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Human and animal microbial source tracking in a tropical river with multiple land use activities

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ABSTRACT

The enhancement and restoration of the water quality of deteriorating surface water resources can be challenging, particularly for rivers with multiple usages, such as agriculture, animal husbandry, human residence, and industries. Recently, the performance of DNA-based microbial source tracking (MST) indicators detected by end-point and quantitative PCR assays for identifying sources of fecal pollution from human sewage, swine, and cattle and non-host-specific (universal) fecal pollution in the Tha Chin River basin, Thailand, was evaluated. The present study monitored these validated MST markers and various physicochemical and microbial water quality parameters in samples collected from twelve stations along the Tha Chin River during four sampling events in the wet and dry seasons. No significant difference in precipitation was observed between the wet and dry samplings. Universal markers (both PCR and qPCR) were detected in all 48 samples, indicating persistent and continuing fecal contamination. The sewage- and swine-specific qPCR marker concentrations did not vary among the sampling events, whereas cattle-specific qPCR markers were detected only in the wet season. Animal-specific markers were detected in the lower Tha Chin River section, which is characterized by intensive animal farming. Sewage-specific markers were also found in the lower section and near an upstream residential area. The high agreement (87.5–100%) between the PCR and qPCR results suggested that PCR could serve as a lower-cost MST screening test that requires less technical expertise. A multivariate analysis conducted using the survival analysis procedure to include censored data also emphasized the high pollution in the lower section of the river at all sampling events. Universal and swine-specific markers showed moderate correlations with microbial indicators, including total coliforms, fecal coliforms, *E. coli*, and enterococci. None of the MST markers or microbial parameters were associated with the measured physicochemical parameters. This study provides the first evaluation of MST markers for monitoring surface freshwater in Thailand, and the findings might aid the pollution surveillance of impaired water bodies and the development of strategies for improving their water quality.

1. Introduction

Population growth and economic development could lead to the expansion of human residential areas and the intensification of animal farming and other industries. These anthropological activities contribute significantly to pollution loads, which could be discharged either directly or indirectly into recipient bodies of water. The problem of water pollution is aggravated by the inadequacy of sanitation and wastewater facilities and the inefficiency of water governance and law enforcement (Pollution Control Department (PCD), 2018). The carrying capacity of a river is thus often exceeded, resulting in the river's inability to return to a natural state. Catchment disturbance, comprising four stressors that threaten water security—cropland, impervious surfaces, livestock density, and wetland disconnectivity—clearly reflects the impact of land use on the health of river systems (Vorosmarty et al., 2010). These crucial underlying factors, including the river health index, have been emphasized in the evaluation criteria for the assessment of environmental water security in the Asia and Pacific region (Asian Development Bank, 2016).

To expedite an abatement of water quality degradation, it is vital to accurately characterize connections between pollution loads and their originating sources. There are generally two types of pollution sources:

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a point source is easily identifiable, e.g., combined sewer overflow and wastewater discharge points from animal farms, factories, or municipal wastewater treatment plants, whereas a nonpoint source is a type of source that cannot be directly identified, including not only rainfall runoff from urban zones, agricultural farmlands, and wildlife areas but also bird guano, vessel wastewater, leaking sewers, and even swimmers (US EPA, 2016). Thus, based on its nature, a nonpoint source is more challenging to retrace. However, fecal water pollution can be tracked to its origin by detecting host-specific gastrointestinal microbiota excreted with feces into polluted environments. This growing area of research and development is called microbial source tracking (MST) (Hagedorn et al., 2011). The anaerobic bacterial order *Bacteroidales* is widely used as an MST indicator using DNA detection techniques to differentiate fecal pollution from humans and various animals, e.g., dogs, goats, sheep, swine, cattle, and birds (Ahmed et al., 2016; Devane et al., 2018). However, the performance of *Bacteroidales* DNA detection techniques appears to vary among certain geographical regions (Somnark et al., 2018a) due to diverse gastrointestinal microbial distribution, which could be attributable to differences in diets, climate, and other environmental and lifestyle factors, such as antibiotic use, drinking water source, and number of siblings (Looft et al., 2014; Mah et al., 2008; Shanks et al., 2011).

Consequently, to facilitate the application of MST in Thailand, the performance of endpoint PCR and real-time PCR assays in detecting the *Bacteroidales* bacterial order and thus tracking nonspecific (universal) and sewage-, swine-, and cattle-specific DNA markers in the Tha Chin river basin was evaluated (Somnark et al., 2018b, 2018a). The set of PCR and qPCR assays that exhibited the best performance in the Tha Chin area was identified, and their performance criteria were characterized. The Tha Chin river in central Thailand has consistently been among the five rivers with the poorest water quality in Thailand, as evaluated by the Thailand Water Quality Index, which includes dissolved oxygen (DO), biochemical oxygen demand (BOD), total coliform bacteria (TCB), fecal coliform bacteria (FCB), and ammonia nitrogen (Pollution Control Department (PCD), 2018). The objective of this study was therefore to utilize *Bacteroidales* MST methods to pinpoint sources of fecal pollution along the Tha Chin River and monitor other water quality parameters. The specific objectives were to (1) investigate both the temporal and spatial distributions of water quality parameters and MST markers along the Tha Chin River and (2) examine the relationship among water quality parameters and MST markers.

2. Materials and methods

2.1. Study area

The 325-km Tha Chin River is situated in a 13,477-sq.km. river basin in Central Thailand that covers the areas of 13 provinces. The Tha Chin River originates from the Chao Phraya River in Chai Nat Province and passes through Suphan Buri and Nakhon Pathom Provinces before flowing into the Gulf of Thailand at Samut Sakhon Province. The inland activities alongside the Tha Chin River mainly include agriculture, livestock farming, and communities, whereas a condense network of industrial activities can be found along the lower Tha Chin River. Twelve water sampling stations from the Pollution Control Department's sampling stations that experienced high TCB and FCB in the past were selected for inclusion in this study (Fig. 1 and Supplemental Table S1). The surface water quality standards in Thailand are classified into five categories: the first category is used for pristine water, and the fifth category is utilized for the most polluted water, which applies only to transportation (National Environment Board, 1994). The upper (stations K and L), middle (stations G-J), and lower (stations A-F) Tha Chin river areas are in categories 2, 3, and 4, respectively (Supplemental Table S2) (Pollution Control Department (PCD), 1994).

