersham, UK), and concentrations of orthophosphate (PO₄-P), ammonium (NH₄-N), nitrate (NO₃-N), and nitrite (NO₂-N) were determined by using a continuous flow autoanalyzer (QuAAtro2-HR; BL TEC K.K., Osaka, Japan). Total phosphorus (TP) was determined with the alkaline persulfate digestion technique (Patton & Kryskalla 2003) with unfiltered samples.

For daily precipitation, data from the Klongtoei area in Bangkok were used (<https://www.tmd.go.th/en/aboutus/department.php>).

Sample filtration and DNA extraction

Five milliliters of each ethanol-fixed sample was filtered through a PTFE (polytetrafluoroethylene) membrane filter (diameter, 25 mm; pore size, 0.2 μ m; type JG; Merck Millipore, Burlington, MA, USA) and the filters were stored at -80 °C until DNA extraction.

DNA was extracted from filter-fixed samples by using an Extrap Soil DNA Kit Plus ver. 2 (Nippon Steel & Sumikin Eco-Tech Corp., Tokyo, Japan). In brief, each filter was cut into small pieces and placed in a bead-beating tube, to which 950 μ l of extraction buffer and 50 μ l of lysis solution were added. Cells on filters were disrupted using a homogenizer (FastPrep; MP Biomedicals, LLC, Santa Ana, CA, USA) at 6.0 m s⁻¹ for 40 s, after which DNA was purified by using magnetic beads to produce 100 μ l of DNA extract. This procedure has been shown to extract DNA with almost 100% recovery rate and with suitable quality for quantitative PCR (qPCRs) (Tsuchiya *et al.* 2019).

Quantification of *Arcobacter* spp., *E. coli*, and *Campylobacter* spp. genes by real-time qPCR

qPCR of *Arcobacter* spp. and *E. coli* was conducted targeting respective 16S rDNA, *Arcobacter* spp. functional gene (*ciaB*), and *E. coli* functional gene (*dut*). The primers used (Table 1) were designed from published DDBJ/EMBL/Genbank *Arcobacter* spp. *E. coli* and *Campylobacter* spp. sequences by using Primer 3 software (National Center for Biotechnology Information). Primer specificity was tested by using NCBI-Primer BLAST and melt curve analysis in real-time qPCR (data not shown). The functional gene *ciaB*, which was used to detect *A. butzleri* and *A. cryaerophilus*, is a pathogenic gene involved in cell invasion (see Introduction). The functional gene *dut*, which was used to detect *E. coli*, encodes dUTPase, an enzyme involved in pyrimidine metabolism (Lundberg *et al.* 1983).

To prepare a standard curve for *Arcobacter* spp. 16S rDNA and *ciaB* gene quantitation, DNA was extracted from a cell suspension of *A. butzleri* strain BON-Feb-2 from the inflow water of the Bongai STP (February 15, 2019). The *E. coli* 16S rDNA and *dut* gene standard curves were prepared from a cell suspension of *E. coli* strain NBRC102203. The numbers of cells in these cell suspensions were determined by using a fluorescence microscope after DAPI (4',6-diamidino-2-phenylindole) staining. *Campylobacter* spp. was quantified by using PCR products of 16S rDNA. To create a standard curve for this purpose, amplicons generated from a partial sequence of the 16S rRNA gene of *Campylobacter jejuni* strain ATCC 29428 were amplified with univ27F and univ1492R primers (Table 1). PCR products were purified by ethanol precipitation. The 16S rDNA copy numbers in the PCR product solution used to create the standard curve were calculated from the molecular weights and densities of the PCR products. Finally, to estimate the number of *Campylobacter* spp. cells per milliliter, the 16S rDNA concentration quantified by qPCR was divided by 3, which is the number of 16S rRNA genes in the genomes of *Campylobacter* spp. Because the external standards of the qPCR were prepared as cell number-based (*Arcobacter* spp. and *E. coli*) or converted to genome copies, the qPCR results are shown with the unit, cells mL⁻¹.

Table 1 | Primers used in this study

Genetic marker	Primer	Sequence (5'-3') ^a	Product size (bp)	Amplified gene	Target(s)	References
Arco 16S	Arco605F	GAAGTGAAATCCTATAGCTTAAC	127	16S rRNA	<i>Arcobacter</i> spp.	This study
	Arco688R	CGCAATCGGTATTCTTCTGAT				
Arco <i>ciaB</i>	ciaB1433F	GAGTTYAAAGCAACAACWGGTGG	140	Invasion gene <i>ciaB</i>	<i>A. butzleri</i> , <i>A. cryaerophilus</i>	This study
	ciaB1531R	ACTTCATCAACTYCCATCCA				
Ecoli 16S	Ecoli489F	ACCTTTGCTCATTGACGTTACC	189	16S rRNA	<i>Escherichia coli</i>	This study
	Ecoli 635R	CTCAAGCTTGCCAGTATCAGAT				
Ecoli <i>dut</i>	dutA159F	TCCTGACCACGGTTCCACAC	190	Deoxyuridine triphosphatase gene <i>dut</i>	<i>Escherichia coli</i>	This study
	dutA312R	CTCCGGGTGACACTACGCT				
Camp 16S	Camp105F	AGCTTGCTAGAAGTGGATTAG	125	16S rRNA	<i>Campylobacter</i> spp.	This study
	Camp182R	TACTCAACTTGTGTTAAGCAGGAG				
Camp cal	univ27F	AGAGTTTGATCCTGGCTCAG	1,477	16S rRNA	<i>Campylobacter</i> spp.	Lane (1991)
	univ1492R	TACGGYTACCTTGTTACGACTT				
Arco seq	univ27F	AGAGTTTGATCCTGGCTCAG	1,375	16S rRNA	<i>Arcobacter</i> spp.	Lane (1991)
	EPSY1384R	CGGTGAGTACAAGACCCG				
<i>ciaB</i> seq	ciaB444F	MWTGGCAAACCTTCTTGAC	1,139	Invasion gene <i>ciaB</i>	<i>Arcobacter</i> spp.	This study
	ciaB1531R	ACTTCATCAACTYCCATCCA				

^aY, T or C; W, A or T.

