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

Noriko Tomioka, Wilasinee Yoochatchaval, Yasuyuki Takemura, Norihisa Matsuur, Tsuyoshi Danshita, Pornpawee Srisang, Noppamas Mungjomkl and Kazuaki Syutsubo

*Regional Environment Conservation Division, National Institute for Environmental Studies, Onogawa 16-2, Tsukuba, Ibaraki 305-8506, Japan
Faculty of Engineering, Department of Environmental Engineering, Kasetsart University, 50 Ngam Wong Wan Rd, Ladyaow Chatuchak, Bangkok 10900, Thailand
Faculty of Geoscience and Civil Engineering, Kanazawa University, Ishikawa 920-1192, Japan
Faculty of Civil Engineering and Architecture, National Institute of Technology, Tokuyama College, Gakuendai Shunan, Yamaguchi 745-8585, Japan
*Corresponding author. E-mail: stubo@nies.go.jp

KS, 0000-0003-4181-2171

**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 (COD) and biochemical oxygen demand (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 (Douidah 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. Douidah 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 5 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.
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 \textit{Arcobacter} spp. were taken at drainage canal Sites 2 and 4 on May 30, 2019. In addition, domestic wastewater (400 m$^3$ 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-$\mu$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, Amsham, UK), and concentrations of orthophosphate (PO$_4$-P), ammonium (NH$_4$-N), nitrate (NO$_3$-N), and nitrite (NO$_2$-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-$\mu$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 μl of extraction buffer and 50 μl 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 μl 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

### Table 1 | Primers used in this study

| Genetic marker | Primer | Sequence (5’–3’) | Product size (bp) | Amplified gene | Target(s) | References |
|----------------|--------|-----------------|------------------|----------------|----------|-----------|
| Arco 16S       | Arco605F | GAAGTGAAATCCTATAGCTTAAC | 127 | 16S rRNA | Arcobacter spp. | This study |
|                | Arco688R | CGCAATCGGTATTCCTTCTGAT | 140 | Invasion gene ciaB | A. butzleri, A. cryaerophilus | This study |
| Arco ciaB      | ciaB1433F | GAGITYYAAAGCAACAAACWGGTGG | 140 | Invasion gene ciaB | A. butzleri, A. cryaerophilus | This study |
|                | ciaB1531R | ACTTCATACAATCCATCCA | 189 | 16S rRNA | Escherichia coli | This study |
| Ecoli 16S      | Ecoli489F | ACCTTGGTCATAGGCTTACC | 189 | 16S rRNA | Escherichia coli | This study |
|                | Ecoli 635R | CTCAAGCTTGGCGATCATGAT | 190 | Deoxyuridine triphosphatase gene dut | Escherichia coli | This study |
| Camp 16S       | Camp105F | AGCTTGCTAGAAGTGGATTAG | 125 | 16S rRNA | Campylobacter spp. | This study |
|                | Camp182R | TACTCACAATCTTCTGCAGG | 125 | 16S rRNA | Campylobacter spp. | This study |
| Camp univ27F   | univ1492R | AGAGTTTTGATCCTGGCCTAG | 1,477 | 16S rRNA | Campylobacter spp. | Lane (1991) |
| Camp univ27F   | univ1492R | TACCGYTAATCCTGGCCTAG | 1,477 | 16S rRNA | Campylobacter spp. | Lane (1991) |
| Camp univ27F   | univ1492R | AGAGGGTTCATCTGGGCTCAG | 1,375 | 16S rRNA | Campylobacter spp. | This study |
| Arco seq       | EPSY1384R | CGGTGAGTACAAGACCC | 1,144 | Invasion gene ciaB | Arcobacter spp. | This study |
| ciaB seq       | ciaB444F | MTWGGCAGAATCTTGGGAC | 1,391 | Invasion gene ciaB | Arcobacter spp. | This study |
|                | ciaB1531R | ACTTCATCAATCCATCCA | 1,391 | Invasion gene ciaB | Arcobacter spp. | This study |

Y, T or C; W, A or T.
(Arcobacter spp. and E. coli) or converted to genome copies, the qPCR results are shown with the unit, cells mL\(^{-1}\).

The real-time qPCRs were performed individually for each primer set. The reaction mixture contained 20 \(\mu\)L of LightCycler 480 SYBR Green I Master (Roche, Mannheim, Germany), 10 pmol of each primer, and 1 \(\mu\)L 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 \(\mu\)L of each diluted solution was spread on Charcoal Cefoperozone Deoxycylolate agar (CCDA) medium (Oxoid Limited, Altrincham, UK) and incubated in a jar at 25 °C for 72 h under microaerophilic conditions (O\(_2\) 6–12% and CO\(_2\) 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 \(-80^\circ\)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 Extrapol 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 (Levican 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.
Sites 1 and 5 were one order of magnitude lower than in the drainage canals, and were below the detection limit from September to December, the second half of the rainy season.