2.2. Sample collection

A total of 48 water samples were collected from 12 sampling stations at four sampling times, each of which was split into two consecutive weeks (Supplemental Table S3). Originally, two sampling events were planned during the rainy season (May to October), and the other two were scheduled during the dry season (November to April). However, during the years 2017–2018, Thailand experienced a dry rainy season but summer storms during the dry season. Therefore, the precipitation records from the rainwater monitoring stations and the river water levels were retrieved from the Hydro and Agro Informatics Institute and the Central Region Irrigation Hydrology Center for analysis. No significant differences in precipitation were observed among the sampling times, whereas water levels showed significant differences between the Feb–Mar and Feb–Jul periods (paired *t*-test, $p < 0.05$; Supplemental Table S4 and Fig. S1). All samples were collected during low tide, according to the Hydrographic Department, Royal Thai Navy. The pH, water temperature, conductivity and salinity were measured onsite with YSI 60 (YSI Inc., USA) and Hach Ion 1 (Hach, Singapore) portable meters. Water samples (2 L) were collected into sterile plastic containers from the middle depth of the river using a submersible water sampler and transported to the laboratory for analysis of the physicochemical water quality parameters. Other 2-L samples were collected at 30 cm below the surface for subsequent analysis of bacterial and MST parameters. Quality control samples, including field duplicates (FD) and field blanks (FB), were collected onsite at all sampling times (Supplemental Table S3). All samples were transported on ice back to the laboratory within 8 h.

2.3. Water quality parameters

The water samples were stored at 5 °C in the laboratory and analyzed the next day to measure the following physicochemical parameters: BOD (American Public Health Association et al., 2017a), total suspended solids (TSS) (American Public Health Association et al., 2017b), total dissolved solids (TDS) (American Public Health Association et al., 2017c), DO (American Public Health Association et al., 2017d), total phosphorus (TP) (American Public Health Association et al., 2017e), and phosphate-phosphorus (American Public Health Association et al., 2017f). Moreover, the TCB and FCB bacterial parameters were measured through the multiple-tube fermentation method (American Public Health Association et al., 2017g). Membrane filtration methods were used to analyze enterococci (US EPA, 2002a) and simultaneously detect TCB and *E. coli* (US EPA, 2002b).

2.4. Water filtration and DNA extraction

Hydrochloric acid (2 N) was added to 1 L of each water sample to adjust the pH to 3.5 ± 0.2 (Ahmed et al., 2015), and 250 mL of the adjusted water sample was filtered through a 0.45-μm-pore-size HAWP membrane (Merck Millipore, Germany). A preliminary experiment was performed to demonstrate that higher recovery of bacterial and viral DNA was achieved with a 0.45-μm-pore-size HAWP membrane and prior pH adjustment to 3.5 than with a 0.22-μm mixed cellulose ester membrane with or without prior pH adjustment to 3.5 (data not shown). DNA extraction was performed from each filtered membrane using a Quick-DNA Fecal Soil Microbe Miniprep kit (Zymo Research, USA) according to the manufacturer's instructions with slight modifications. To prevent experimental bias, a fixed supernatant volume of 350 μL obtained after bead beating was transferred to a subsequent filter for genomic lysis. Furthermore, in the final step, elution steps were repeated (50 μL of DNA elution buffer was added before each step) to obtain 100 μL of DNA extract per membrane filter. Therefore, each water sample yielded 400 μL of DNA extract. The DNA concentration was measured with a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA), and the DNA was stored at −80 °C until use. Filtration

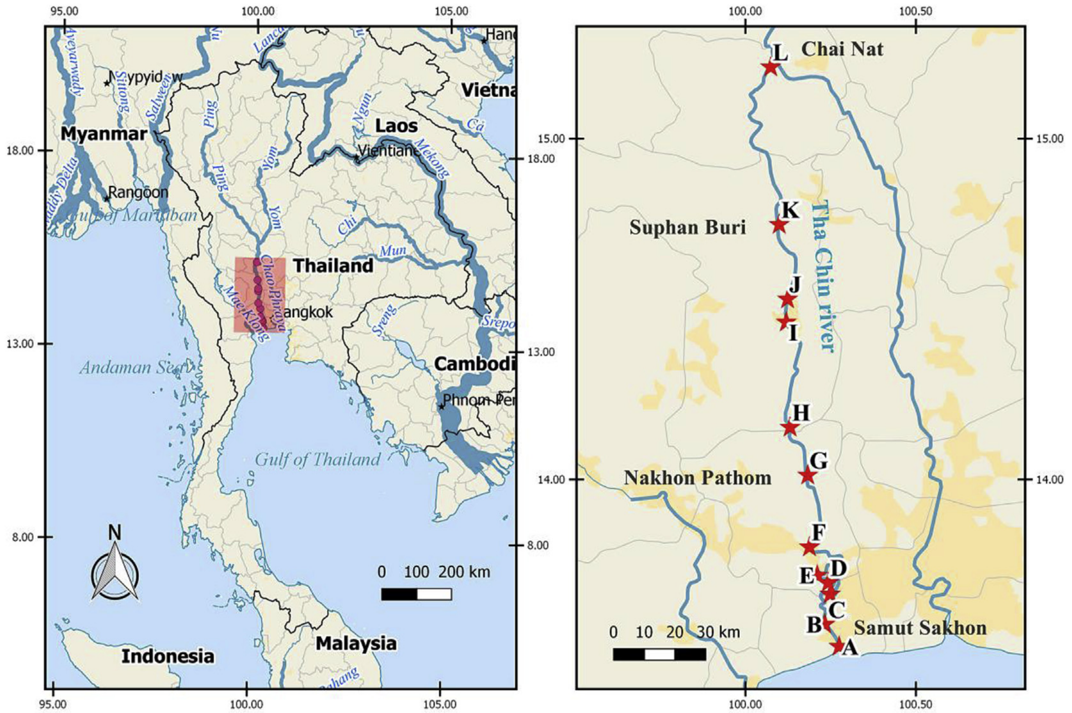


Fig. 1. Map of sampling stations in the Tha Chin River.

blanks (also known as method blanks) were analyzed by filtering 1-L of sterile reverse osmosis (RO) water prior to the abovementioned protocol. Extraction blanks were obtained by processing 400 μL of sterile RO water using the Quick-DNA Fecal Soil Microbe Miniprep kit.