The real-time qPCRs were performed individually for each primer set. The reaction mixture contained 20 µl of LightCycler 480 SYBR Green I Master (Roche, Mannheim, Germany), 10 pmol of each primer, and 1 µl of the DNA extract. Denaturation was performed for 5 min at 95 °C, followed by 40 cycles of repeated denaturation (10 s at 95 °C), annealing (10 s at 60 °C) with fluorescence acquisition (wavelength, 530 nm), and extension (10 s at 72 °C). The temperature transition rate was 4.4 °C s⁻¹ for denaturation and extension and 2.2 °C s⁻¹ for annealing. After the PCR procedure, melting curves were obtained by heating from 65 to 97 °C at a rate of 0.11 °C s⁻¹.

Isolation of *Arcobacter* spp. by culture methods and counting viable cell of *E. coli*

For the isolation of *Arcobacter* spp., 100 µl of each diluted solution was spread on Charcoal Cefoperazone Deoxycholate agar (CCDA) medium (Oxoid Ltd, Altrincham, UK) and incubated in a jar at 25 °C for 72 h under microaerophilic conditions (O₂ 6–12% and CO₂ 5–8%) created using an AnaeroPack-MicroAero system (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan). Circular white or gray colonies were selected and identified by real-time qPCR using Arco 16S primers (Table 1). Positive colonies were inoculated on Bolton agar medium (Oxoid Limited), and cells were frozen with 20% (v/v) glycerol and stored at –80 °C.

CompactDry EC plates (Nissui Pharmaceutical Co., Ltd, Tokyo Japan) were used for counting viable cells of *E. coli*. Because a maximum of 250 colonies can be counted on one plate, each fresh sample was diluted to an appropriate concentration with sterile water. One milliliter of each diluted solution was applied to a CompactDry EC plate, and the plate was incubated for 24 h at 37 °C. After incubation, blue colonies were counted as *E. coli*.

Phylogenetic analysis of *Arcobacter* spp. isolated from the Bangkok canals and sewage

DNA was extracted from *Arcobacter* spp. cells harvested from the surface of the Bolton agar medium by using an Extrap Soil DNA Kit Plus ver. 2, as described in the section ‘Sample filtration and DNA extraction’. The partial 16S rDNA sequence was amplified with Arco seq primers (Table 1), and the partial *ciaB* sequence was amplified with *ciaB* seq primers (Table 1). The PCR products were sequenced with a BigDye Terminator Kit and an Applied Biosystems 3500/3500xL Genetic Analyzer (Thermo Fisher Scientific, Richardson, TX, USA).

For the 16 strains isolated from the Bangkok sewage in February 2019, DNA extraction was performed, and then genome analysis by next-generation sequencing was performed. The extracted DNA was cleaved to fragments of about 550 bp by ultrasonic cleavage. DNA libraries were prepared using an Illumina TruSeq kit and were subsequently sequenced on an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA).

To study the phylogenetic relationships of *Arcobacter* spp. between sampling points, 16S rDNA and the invasion gene *ciaB* were used to construct phylogenetic trees by the maximum-likelihood method using the MEGA X computer program (Levicán *et al.* 2013; Kumar *et al.* 2018). The statistical significance of phylogenies was estimated by using MEGA X for bootstrap analysis with 1,000 pseudoreplicate datasets; only those grouped with bootstrap values of >50% were considered significant. The 16S rRNA gene sequences obtained in the present study were deposited in DDBJ/EMBL/GenBank with accession numbers LC574011–LC574020 and LC574932–LC574968, and the *ciaB* gene sequences obtained in the present study were deposited in DDBJ/EMBL/GenBank with accession numbers LC581284–LC581330.

Statistical analysis

All statistical analyses were performed with the Bellcurve statistics package for Excel (version 3.2.0; SSRI Co., Ltd, Tokyo, Japan). Data from the real-time qPCR assays and culture methods for *E. coli* were log-transformed for all analyses. All statistical tests on Pearson’s correlation outcomes were regarded as significant at $P < 0.01$. The difference in environmental factors between sampling sites was analyzed using nonparametric Kruskal–Wallis one-way analysis of variance, with $P < 0.05$ regarded as statistically significant.

RESULTS

Water quality at the test sites

Water quality items of drainage canals (Sites 2, 3, and 4), the end of an agricultural irrigation canal (Site 1), and the Chao Phraya River (Site 5) are listed in Table 2.

ORP was high at Site 1 on the irrigation canal and at Site 5 on the Chao Phraya River. On the other hand, in the drainage canals at Sites 2, 3, and 4, although ORP sometimes showed extremely high values, in general the values were low. DO was

Table 2 | Water quality items (mean \pm SD) at the sampling points (Sites 1–5) during the study period

Sampling site	Temperature (°C)	pH	ORP _{NHE} (mV)	DO (mg L ⁻¹)	Conductivity (μ S cm ⁻¹)	SS (mg L ⁻¹)	COD (mg L ⁻¹)
Site 1	29.1 \pm 2.1	7.1 \pm 0.4	175.1 \pm 57.1	2.6 \pm 1.0	867 \pm 125.5	19.8 \pm 7.6	45.8 \pm 8.9
Site 2	29.2 \pm 3.1	7.4 \pm 0.5	3.7 \pm 85.4	1.5 \pm 0.5	728 \pm 111.2	35.1 \pm 12.7	73.5 \pm 31.7
Site 3	30.3 \pm 2.1	7.1 \pm 0.2	-4.3 \pm 68.5	1.4 \pm 0.6	692 \pm 100.2	29.3 \pm 18.2	75.0 \pm 21.3
Site 4	30.3 \pm 2.2	7.1 \pm 0.2	-17.2 \pm 63.9	1.6 \pm 0.8	829 \pm 180.9	66.8 \pm 35.3	169.7 \pm 78.6
Site 5	31.5 \pm 2.8	7.1 \pm 0.3	240.8 \pm 30.3	3.0 \pm 0.7	371 \pm 187.6	32.7 \pm 22.0	16.2 \pm 5.3
	BOD (mg L ⁻¹)	TN (mg L ⁻¹)	NH ₄ -N (mg L ⁻¹)	NO ₂ -N (mg L ⁻¹)	NO ₃ -N (mg L ⁻¹)	PO ₄ -P (mg L ⁻¹)	TP (mg L ⁻¹)
Site 1	6.9 \pm 2.0	18.0 \pm 5.3	4.5 \pm 1.4	0.06 \pm 0.14	0.03 \pm 0.09	0.45 \pm 0.09	0.54 \pm 0.13
Site 2	29.1 \pm 7.6	24.7 \pm 9.1	12.0 \pm 3.2	0.28 \pm 0.76	0.03 \pm 0.08	0.94 \pm 0.31	1.27 \pm 0.30
Site 3	25.4 \pm 6.2	25.8 \pm 6.8	13.1 \pm 3.6	0.03 \pm 0.04	0 \pm 0	0.91 \pm 0.33	1.26 \pm 0.34
Site 4	66.8 \pm 35.8	27.5 \pm 6.1	18.1 \pm 6.4	0.06 \pm 0.09	0.02 \pm 0.05	1.51 \pm 0.81	2.08 \pm 1.65
Site 5	4.6 \pm 1.1	13.2 \pm 4.7	1.3 \pm 0.9	0.53 \pm 0.24	0.15 \pm 0.13	0.26 \pm 0.09	0.26 \pm 0.13