The limit of detection for each real-time qPCR protocol was as follows: Arcobacter spp. 16S rDNA and Arcobacter spp. 16S rDNA, 1.4 × 10^4 cells mL^-1; A. butleri and A. cryaerophilus ciaB, 1.0 × 10^5 cells mL^-1; E. coli 16S rDNA, 1.2 × 10^5 cells mL^-1; E. coli dut, 3.0 × 10^5 cells mL^-1, and Campylobacter spp. 16S rDNA, 2.7 × 10^5 cells mL^-1.

Arcobacter spp. 16S rDNA and A. butleri 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. butleri 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. butleri and A. cryaerophilus ciaB at Site 5 tended to decrease from September to December, the second half of the rainy season.

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. butleri 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 × 10^5 cells mL^-1; A. butleri and A. cryaerophilus ciaB, 4.0 × 10^6 cells mL^-1; E. coli 16S rDNA, 1.2 × 10^6 cells mL^-1; E. coli dut, 3.0 × 10^6 cells mL^-1, and Campylobacter spp. 16S rDNA, 2.7 × 10^6 cells mL^-1.

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

| Sampling site | Temperature (°C) | pH | ORP (mV) | DO (mg L^-1) | Conductivity (μS cm^-1) | SS (mg L^-1) | COD (mg L^-1) |
|---------------|-----------------|----|----------|-------------|-------------------------|--------------|--------------|
| Site 1        | 29.1 ± 2.1      | 7.1 ± 0.4 | 175.1 ± 57.1 | 2.6 ± 1.0 | 867 ± 125.5 | 19.8 ± 7.6 | 45.8 ± 8.9 |
| Site 2        | 29.2 ± 3.1      | 7.4 ± 0.5 | 37.5 ± 85.4  | 1.5 ± 0.5 | 728 ± 111.2 | 35.1 ± 12.7 | 73.5 ± 31.7 |
| Site 3        | 30.3 ± 2.1      | 7.1 ± 0.2 | -4.3 ± 68.5 | 1.4 ± 0.6 | 692 ± 100.2 | 29.3 ± 18.2 | 75.0 ± 21.3 |
| Site 4        | 30.3 ± 2.2      | 7.1 ± 0.2 | -17.2 ± 63.9 | 1.6 ± 0.8 | 829 ± 180.9 | 66.8 ± 55.3 | 169.7 ± 78.6 |
| Site 5        | 31.5 ± 2.8      | 7.1 ± 0.3 | 240.8 ± 30.3 | 3.0 ± 0.7 | 371 ± 187.6 | 32.7 ± 22.0 | 16.2 ± 5.3 |

ORN 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 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.
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 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 \( 7.3 \times 10^2 \) cells \( mL^{-1} \).

Boxplots of monthly data of specific bacterial concentrations estimated from \( Arcobacter \) 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.

\( Arcobacter \) spp. were present in high concentrations in drainage canals (annual averages from 16S rDNA at Sites 2, 3, and 4 were \( 2.3 \times 10^6 \), \( 2.2 \times 10^6 \), and \( 1.5 \times 10^6 \) cells \( mL^{-1} \), respectively; annual averages from ciaB at Sites 2, 3, and 4 were \( 1.6 \times 10^5 \), \( 1.5 \times 10^5 \), and \( 9.0 \times 10^5 \) 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, \( 5.7 \times 10^5 \) cells \( mL^{-1} \) at Site 1 and \( 1.4 \times 10^5 \) cells \( mL^{-1} \) at Site 5; estimate from ciaB, \( 2.6 \times 10^3 \) cells \( mL^{-1} \) at Site 1 and \( 1.2 \times 10^3 \) 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 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.
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 increase, 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.

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, $20 \text{ mg L}^{-1}$); in particular, Site 4 exceeded the Bangkok market effluent standards (BOD, $50 \text{ mg L}^{-1}$). 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 $1.0 \times 10^2 \text{ cells mL}^{-1}$ 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 $2.0 \times 10^2$ to $3.2 \times 10^2 \text{ cells mL}^{-1}$. This concentration range is close to the average concentration of cultivatable E. coli CFU ($1.5 \times 10^2 \text{ cells mL}^{-1}$) that we found in the current survey in the Chao Phraya River in Bangkok 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 $1.1 \times 10^3 \text{ cells mL}^{-1}$ 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

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 700619) 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.

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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 × 10⁶ 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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