2.5. Conventional PCR and qPCR assays for Bacteroidales MST markers

GenBac3, HF183/BFDrev, Pig-2-Bac, and Bac3 assays were used for both PCR and qPCR detection of universal and human sewage-, swine-, and cattle-specific *Bacteroidales* markers, respectively (Table 1). These assays were selected based on their conventional PCR performance evaluated in Thailand, as previously reported (Somnark et al., 2018b). A 10-μL PCR was composed of 0.5 μL of each forward primer at 10 μM and reverse primer at 10 μM, 1 μL of DNA template (corresponding to 20 ng of total DNA), 5 μL of DreamTaq PCR Master Mix (2 × ; Thermo Fisher Scientific, USA), and sterile water. The PCR cycle comprised an initial denaturation at 95 °C for 3 min; 30 cycles of a denaturation step at 95 °C for 30 s, an annealing step at 60 °C for 30 s, and an elongation step at 72 °C for 30 s; and a final extension at 72 °C for 10 min. The PCRs were processed using a Mastercycler Pro thermocycler (Eppendorf, Germany). DNA bands were visualized with a Gel Doc XR system (BIO-RAD, USA) after agarose electrophoresis and ethidium bromide staining. Each sample was run in duplicate by two different lab personnel on different days to demonstrate the reproducibility of the results (interassay consistency). If the results were not in agreement, an additional run was performed. Moreover, no-template controls (NTCs) and plasmid standards were included in every instrumental run as quality controls.

In the qPCR analysis, previously designed probes were used, with the exception of the probe for the Bac3 marker, which was originally designed for use in an endpoint PCR assay (Shanks et al., 2006). Therefore, a hydrolysis probe was designed in this study using Primer Express Software (Applied Biosystems, Thermo Fisher Scientific, USA). In addition, all primers and probes were subjected to BLAST analysis to reconfirm their specificity. The qPCR protocol was conducted according to the MIQE guidelines (Bustin et al., 2009). Each 20-μL reaction mixture was composed of 0.8 μL of each 10 μM forward and reverse primers, 0.4 μL of 10 μM probe, 4 μL of DNA template, 10 μL of the 2X iTaq

Table 1
Primers and experimental conditions used for the PCR and qPCR assays performed in this study.

Assay	Primer name	Nucleotide sequence (5'→ 3′)	Tm (°C)	Reference
Universal	GenBac3F	GGG-GTT-CTG-AGA-GGA-AGG-T	60	Siefring et al. (2008)
	GenBac3R	CCG-TCA-TCC-TTC-ACG-CTA-CT		
	GenBacProbe	FAM-CAA-TAT-TCC-TCA-CTG-CTG-CCT-CCC-GTA-TAMRA		
Sewage	HF183	ATC-ATG-AGT-TCA-CAT-GTC-CG	60	Haugland et al. (2010)
	BFDrev	CGT-AGG-AGT-TTG-GAC-CGT-GT		
	BFDFAM	FAM-CTG-AGA-GGA-AGG-TCC-CCC-ACA-TTG-GA-TAMRA		
Swine	Pig-2-Bac41F	GCA-TGA-ATT-TAG-CTT-GCT-AAA-TTT-GAT	60	Mieszkin et al. (2009)
	Pig-2-Bac163Rm	ACC-TCA-TAC-GGT-ATT-AAT-CCG-C		
	Pig-2-Bac113	FAM-TCC-ACG-GGA-TAG-CC-TAMRA		
Cattle	Bac3F	CTA-ATG-GAA-AAT-GGA-TGG-TAT-CT	60	Shanks et al. (2006)
	Bac3R	GCC-GCC-CAG-CTC-AAA-TAG		
	Bac3Probe	FAM-CCT-TAT-ACA-TTG-AGC-ATC-GAG-GCC-TAMRA		This study
Salmon	Forward primer	GGT-TTC-CGC-AGC-TGG-G	60	Haugland et al. (2010)
Sketa22	Reverse primer	CCG-AGC-CGT-CCT-GGT-C		
	TaqMan® probe	FAM-AGT-CGC-AGG-CGG-CCA-CCG-T-TAMRA		