also relatively high at Sites 1 and 5 and low at Sites 2, 3, and 4; however, even at Site 5, the average of 3.0 mg L⁻¹ was about 40% of the saturated oxygen concentration calculated from the water temperature and was lower than DO measured upstream (Singkran *et al.* 2019). DO at Sites 1 and 5 showed high values in September (the rainy season) and tended to decrease in the dry season, but no seasonal change was observed at Sites 2, 3, or 4 (data not shown). Both COD and BOD were high in drainage canals, being more than twice as high at Site 4 than at Sites 2 or 3 (BOD, $P < 0.01$; COD, $P < 0.05$ by the Kruskal–Wallis test). The BOD and COD at Sites 1 and 5 were lower than those at the drainage canal sites, but the BOD at Site 5 was about three times the value measured at a point upstream of Bangkok (Singkran *et al.* 2019). These results indicate that a substantial quantity of organic pollutants flows into the mainstream of the Chao Phraya River in Bangkok city. The ratio of BOD to COD was high (approximately 40%) at Sites 2 and 4. We consider that this is due to direct inflows of wastewater from slums and markets. On the other hand, the ratio of BOD to COD was lower at Site 3 (33%). NH₄-N was highest at Site 4 (annual average, 18.1 mg L⁻¹), which is adjacent to the Klong Toei Market, and was also high at ≥ 12 mg L⁻¹ at Sites 2 and 3. NO₂-N and NO₃-N were low in drainage canals, especially at Sites 3 and 4. We consider that low NO₂-N and NO₃-N may be associated with the low ORP and relatively high BOD. Both TP and PO₄-P were detected in high concentrations in drainage canals.

Concentrations of *Arcobacter* spp., *E. coli*, and *Campylobacter* spp. as estimated by real-time qPCR and culture-based counting of *E. coli*

The concentrations of *Arcobacter* spp., *E. coli*, and *Campylobacter* spp. genes in water samples were determined by real-time qPCR of *Arcobacter* spp. 16S rDNA, *A. butzleri* and *A. cryaerophilus ciaB*, *E. coli* 16S rDNA, *E. coli dut*, and *Campylobacter* spp. 16S rDNA. In addition, the concentrations of cultivatable *E. coli* were determined by CFU. The seasonal changes in the above bacterial concentrations in the water samples are shown in Figure 2. The limit of detection for each real-time qPCR protocol was as follows: *Arcobacter* spp. 16S rDNA, 6.0×10^2 cells mL⁻¹; *A. butzleri* and *A. cryaerophilus ciaB*, 4.0×10^2 cells mL⁻¹; *E. coli* 16S rDNA, 1.2×10^3 cells mL⁻¹; *E. coli dut*, 3.0×10^2 cells mL⁻¹, and *Campylobacter* spp. 16S rDNA, 2.7×10^1 cells mL⁻¹.

Arcobacter spp. 16S rDNA and *A. butzleri* and *A. cryaerophilus ciaB* were detected in all samples, and the changes in the two detection results showed similar trends. For both *Arcobacter* spp. 16S rDNA and *A. butzleri* and *A. cryaerophilus ciaB*, there was little monthly change in the drainage canals or at Site 1, whereas the concentration of *Arcobacter* spp. 16S rDNA and *A. butzleri* and *A. cryaerophilus ciaB* at Site 5 tended to decrease from September to December, the second half of the rainy season. *E. coli* 16S rDNA and *E. coli dut* were detected all year round in the drainage canals (Sites 2, 3, and 4). The concentration of *E. coli* 16S rDNA and *E. coli dut* at Sites 1 and 5 were one order of magnitude lower than in the drainage canals, and were below the detection limit in the rainy season and the dry season (from June to February). Concentrations of cultivatable *E. coli* CFU (cells mL⁻¹) also tended to decrease from the rainy season to the cool season (from June to February); the one exception was Site 4, which has the highest BOD concentration, where cultivatable *E. coli* increased from