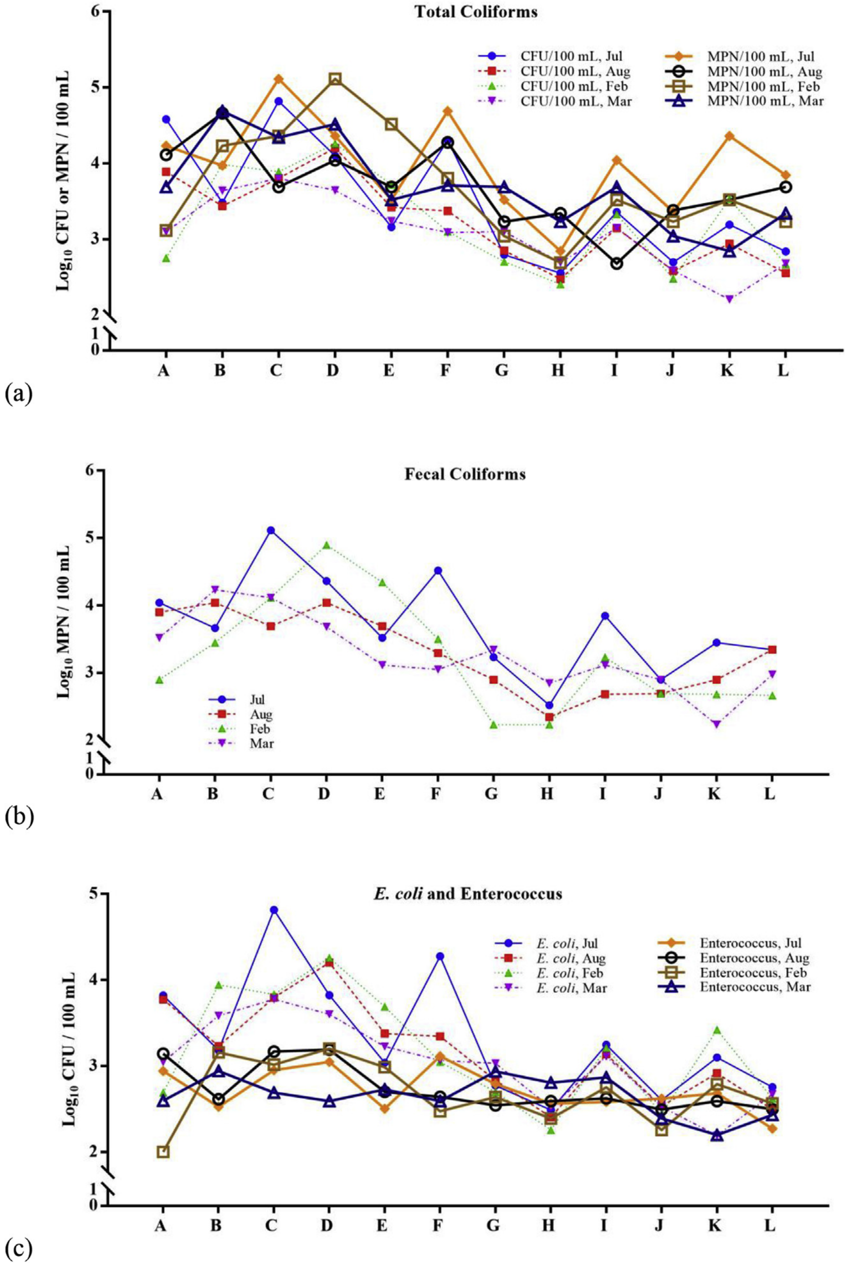
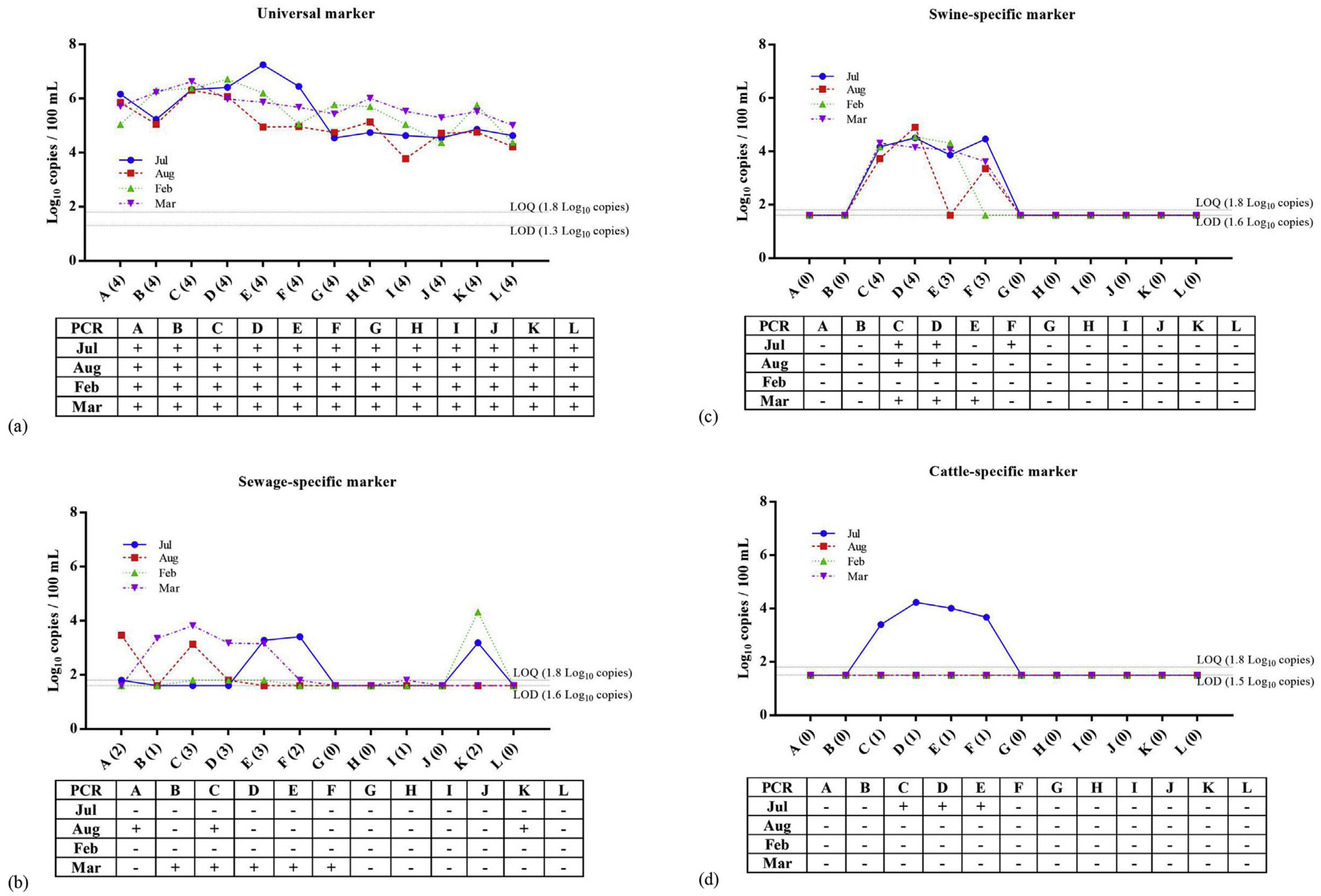


Fig. 2. Spatial and temporal distributions of total coliforms (a), fecal coliforms (b), and *E. coli* and enterococci (c).

Universal Probes Supermix (BioRad, USA), and 4 µL of 1 µg/µL bovine serum albumin (BSA). The qPCR reactions were performed with an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, USA) according to the following steps: an initial denaturation at 95 °C for 3 min followed by 40 cycles of a denaturation step at 95 °C for 20 s and a combined annealing and elongation step at 60 °C for 1 min. The qPCR runs were conducted in eight-tube strips and caps (Applied Biosystems, Thermo Fisher Scientific, USA). Three dilutions of each sample, corresponding to 10, 20 and 40 ng of total DNA, were run in duplicate in the universal qPCR assay to investigate a potential inhibitory effect. The cycle threshold (C_q) values for each dilution were plotted against the DNA concentration, and $R^2 < 0.9$ indicated the presence of inhibition.

To obtain the standard curves, a 521-bp linear DNA fragment was designed by aligning the DNA sequences from the PCR products of each qPCR assay and subsequently synthesized (Invitrogen, Thermo Scientific, USA). A map presenting the synthetic DNA standard with annealing regions to sets of primers and probes is illustrated in Fig. S2. The synthetic DNA standard was aliquoted and frozen at −20 °C according to the manufacturer's recommendation, and the DNA concentration in the standard was measured with a NanoDrop 2000 spectrophotometer prior to the run. The standard curves were obtained from four replicates of individual instrumental runs. For each instrumental run, the qPCR reactions were performed with six concentrations obtained from ten-fold dilution, ranging from 10^1 to 10^6 gene copy numbers per µL, and each of these concentrations was run in triplicate. The PCR amplification efficiency (E_p), which reflects the primer-probe amplification efficiency under standard conditions, was derived from the common slope of the corresponding standard curve. For each instrumental run containing unknown samples, the DNA standard at a concentration of 5×10^4–5×10^5 copies per reaction was run in triplicate as a calibration control. The qPCR results were analyzed using StepOne Software v2.3 with an automatic baseline and manual adjustment of the threshold values (0.15 for the universal and sewage-,