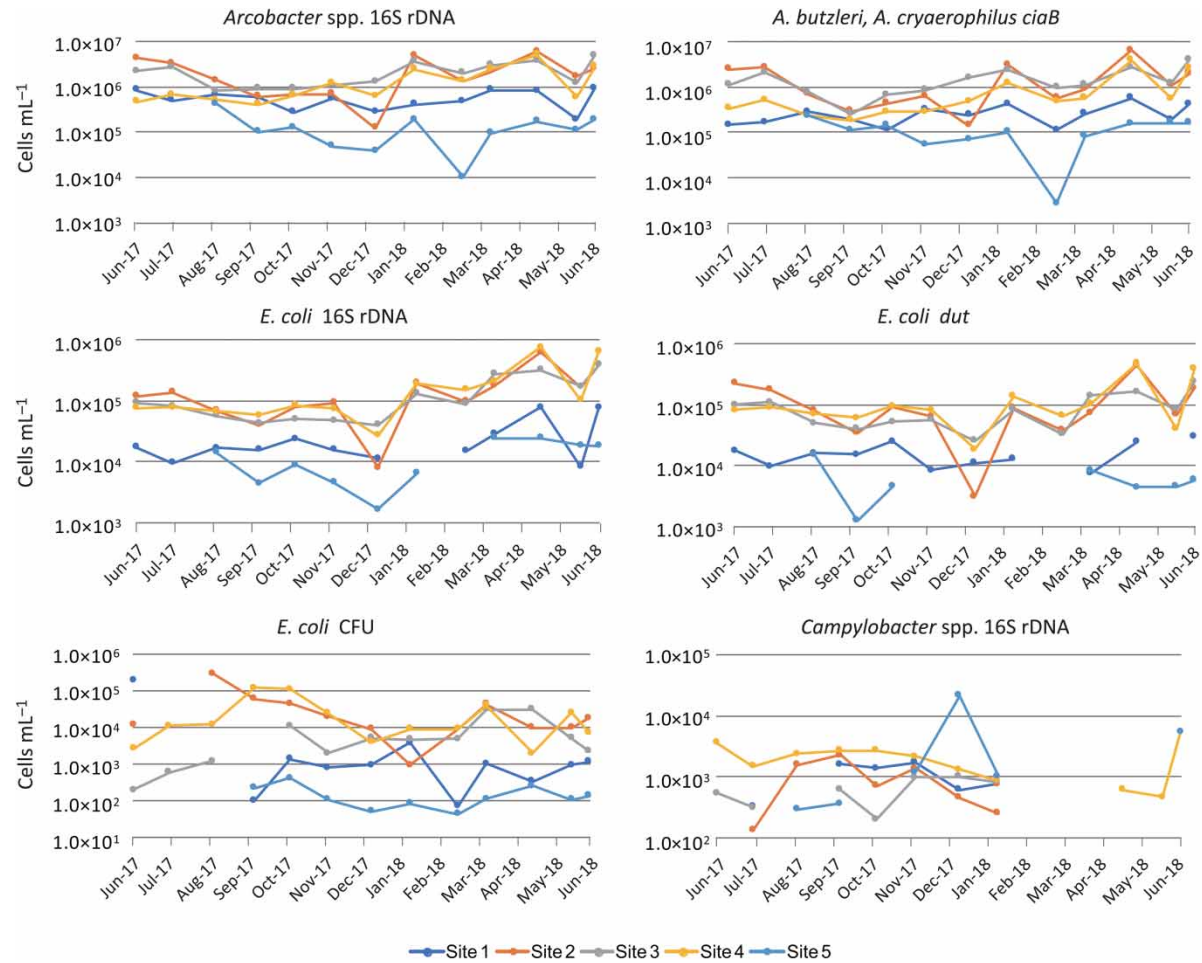


Figure 2 | Seasonal changes in specific bacterial concentrations estimated from real-time qPCR analysis of the abundance of *Arcobacter* spp. 16S rDNA, *A. butzleri* and *A. cryaerophilus ciaB*, *Escherichia coli* 16S rDNA, *E. coli dut*, and *Campylobacter* spp. 16S rDNA (cells mL⁻¹) and from counting culturable *E. coli* CFU (cells mL⁻¹). The water samples tested were collected at the five sites described in Figure 1 over a 13-month period.

September to October in the rainy season. *Campylobacter* spp. 16S rDNA was detected in the drainage canals, except for in the later part of the dry season (February to March). At Sites 1 and 5, *Campylobacter* spp. were often not detected by the 16S rDNA qPCR analysis; however, at Site 5 in December 2017, *Campylobacter* spp. were detected at 7.3×10^4 cells mL⁻¹.

Boxplots of monthly data of specific bacterial concentrations estimated from *Arcobacter* spp. 16S rDNA, *A. butzleri* and *A. cryaerophilus ciaB*, *E. coli* 16S rDNA, *E. coli dut*, and by counting *E. coli* CFU are shown in Figure 3. *Campylobacter* spp. were not detected by the 16S rDNA qPCR analysis in many samples and were excluded from the analysis. *Arcobacter* spp. were present in high concentrations in drainage canals (annual averages from 16S rDNA at Sites 2, 3, and 4 were 2.3×10^6 , 2.2×10^6 , and 1.5×10^6 cells mL⁻¹, respectively; annual averages from *ciaB* at Sites 2, 3, and 4 were 1.6×10^6 , 1.5×10^6 , and 9.0×10^5 cells mL⁻¹, respectively).

The average concentration of *Arcobacter* spp. at Site 5 was about one-tenth that in the drainage canals (estimate from 16S rDNA, 5.7×10^5 cells mL⁻¹ at Site 1 and 1.4×10^5 cells mL⁻¹ at Site 5; estimate from *ciaB*, 2.6×10^5 cells mL⁻¹ at Site 1 and 1.2×10^5 cells mL⁻¹ at Site 5). Moreover, the distribution of concentrations between the sampling points for *Arcobacter* spp. 16S rDNA was similar to that for *A. butzleri* and *A. cryaerophilus ciaB*, and the average ratio of *ciaB*/16S rDNA was 0.69. From this, we consider that most of the *Arcobacter* spp. detected by 16S rDNA in Bangkok belonged to *A. butzleri* and *A. cryaerophilus*, which are known to be pathogenic.

The box plots for *E. coli* concentration determined by the two sets of primers were similar to each other, and 74% of the *E. coli* detected by using 16S rDNA were also detected by using *dut*. This suggests that either of these two sets of primers is