PCR	A	B	C	D	E	F	G	H	I	J	K	L
Jul	+	+	+	+	+	+	+	+	+	+	+	+
Aug	+	+	+	+	+	+	+	+	+	+	+	+
Feb	+	+	+	+	+	+	+	+	+	+	+	+
Mar	+	+	+	+	+	+	+	+	+	+	+	+

(a)

PCR	A	B	C	D	E	F	G	H	I	J	K	L
Jul	-	-	-	-	-	-	-	-	-	-	-	-
Aug	+	-	+	-	-	-	-	-	-	-	+	-
Feb	-	-	-	-	-	-	-	-	-	-	-	-
Mar	-	+	+	+	+	+	-	-	-	-	-	-

(b)

PCR	A	B	C	D	E	F	G	H	I	J	K	L
Jul	-	-	+	+	-	+	-	-	-	-	-	-
Aug	-	-	+	+	-	-	-	-	-	-	-	-
Feb	-	-	-	-	-	-	-	-	-	-	-	-
Mar	-	-	+	+	+	-	-	-	-	-	-	-

(c)

PCR	A	B	C	D	E	F	G	H	I	J	K	L
Jul	-	-	+	+	+	-	-	-	-	-	-	-
Aug	-	-	-	-	-	-	-	-	-	-	-	-
Feb	-	-	-	-	-	-	-	-	-	-	-	-
Mar	-	-	-	-	-	-	-	-	-	-	-	-

(d)

Fig. 3. Spatial and temporal distributions of PCR and qPCR universal (a), and sewage-specific (b), swine-specific (c), and cattle-specific markers (d).

and cattle-specific assays, and 0.009 for the swine-specific assay). A mixed model method was used for the absolute quantification of the unknown samples, as described elsewhere (Sivaganesan et al., 2010).

2.6. Bacteroidales recovery efficiency

Bacteroides fragilis strain ATCC700786 was cultured in *Bacteroides* Phage Recovery Medium (BPRM) under anaerobic conditions (Chyerochana et al., 2018; Sirikanchana et al., 2014) and stored at − 80 °C until use. The pH of 1 L of each water sample was adjusted to 3.5, and the sample was then spiked with 10^7 CFU/mL *B. fragilis* culture. The recovery control samples were filtered, and DNA was extracted according to the above-mentioned protocol. The GenBac qPCR assay was performed. The increase in the number of recovered DNA copies from the spiked and nonspiked samples was divided by the number of DNA copies in the bacterial stock to calculate the recovery efficiency.

2.7. Sample processing controls

Salmon testes DNA (Sigma-Aldrich, USA) was prepared, and 160 ng of the salmon DNA was spiked into each sample and method blanks prior to DNA extraction. A Sketa22 qPCR assay (Table 1) (Haugland et al., 2010) was used to detect salmon DNA concentrations with 4 μL of DNA template. The acceptance threshold value was the averaged C_q value from three method blanks + (3 × standard deviation) (Shanks et al., 2016).

2.8. Statistical analysis

Statistical analyses were conducted in R (R Core Team, 2017). The normality of the data was analyzed using the Shapiro-Wilk normality test. The equality of the variances was tested with Levene's test. The significance of the differences was analyzed by one-way ANOVA with Tukey's multiple comparison test (for normal data) and by the Kruskal-Wallis test with Bonferroni's multiple comparisons test (for nonnormal data). Nonparametric Mann-Whitney and Wilcoxon signed-rank tests were used for independent- and paired-sample comparisons, respectively.

For the water quality parameters that contained data below the detection limit (non-detected results), a nonparametric survival analysis procedure was used (Helsel, 2012). Descriptive statistics were calculated using Kaplan-Meier estimates. Normality was tested using the maximum likelihood estimation method with an adjusted Anderson-Darling value. The significance of the differences was tested using the paired Prentice–Wilcoxon test for paired comparisons and the Generalized Wilcoxon test for multiple comparisons. All correlation analyses were conducted with Kendall's tau (rank correlation) using U-Score. A multivariate analysis of the U-Score ranks was performed, and hierarchical clustering performed using the Euclidean distance and Ward's method. In addition, the model-based clustering was analyzed with the Expectation-Maximization (EM) algorithm.

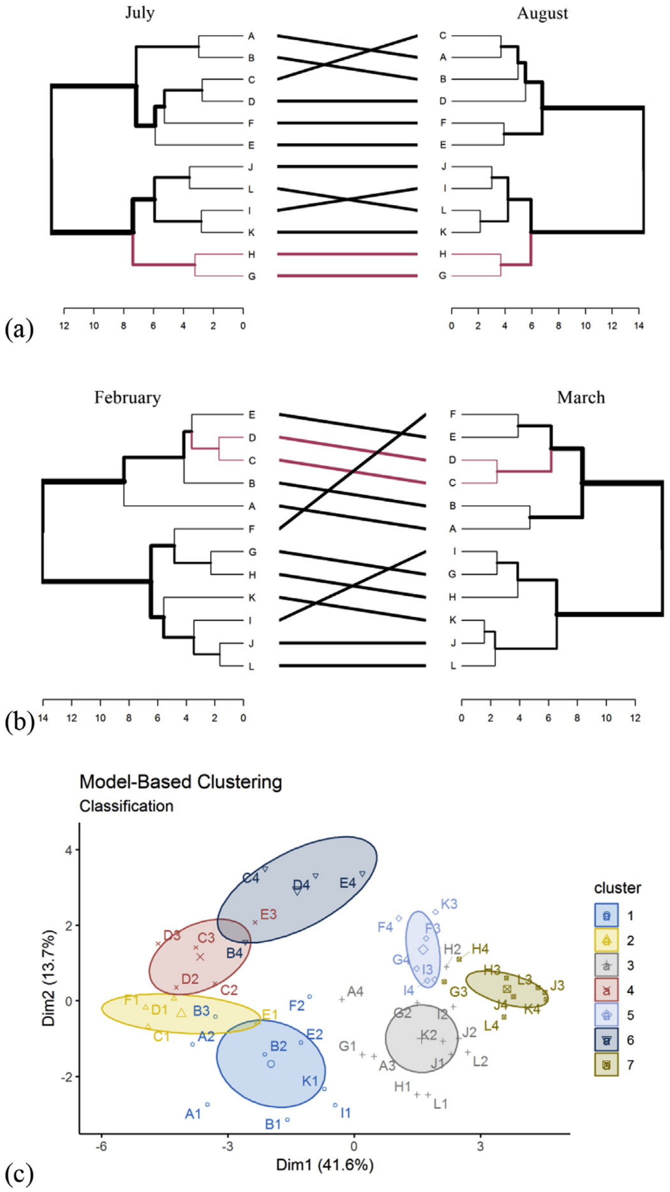


Fig. 4. Multivariate clustering analysis of water samples with hierarchical clustering of sampling stations comparing between two wet seasons (a), and two dry seasons (b), model-based clustering analysis of samples from all twelve stations and all four events (c).