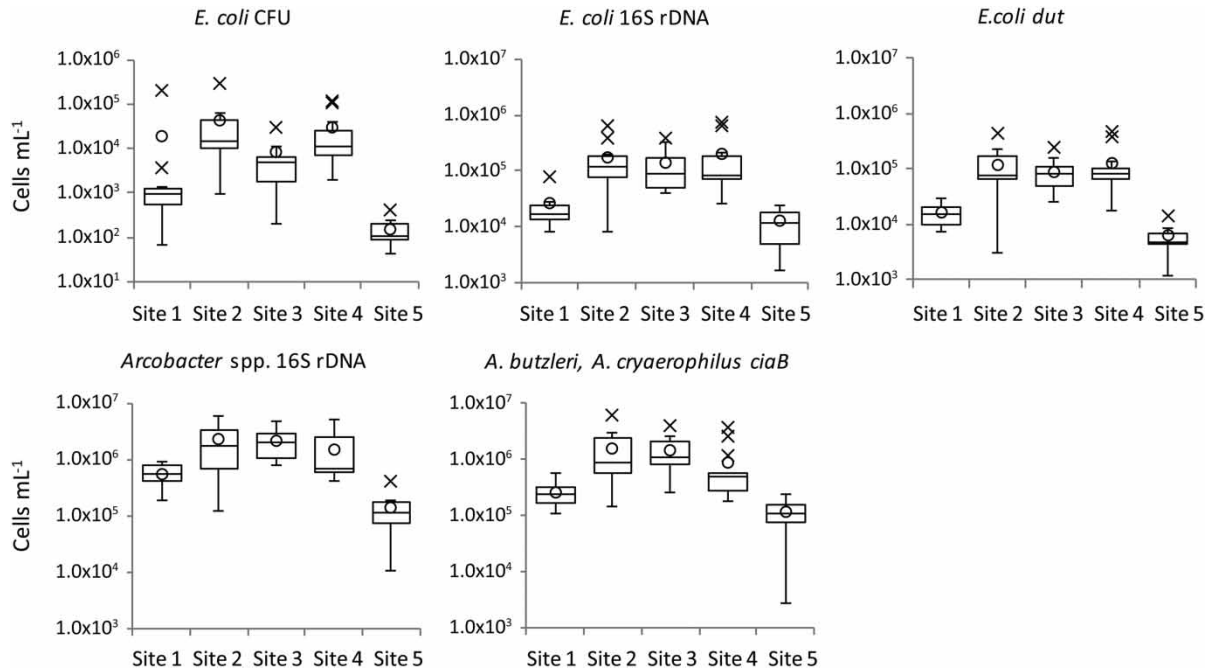


Figure 3 | Box plots of monthly data of the specific bacterial concentrations estimated from the abundance of *Arcobacter* spp. 16S rDNA, *A. butzleri* and *A. cryaerophilus* *ciaB*, *Escherichia coli* 16S rDNA, and *E. coli dut*, and from the number of *E. coli* CFUs (based on data in Figure 2). The box represents the interquartile range (IQR), the line in the box indicates the median, the circle indicates the geometric mean, the lines extending from the box show the maximum value (≤ 3 rd quartile + $1.5 \times$ IQR) and the minimum value (≥ 1 st quartile - $1.5 \times$ IQR), and crosses indicate outliers.

suitable for detecting *E. coli*. The concentration of cultivatable *E. coli* was high in the drainage canals and low in the irrigation canal and the Chao Phraya River, and it tended to be lower at Site 3 near the water gate of drainage canal exit than at Sites 2 and 4 (statistically not significant).

Relationship between water quality item and *Arcobacter* spp., *E. coli*, and *Campylobacter* spp. concentrations

Table 3 shows the relationship between water quality items and the concentrations of *Arcobacter* spp., *E. coli*, and *Campylobacter* spp. estimated by real-time qPCR and the concentration of cultivatable *E. coli* measured by counting colonies on plates. Measurements whose values were below the detection limit were excluded from the statistical analyses. ORP, DO, NO₂-N, and NO₃-N showed significantly negative correlations ($P < 0.01$) with the concentrations of *Arcobacter* spp. and *E. coli* measured by real-time qPCR and that of cultivatable *E. coli* measured by colony counting, whereas NH₄-N had significantly positive correlations ($P < 0.01$) with these concentrations. This suggests that *Arcobacter* spp. and *E. coli* concentrations are related to the degree of redox potential (anaerobic level). On the other hand, COD, BOD, and TP were correlated only with *E. coli* concentrations (measured by real-time qPCR or colony counting) ($P < 0.01$), i.e., not the other species tested.

Relationships between the qPCR-based concentrations of *Arcobacter* spp. and *Campylobacter* spp. and the qPCR- and culture-based concentrations of *E. coli*

The relationships among the estimated concentrations of *Arcobacter* spp., *Campylobacter* spp., and *E. coli* are shown in Table 4. There was a very high positive correlation between *Arcobacter* spp. and *E. coli* in the qPCR results ($r > 0.82$, $P < 0.01$). The correlation between *Arcobacter* spp. and cultivatable *E. coli* estimated from colony counting was also significant, although the correlation coefficient was not as high ($r = 0.45$, $P < 0.01$). These results indicate that the sources of *Arcobacter* spp. and *E. coli* are likely to be similar. On the other hand, no correlation was found between *Campylobacter* spp. concentrations determined by qPCR and *E. coli* concentrations determined by either method, suggesting that *Campylobacter* spp. and *E. coli* have different sources.

Table 3 | Pearson's correlation coefficients between bacterial concentrations and water quality

	Log_{10} cells mL^{-1a}					
	<i>E. coli</i> CFU	<i>E. coli</i> 16S rDNA	<i>E. coli</i> <i>dut</i>	<i>Arcobacter</i> spp. 16S rDNA	<i>A. butzleri</i> , <i>A. cryaerophilus</i> <i>ciaB</i>	<i>Campylobacter</i> spp. 16S rDNA
Rainfall 24 h (mm)	0.20	0.11	0.09	0.07	0.10	0.15
Rainfall 48 h (mm)	0.16	0.05	-0.04	0.02	0.04	0.07
Temperature ($^{\circ}\text{C}$)	-0.01	0.20	0.09	-0.01	-0.06	0.01
pH	0.10	0.09	-0.09	0.04	0.09	-0.40
ORP _{NHE} (mV)	-0.67*	-0.67*	-0.72*	-0.67*	-0.60*	-0.01
DO (mg L^{-1})	-0.53*	-0.54*	-0.50*	-0.46*	-0.44*	-0.01
Conductivity ($\mu\text{S cm}^{-1}$)	0.40*	0.30	0.36	0.50*	0.38*	-0.02
SS (mg L^{-1})	0.32	0.41*	0.35	0.11	0.02	0.15
COD (mg L^{-1})	0.54*	0.42*	0.47*	0.30	0.19	0.28
BOD (mg L^{-1})	0.60*	0.43*	0.45*	0.29	0.20	0.29
TN (mg L^{-1})	0.41*	0.45*	0.42*	0.40*	0.32	0.23
NH ₄ -N (mg L^{-1})	0.65*	0.57*	0.63*	0.53*	0.44*	0.18
NO ₃ -N (mg L^{-1})	-0.28	-0.38*	-0.46*	-0.46*	-0.32	0.02
NO ₂ -N (mg L^{-1})	-0.43*	-0.36*	-0.45*	-0.58*	-0.52*	0.08
PO ₄ -P (mg L^{-1})	0.51*	0.29	0.41*	0.25	0.20	0.33
TP (mg L^{-1})	0.38*	0.37*	0.42*	0.31	0.27	0.17

^aBacterial concentrations were estimated by qPCR of the indicated gene or by colony counting (CFU).

*Significant correlation ($P < 0.01$).

Table 4 | Pearson's correlation coefficients between bacterial concentrations of *Arcobacter* spp., *Campylobacter* spp., and *E. coli*

	Log cells mL^{-1}		
	<i>Arcobacter</i> spp. 16S rDNA	<i>A. butzleri</i> , <i>A. cryaerophilus</i> <i>ciaB</i>	<i>Campylobacter</i> spp. 16S rDNA
<i>E. coli</i> CFU (log cells mL^{-1})	0.54*	0.45*	-0.02
<i>E. coli</i> 16S rDNA (log cells mL^{-1})	0.88*	0.87*	-0.13
<i>E. coli</i> <i>dut</i> (log cells mL^{-1})	0.87*	0.82*	0.21

*Significant correlation ($P < 0.01$).

Plotting the ratio of *dut* of *E. coli* to *ciaB* of *Arcobacter* spp. (*dut/ciaB*) against BOD (Figure 4(a)) and COD (Figure 4(b)) shows that when COD and BOD increased, *dut/ciaB* tended to increase. This indicates that as COD and BOD decrease, *E. coli* concentrations decrease sharply, while *Arcobacter* spp. concentrations decrease slowly, i.e., the behaviors of these species after release into the environment differ from each other.

Phylogenetic analysis of *Arcobacter* spp.

The source of *Arcobacter* spp. detected in the drainage canals is likely to be untreated wastewater from slums and markets, and most *Arcobacter* spp. are considered to belong to the potentially pathogenic *A. butzleri* and *A. cryaerophilus* because the changes of 16S rDNA and *ciaB* detection results showed similar trends. Therefore, we cultivated *Arcobacter* spp. from drainage canal Sites 2 and 4 and from the inflow to the Bongai STP in Bangkok. We obtained 7 strains at Site 2, 19 strains at Site 4, and 21 strains at the Bongai STP.

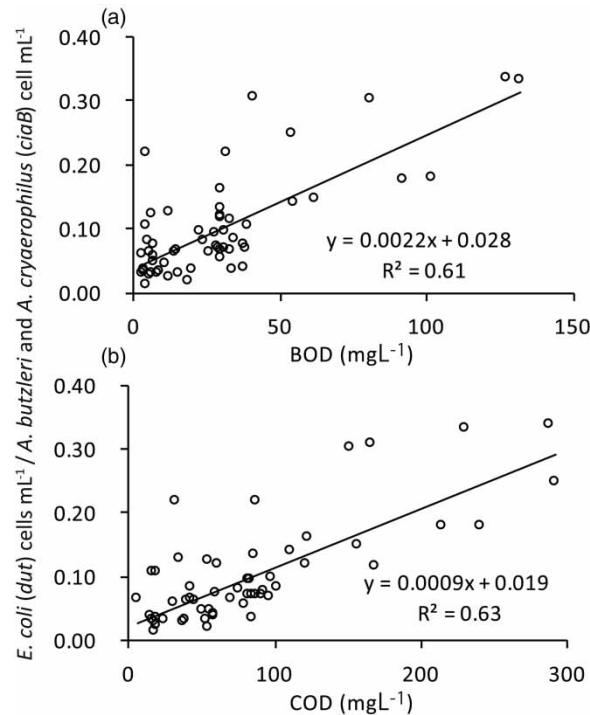


Figure 4 | Correlation curves of the ratio of *E. coli dut* over *A. butzleri* and *A. cryaerophilus ciaB* against indexes of organic pollution: (a) BOD and (b) COD.

The phylogenetic tree based on 16S rDNA is shown in Figure 5(a). *A. butzleri* was detected in the inflow domestic wastewater of the Bongai STP; *A. butzleri* and *A. cryaerophilus* were detected at Site 4, likely due to significant pollution from domestic and market wastewater; and only *A. cryaerophilus* was detected at Site 2.

The phylogenetic tree based on *ciaB* is shown in Figure 5(b). In this tree, as in the above phylogenetic tree, *A. butzleri* was detected from the Bongai STP, *A. butzleri* and *A. cryaerophilus* were detected at Site 4, and only *A. cryaerophilus* was detected at Site 2. *A. cryaerophilus ciaB* detected at Sites 2 and 4 fell into the same group, indicating that *A. cryaerophilus* may be supplied from Site 2 to 4. On the other hand, *A. butzleri* isolated from the Bongai STP was divided into three groups regardless of the season, and the strains isolated from Site 4 were also divided into four groups, indicating that the *ciaB* gene had diversity. Since the phylogenetic analysis in this study revealed the diversity of *ciaB* in *A. butzleri*, we consider that analysis of *ciaB* will be a powerful tool for identifying the source of *Arcobacter* spp. contamination.

DISCUSSION

In the city center of Bangkok, the Chao Phraya River has higher levels of both BOD and TP than in the waters further upstream (Singkran *et al.* 2019), revealing organic matter pollution due to untreated wastewater. In our survey, we found that the average BOD of drainage canals in the city was higher than allowed under the Bangkok sewage discharge standards (BOD <20 mg L⁻¹); in particular, Site 4 exceeded the Bangkok market effluent standards (BOD <50 mg L⁻¹). This indicates that the organic pollution due to human-derived wastewater is a serious problem in the drainage canals.