3. Results

3.1. Temporal distribution of water quality parameters and MST markers

The water quality parameters of 48 water samples from 12 stations collected during four sampling events were measured. All physicochemical and biological water quality parameters were detectable in all samples (Fig. 2 and Fig. S3). In contrast, MST markers were found only in certain samples. The qPCR results demonstrated that universal MST markers were detected in all samples (Fig. 3). The sewage- and swine-specific qPCR markers showed positive rates of 20.8% and 29.2% (n = 24 in each season), respectively, and similar values were obtained in the wet (Jul and Aug) and dry seasons (Feb and Mar). In contrast, the cattle-specific qPCR markers were detected only in the Jul samples (16.7%; n = 24 in the wet season). The presence of MST markers detected by conventional PCR is also shown in Fig. 3. A comparison showed 100%, 93.8%, 87.5%, and 97.9% agreement between the PCR and qPCR results for universal and sewage-, swine-, and cattle-specific markers, respectively. Notably, sewage markers at low concentrations close to the limit of quantification (LOQ) were also not detectable by the sewage-specific PCR assay.

The following parameters showed no significant difference among the sampling times ($p > 0.05$): TSS, *E. coli*, TC (CFU), enterococci, FC, TC (MPN), and sewage- and swine-specific qPCR markers (Supplemental Table S5). The remaining physicochemical parameters and universal and cattle-specific qPCR markers showed significant differences among various pairs of sampling events ($p < 0.05$; Supplemental Table S6). In addition, the universal qPCR markers showed significantly lower concentrations in the Aug samples (wet season) than in the Feb and Mar samples (dry season) ($p < 0.05$).

3.2. Spatial distribution of water quality parameters and MST markers

The water quality parameters tended to show higher concentrations in the downstream and river mouth stations (Fig. 2 and Fig. S3). The MST analyses showed that universal markers were present at all stations along the Tha Chin River (Fig. 3). Sewage-specific PCR and qPCR markers were detected at stations located in the lower part (A-F) and at station K, and swine-specific PCR and qPCR, and cattle-specific PCR and qPCR markers were detected at stations C, D, E, and F. A multivariate analysis of all water quality parameters and MST markers was performed. The hierarchical clustering of the sampling stations demonstrated a strongly consistent trend between the two wet season samplings, with a moderately consistent trend between the two dry season samplings (Fig. 4a and b). Stations G-L presented better water quality at all four sampling times. Model-based clustering revealed that the optimal number of clusters was seven (Fig. S4a). The findings showed that the samples from adjacent stations tended to cluster together within those from the lower section and the middle-upper section, indicating that these stations exhibited identical water quality conditions at each sampling event (Fig. 4c).

3.3. Relationship among parameters

A multivariate analysis using model-based clustering showed the relationships of water quality parameters with the most appropriate clustering (Fig. S4b), which consisted of nine groups (Fig. 5). The bacterial parameters, namely, TC (MPN), TC (CFU), FC, *E. coli*, and enterococci, were grouped together, and the MST qPCR markers were clustered into the same group. The BOD, pH, temperature, DO and TSS were individually separated, whereas the salinity, conductivity and TDS were clustered together, and phosphate and TP formed another group.

Moreover, strong correlations ($\tau > 0.55$) among most biological parameters were found, including TC (CFU), TC (MPN), FC, *E. coli*, and enterococci (Fig. 6a). The universal qPCR marker showed moderate correlations ($0.31 < \tau < 0.55$) with other source-specific markers. Interestingly, both the universal and swine-specific markers presented moderate correlations with all biological parameters. Among the physicochemical parameters, strong correlations between salinity and conductivity, between conductivity and TDS, and between TP and phosphate, were found (Fig. 6b). No strong correlation was observed between a physicochemical parameter and a biological parameter (Supplemental Table S7).

3.4. Quality controls and standards

Of the 12 sampling stations, site F was selected as a representative for field duplicate sampling at each of the four sampling events. The biological parameters and MST markers were analyzed to assess the repeatability of the results, and coefficients of variation (CVs) were calculated (Supplemental Table S8). Furthermore, the three field blanks and seven method blanks showed no false positive detection of the biological parameters and MST markers.

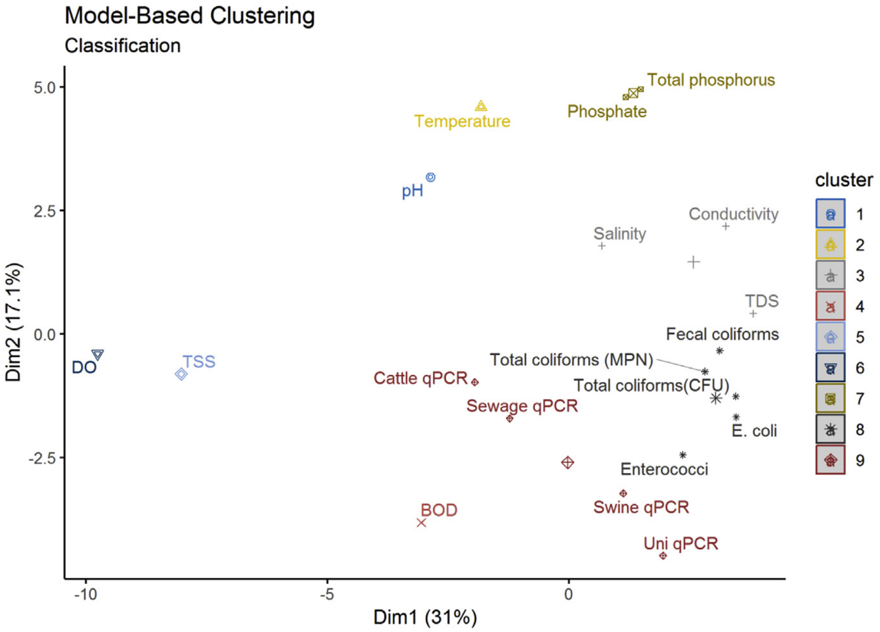


Fig. 5. Model-based clustering analysis of water quality parameters.