Data from the Pollution Control Department of Bangkok over 28 years from 1990 to 2017 show that the concentration of fecal coliform bacteria has increased sharply in the urban area of Chao Phraya River (Singkran *et al.* 2019). In our survey, around 1.0×10^2 cells mL⁻¹ of *E. coli* CFU was constantly detected in the mainstream of the Chao Phraya River during the 13-month survey period, indicating that contamination with fecal pollution of the Chao Phraya River flowing through Bangkok city continues throughout the year. Diallo *et al.* (2009) investigated a canal that merges with the Chao Phraya River about 40 km upstream of Bangkok and showed that *E. coli* concentration determined using the most probable number method (MPN) was 2.0×10^2 to 3.2×10^2 cells mL⁻¹. This concentration range is close to the average concentration of cultivatable *E. coli* CFU (1.5×10^2 cells mL⁻¹) that we found in the current survey in the Chao Phraya River in Bangkok

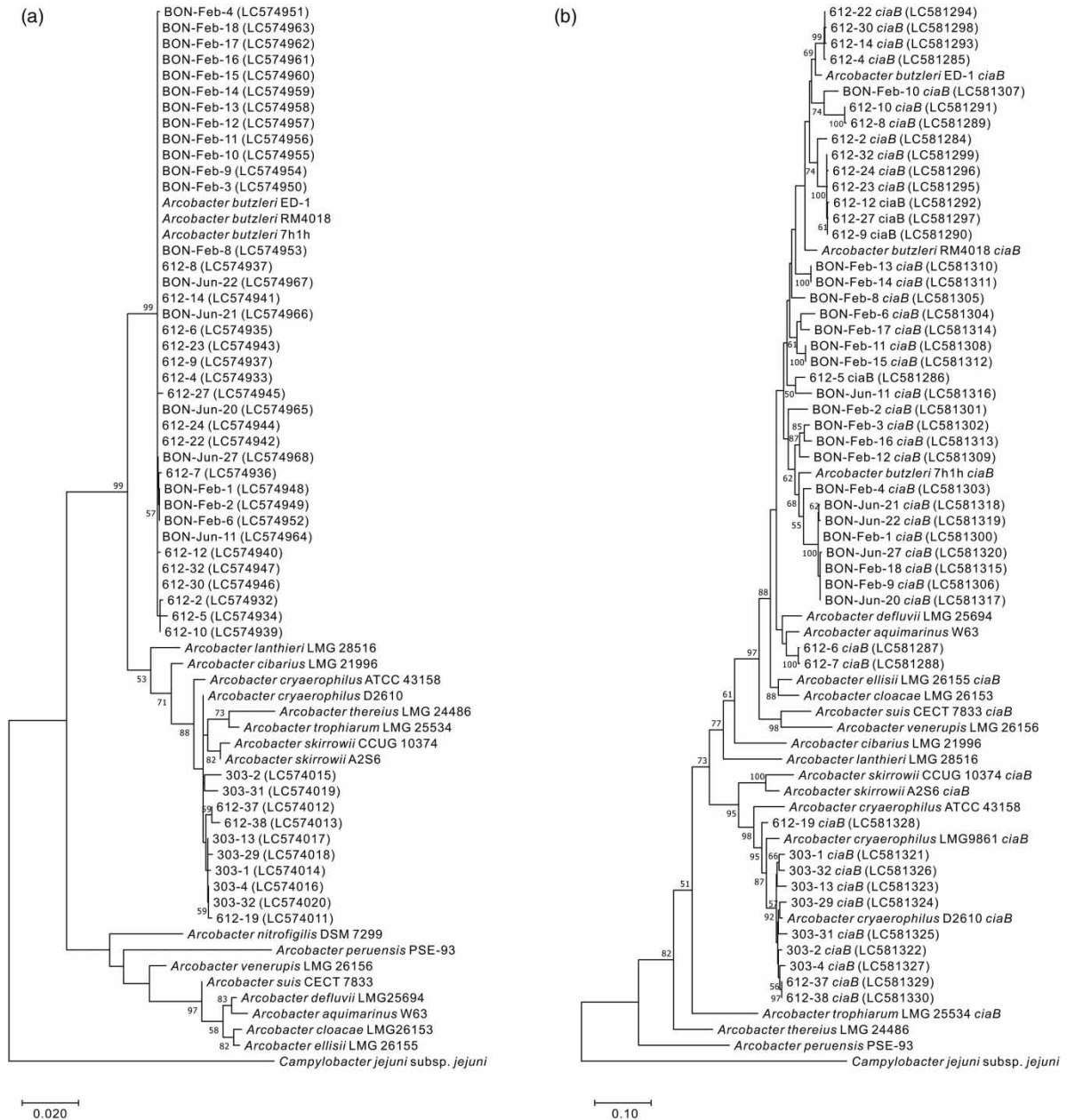


Figure 5 | Phylogenetic analysis of 47 *Arcobacter* spp. isolates based on partial 16S rRNA gene sequences (a) and the partial *ciaB* gene sequences (b) *Campylobacter jejuni* subsp. *jejuni* (ATCC 700819) was used as an outgroup. Branches with bootstrap values of greater than 50% are shown. The scale bars correspond to 0.02 and 0.1 substitutions per nucleotide position on 16S rRNA gene sequences and partial *ciaB* gene sequences, respectively. The DDBJ/EMBL/GenBank sequence accessions are shown in parentheses. Samples for the isolation of *Arcobacter* spp. were taken at drainage canal Site 2 (strains prefixed with 303) and Site 4 (strains prefixed with 612), and domestic wastewater flowing into the Bongai STP (strains prefixed with BON). The lengths of the sequence fragments of 16S rDNA and *ciaB* were 1,015–1,573 and 1,008–1,893 bp, respectively.

city. Taken together, these results indicate that the *E. coli* contamination of Chao Phraya River starts upstream from Bangkok. In addition, the annual average value of cultivatable *E. coli* was 1.1×10^3 cells mL⁻¹ at Site 1 in the downstream reaches of a canal supplying agricultural water. Agricultural water from this site was also shown to be contaminated with *E. coli*. In addition, since there is a pump facility for rainwater drainage near Site 1, the effect of human pollution cannot be excluded. Higher concentrations of cultivatable *E. coli* were detected in the drainage canals in Bangkok, and it is likely that wastewater

from slums, houses, and markets flows directly into the canals. On the other hand, compared with Sites 2 and 4, which are close to the sources of *E. coli* such as slums and markets, the number of cultivatable *E. coli* was lower at Site 3 near the water gate (not statistically significant). The water of the drainage canals flows from Site 2 to Site 4 to Site 3, and it is possible that *E. coli* discharged at Sites 2 and 4 became partially uncultivable (decreased activity) while flowing down the drainage canals. Also, the ratio of BOD to COD at Site 3 was lower than that at Sites 2 and 4. Since Site 3 is near the water gate, BOD may have been attenuated during transport along the canals, which may indicate that canals have a function of removing contaminating microorganisms and organic substances (Diallo *et al.* 2009). In the future, it will be necessary to identify the sources of pollution and examine in detail how the flow of the canals influences the concentrations of enterobacteria.

In Thailand, the rainy season is from July to October, and it is thought that organic matter flows in from the basin during this season. In Site 4, which has the highest average BOD, an increase in BOD concentration was observed from September to October (data not shown). The concentration of cultivatable *E. coli* CFU (cells mL⁻¹) increased at that time. It is possible that the survival of *E. coli* during this period increased. On the other hand, no seasonal fluctuation in the concentrations of *Arcobacter* spp. estimated by qPCR was observed in the canal and irrigation canals. Furthermore, the concentration of *Arcobacter* spp. tended to decrease only in the Chao Phraya River from September to December. This is thought to be due to the increase in the flow rate of the Chao Phraya River.

In Nepal, which has a monsoon climate, 16S rDNA of the *Arcobacter* spp. have been detected as major fecal bacteria in the Bagmati River during the monsoon season (Pantha *et al.* 2021). Furthermore, it has been reported that the detection of *A. butzleri* by culture-based method increases during rainfall in Canadian rivers (Webb *et al.* 2017). In these relatively high-flow rivers, flush out is thought to contribute to the increase in *Arcobacter* numbers. In these areas, the rainy season falls from spring to summer, and the resultant change in water temperature may increase survivability of *Arcobacter* spp.

The foodborne bacterial pathogen *C. jejuni* is a leading cause of infectious bacterial enteric infections worldwide (Mousavi *et al.* 2020). *Campylobacter* spp. were detected by qPCR analysis of 16S rRNA at relatively high frequencies at Sites 2 and 4, but were detected only a few times at Sites 1, 3, and 5, and had no correlation with *E. coli*. Considering the low oxygen tolerance of *Campylobacter* spp. (Oh *et al.* 2015), we presumed that they would die relatively quickly after discharge into the environment. However, a high concentration of *Campylobacter* spp. (7.3×10^4 cells mL⁻¹ based on 16S rDNA) was detected on one occasion in the Chao Phraya River in December 2017, showing that high levels of *Campylobacter* spp. contamination can occur. It is reported that as few as 800 *C. jejuni* cells are sufficient to cause diarrheal illnesses in humans (Black *et al.* 1988), and it should be noted that *Campylobacter* spp. were detected within areas of human habitation such as in canals and the Chao Phraya River.

Arcobacter spp. were detected by qPCR analysis in all the samples investigated in the current study. The concentrations were very high in the drainage canals, but were also high in samples from Sites 1 and 5 which had relatively low BOD. Because a high correlation was found between concentrations of *Arcobacter* spp. and *E. coli*, the source of *Arcobacter* spp. is likely to be human-derived sewage (domestic wastewater) (Lee *et al.* 2012).

Furthermore, the ratio of *E. coli* to *A. butzleri* and *A. cryaerophilus* concentrations calculated with *dut* and *ciaB* genes increased with increasing COD and BOD (Figure 4). The difference between *E. coli* concentration and that of *A. butzleri* and *A. cryaerophilus* tended to widen as the organic matter concentration decreased. Due to these findings and the high concentrations of *Arcobacter* spp. detected in all samples, we consider that *Arcobacter* spp. are more likely to remain in the environment for a longer period of time than *E. coli*. *Arcobacter* spp. are reported to survive for a long period of time in aseptic culture when the organic matter concentration is high (Van Driessche & Houf 2008). The high concentration of organic matter in the drainage canals is likely to contribute to survival of *Arcobacter* spp. Therefore, improvement of canal water quality, such as control of wastewater inflow by installation of wastewater treatment facilities, is necessary from a hygienic point of view.

Furthermore, *Arcobacter* spp. can form biofilms (Ferreira *et al.* 2013), and if they grow in water-supply pipes, they may remain even after disinfection with chlorine, meaning that there is a risk of contamination of the purified tap water (Assanta *et al.* 2002). In particular, *A. butzleri*, which was detected in canals and sewage (domestic wastewater) in the current study, is often reported to be pathogenic (Vandenberg *et al.* 2004; Soelberg *et al.* 2020). Therefore, the existence of high concentrations of *A. butzleri* in canals and sewage is a big problem. *Arcobacter* spp. were also detected in effluent from a sewage treatment plant in Bangkok (data not shown). In Bangkok, the treated water is not chlorinated and is used for irrigation of street trees and road surface cleaning (private communication), which is a potential sanitary risk.

CONCLUSION

We investigated seasonal changes in the concentrations of enterobacteria and *Arcobacter* and *Campylobacter* spp. quantified by 16S rDNA and virulence gene-specific qPCR in the surface water of four canals and the Chao Phraya River in Bangkok. High levels of potentially pathogenic *Arcobacter* spp. were detected at all the sites in all seasons. The concentrations of potentially pathogenic *Arcobacter* spp. were highly correlated with those of *E. coli* in all samples, indicating that the source of *Arcobacter* spp. is likely to be human-derived domestic wastewater. The high levels of potentially pathogenic *Arcobacter* spp. in surface water in areas of human habitation in Thailand are a major concern for public health and should be considered in plans to restore a safe, sanitary environment. Installation of wastewater treatment facilities is desirable to reduce the inflow of *Arcobacter* spp. to the water environment and to prevent *Arcobacter* survival by improving water quality.

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DATA AVAILABILITY STATEMENT

Data cannot be made publicly available; readers should contact the corresponding author for details.

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