Conventional PCR analysis was performed in duplicate for each water sample. Six percent of the 476 PCRs presented non-agreeing results, and therefore, one additional run of these samples was conducted. The quality controls for PCR, including positive controls and NTCs, demonstrated that 100% of the instrumental runs (n = 43) worked properly and were free of contaminated DNA. The three extraction blanks were also negative for the GenBac PCR marker. In the qPCR assay, the standard curves for universal, sewage-, swine- and cattle-specific assays showed 101.1, 96.0, 90.5, and 94.2% amplification efficiencies, respectively. The LOQ for each assay was 50 gene copies/reaction. Only one out of 62 samples indicated an inhibitory effect. The sample processing controls showed that the Sketa22 C_q values of all representative samples (50% of the total environmental samples; n = 52) were below the acceptance criteria, indicating that acceptable DNA isolation efficiency was achieved in this study (Supplemental Table S9). Six representative samples spiked with *B. fragilis* demonstrated no loss during the filtration and DNA extraction processes, as demonstrated with the GenBac qPCR assay (Supplemental Table S10).

4. Discussion

This study monitored multiple water quality parameters in a tropical river associated with various land use activities. The Tha Chin River has been considered one of the most polluted rivers in Thailand for more than several decades, and the lower section of this river presents the most deteriorated conditions (Pollution Control Department (PCD), 2018). The various water quality parameters monitored during the four sampling events in this study demonstrated the deteriorating quality in the lower Tha Chin River, which is located in Samut Sakhon and Nakhon Pathom Provinces. As supported by the multivariate analyses, the spatial similarities within the lower and middle-upper sections were more pronounced than the temporal similarities among four sampling events. The MST analysis indicated that swine and cattle DNA marker concentrations were detected in the lower Tha Chin River sampling stations (Fig. 3). The swine populations in the downstream to upstream provinces, namely, Samut Sakhon, Nakhon Pathom, and Suphan Buri, include 2, 2.06×10^5, and 2.79×10^5 individuals, respectively, whereas the cattle populations in these three provinces consist of 3.07×10^2, 6.35×10^4, and 1.43×10^5 individuals, respectively (Information and Communication Technology Center, 2017a; 2017b, 2017c). It is therefore likely that pollution sources from animals could be derived from immediate and upstream sites. Moreover, there are numerous waste management methods used on farms, such as allowing feces droppings on open land, sweeping away dry feces in animal stalls, watering the floor to collect slurry, or opting for disinfectant application. Additionally, wastewater treatment facilities are required for swine farms raising more than 500 individuals, and more stringent effluent standards for pH, BOD, chemical oxygen demand (COD), TSS, and total Kjeldahl nitrogen (TKN) are required for farms with more than 5000 individuals. Consequently, different farm waste and wastewater management approaches could also affect the fate and transport of contaminants into the main river. Sewage markers were detected in the lower Tha Chin River along condensed residential areas and at the upstream site located next to a community fresh market (site K). Overall, MST marker occurrence was related to the land use activities along the river, concurring with previous reports that demonstrated well-correlated land use patterns with microbial sources (Jent et al., 2013; Wu, 2019). Universal markers were found at all stations along the river at all sampling events, even at sites where no sewage, swine, and cattle markers were found. As tested in this study, chicken, goat, sheep, buffalo, and duck also contributed to universal marker abundance. Other possible sources of fecal *Bacteroidales* include migrating birds, waterfowl, and wildlife (Devane et al., 2013; Lu et al., 2008; Nguyen et al., 2018). Although certain sewage-specific *Bacteroidales* conventional PCR and qPCR assays have been reported for cross detection with fish feces (McLain et al., 2009), the sewage-PCR, swine-PCR, and cattle-PCR assays used in this study were tested with fish feces from Nile tilapia, red tilapia, and carp, and no false amplification was detected (n = 8; data not shown).

In this study, no significant difference in precipitation was found among the four sampling events. Considering that the 24-h precipitation data only indicated light rain (less than 10.0 mm/m^2-h), it is not very likely that a significant amount of runoff occurred prior to sampling. Parameter comparisons revealed that the following parameters demonstrated differences among the samplings: pH, BOD, temperature, salinity, conductivity, TP, phosphate, TDS, DO, and universal and cattle-specific qPCR markers. In contrast, the following parameters were detected at consistent concentrations across the various sampling events: TSS, TC (CFU), TC (MPN), FC, *E. coli*, enterococci, and sewage- and swine-specific qPCR markers. Intact microbial cells and the

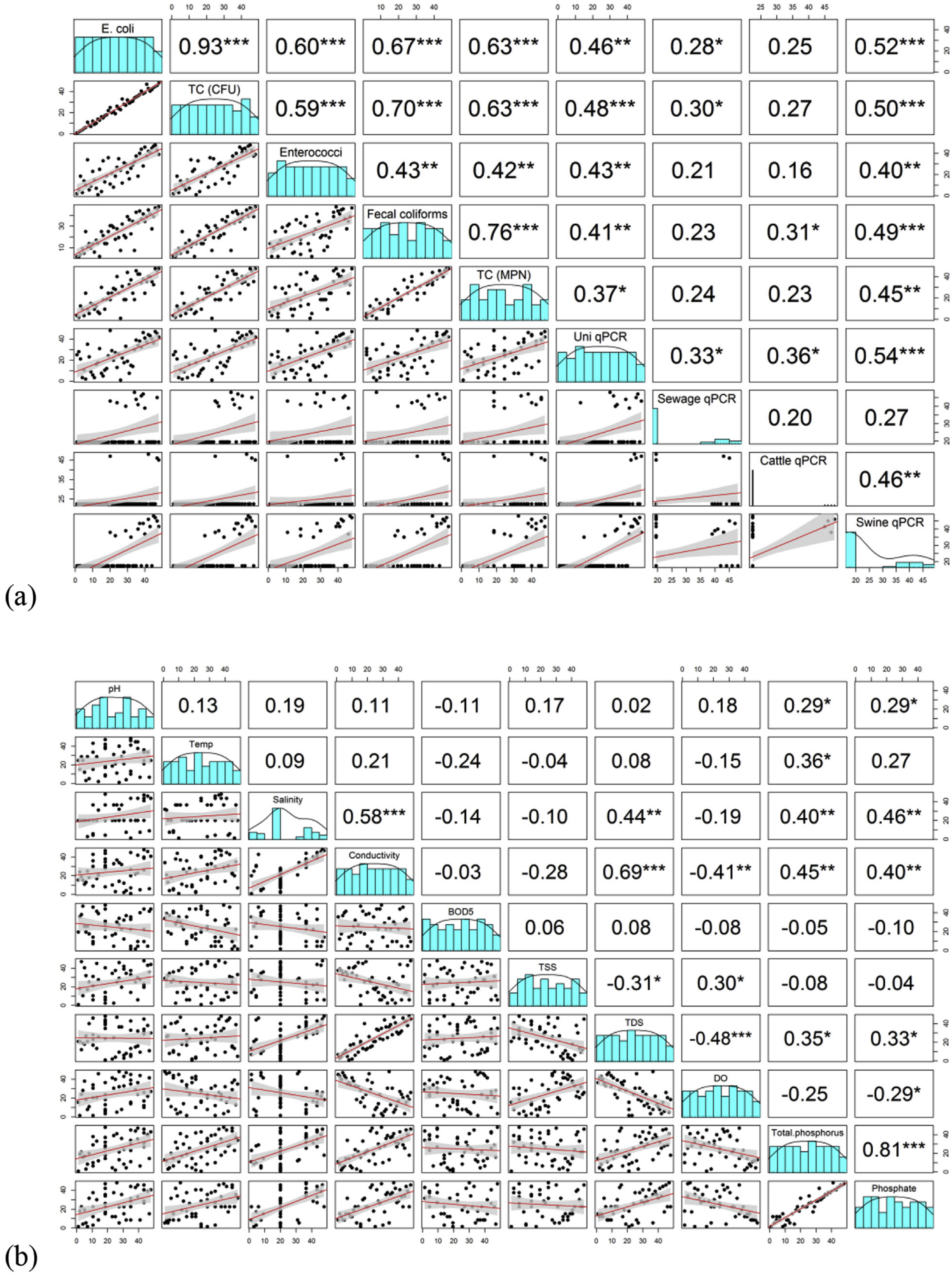


Fig. 6. Kendall-tau correlation analysis of biological parameters (a) and physicochemical parameters (b). Asterisks indicate significant levels, with *** meaning $p < 0.001$, ** meaning $p < 0.01$, and * meaning $p < 0.05$. Histograms show the kernel density estimation of each parameter. Scatter plots with the fitted line and confidence intervals display the statistical associations of each pair of variables.

corresponding DNA exhibited different decay rates that could also be affected by many factors, including the type of microorganism, temperature, salinity, sunlight, pH, DO, organic matter, and solid adsorption (Bae and Wuertz, 2014; Booncharoen et al., 2018; Chen and Liu, 2017; Cho et al., 2016; Wangkahad et al., 2015). Traditional fecal indicators, i.e., TC, FC, *E. coli*, and enterococci, could potentially regrow in tropical environmental water (Desmarais et al., 2002; Fujioka et al., 2015), whereas the strictly anaerobic *Bacteroidales* and its DNA material are unable to regrow. Recent research reported that river sediments and biofilms could serve as reservoirs for microbes and DNA markers to be reintroduced into overlying water (Devane et al., 2019; Li et al., 2018; Mackowiak et al., 2018). Moreover, various hydrological factors, including the river flow rate and direction and the tidal effect at the river mouth, could affect the fate and transport of contaminants in recipient waters (Sirikanchana et al., 2008).

For the management of a polluted water body, it is important to quantify the fecal loads from each pollution source in order to comply with the carrying capacity of the water body. Our unpublished data revealed that sewage-, swine-, and cattle-specific qPCR markers were present at median concentrations of 6.09 log DNA copies/100 mL of sewage, 8.55 log DNA copies/g of swine feces, and 5.92 log DNA copies/g of cattle feces, respectively. Universal markers were detected at a median concentration of 9.33 log DNA copies/g of animal feces, including cattle, swine, chicken, duck, buffalo, sheep and goat feces, or at a median concentration of 8.02 log DNA copies/100 mL of human sewage. According to this study, the maximum loads of river contamination could have derived from approximately 1.7 mL of human sewage, 0.23 mg of swine manure, and 20.4 mg of cattle manure per 100 mL of river water. The maximum load from all fecal contamination (universal marker) was estimated to equal 134.9 g of animal feces (or 17.0 mL of sole sewage source) per 100 mL of river water.

The current surface water quality standards for freshwater in Thailand list only total and fecal coliforms as biological indicators, which need to be detected using a multiple-tube fermentation technique and are reported as most probable number (MPN) per volume of water (National Environment Board, 1994). However, much research has been conducted since 1994, and therefore, recommendations from that time might be slightly outdated. Our results, which showed that the MPN method resulted in a higher variance than the membrane filtration method (CFU units), agreed with previous reports (Gronewold and Wolpert, 2008). Furthermore, TC (CFU) showed a stronger correlation with *E. coli* and enterococci ($\tau = 0.93$ and 0.59, respectively) (Wangkahad et al., 2015) than TC (MPN) ($\tau = 0.63$ and 0.42, respectively; Fig. 6). *E. coli* and enterococci, which are included in the water quality standards in the US and Europe, were measured in this study to evaluate the appropriateness of their inclusion in the water quality standards of Thailand (European Union, 2006; US EPA, 2012). Additionally, the measurement of TC (CFU) requires less analysis time than that of TC (MPN), which supports the use of TC (CFU) over TC (MPN) for rapidly obtaining water quality information.

The use of MST markers to differentiate sources of fecal pollution has successfully facilitated the water resource management and water quality restoration of polluted water bodies in many areas, such as the UK and the United States (Benham et al., 2011; Environment Agency, 2008; US EPA, 2011). None of these applications have included MST markers in regional water quality standards but have supported the use of bacterial indicators for providing bacterial contamination sources (Porter, 2017). Water samples can be processed by filtration when the samples arrive at the laboratory, and the filters can then be stored. However, only the samples with bacterial indicators higher than the standards can be used for MST analysis. The 87.5–100% agreement between the MST-PCR and MST-qPCR results obtained in this study also emphasizes that PCR application is a screening step that requires less technical skills and has lower costs. Our study estimated that both the operational and fixed instrumental costs for qPCR were approximately 1.8-fold higher than those for PCR. The universal and sewage-, swine-, and cattle-specific markers obtained from the PCR and qPCR assays using the same pairs of primers demonstrated comparable accuracies, with zero-to two-orders-of-magnitude differences in the limits of detection (Somnark et al., 2018b, 2018a). If more information is preferred, such as the relative quantification of contamination from more than one source or low contamination levels, a corresponding qPCR assay can be subsequently performed with the desired sample. DNA-based detection methods offer the advantage of sample storage, and the stored samples can be reanalyzed if replicated data or new assays are needed.

5. Conclusion

This study monitored physicochemical and biological indicators in the Tha Chin River for water quality analysis. MST markers were also analyzed to provide information on fecal pollution sources in multi-activity surface water in Thailand. This study provides insights into the spatial and temporal distributions of water quality parameters and MST markers and the correlations among these variables, which could facilitate water quality management and the restoration of tropical river systems.

Declaration of interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2019.01.005.

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