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Biomonitoring by using Rapid-Read Pathogenic Bacteria Indicator in Sediments and Bivalve Mollusks: Southern Gulf of Thailand, a Mangrove Area Case Study

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Abstract

Pathogenic bacteria groups can be applied to be rapid bioindicator for environmental assessment. The objective of study was to monitor 5 groups of indicator and pathogenic bacteria (*Escherichia* coli/ total coliform, *Salmonella* spp., *Vibrio* spp., *Bacillus* spp. and *Clostridium* spp.) in surface water sediments collected before monsoon and after monsoon season from 6 areas located in a Thepha District, Songkhla province mangrove closed to Southern gulf of Thailand. Each collected area were selected in criteria of totally different in environment parameter and tide. The relative levels of total coliforms, and *E. coli*, were typically increased in rainy season (July) compared with winter and summer at most sites. In parallel, the highest number of *Vibrio* spp. was found in summer (April). The highest number of *E. coli* and Coliform were detected during rainy season at the estuary area with low oxygen dissolved, the average numbers were 6.6×10^3 and 3.8×10^5 CFU/g, respectively. Coliform number shows negative correlate with dissolved oxygen ($r^2 = 0.756$, $p < 0.05$). In addition, total coliform, *Bacillus* spp. and *Clostridium* spp. were found in bivalve mollusks during rainy season but the total was not over standard. A very strong positive correlation was found between the temperature and the number of *Vibrio* spp. and *Bacillus* spp. The number presence of *Vibrio* spp. has a strong positive correlation with pH, the number of *Bacillus* spp. and number of *Clostridium* spp. In contrast, *Vibrio* spp. number was strongly negative correlated with water salinity ($r^2 = 0.632$, $p < 0.05$). *Salmonella* species were detected only 2 area around shrimp farm in between December of a year-round. In this study area, there are no evidence that the number of *Salmonella* and *Clostridium* species founded related to changing of season. This is the first baseline data as an alternative guide concept for the determination of bacterial indicators. Pathogenic bacteria number can be applied to be a key factors for a rapid health assessment for villager around study area that related to EIA in the near future.

Keywords: Bioindicator, Pathogenic bacteria, Sediments, Bivalve mollusk, Southern Gulf of Thailand, Mangrove, Environmental impact assessment

Introduction

Thepha District, Songkhla Province, in the Southern part of the Gulf of Thailand (GOT) coast has a large mangrove forest and coastal area. The mangrove forest in Thepha is located near a riverine and covers an area of 3.4 km^2 to the GOT seashore. The area is also the location of a small-fisheries village and some agriculture areas (4.09 km^2). However, it also is viewed as a potential site to develop industries nearby the bay. Thus, there is a need to characterize the current environment before the construction of any project to assess the areas of potential conservation or health risks and to get the data to predict, prevent, correct and monitor these aspects during and after construction and operation of industries or any project.

Recently, a researcher used environmental microbes as an indicator for pollution [1]. Bio-indicators bacteria have been discussed continually and accepted to be one type of indicator for environmental or ecosystem monitoring. Bio-indicator bacteria can reveal important signs of environmental change because of their fast response to environmental changes, such as low level of contaminants and other physiochemical and biological change [2,3]. In some cases, bacteria can be increased rapidly by human activities, such as industries, agricultures, fisheries and other household waste. Pathogenic bacterial populations can be

induced and play an important role for human or other animal health through water and food contamination [4-6].

Fecal coliform bacteria is a group of bacteria that can be used worldwide as indicators of fecal contamination and waterborne diseases [7]. However, in each environment, there is a variety of different bacteria. It is not logistically feasible to determine all the bacteria in a large number of samples, and so typically representative groups or individual marker species are measured to determine the environmental quality in every environment. Thereby, monitoring bacteria as a means to assess the aquatic environment and health in the Thepha District should be focused on the potential of human health risk in terms of waterborne diseases. Cabral [8] discussed about the species which are always found to be the cause of waterborne infections disease that are transmitted through water, including those that cause cholera, typhoid fever, bacillary dysentery, diarrhea and gastroenteritis, and therefore can be a serious problem to human health. Thus, the communicable diseases that cause, for instance, *Vibrio* spp, *Escherichia coli*, *Shigella* spp, *Salmonella* spp. and *Clostridium* spp. are required for appropriate monitoring and the control process. Moreover, Bal *et al.* [9] and Goja [10] revealed that surface water sources can be a prime reservoir for *Bacillus* spp., a causative agent in both non-pathogenic and pathogenic species such as *B. cereus*, *B. subtilis*, *B. licheniformis* and *B. penibacillus* etc.

Monitoring pathogenic bacteria in sediments is relevant for assessing water quality that impacts on human health and the environment. A diverse range of studies have reported that aquatic sediments can provide a stable essential microenvironment for bacteria accumulation [11-13]. The ability of aquatic sediments to act as reservoirs for bacteria is illustrated by the findings that sediments may contain 100 to 1000-fold higher bacteria levels than overlying water [14,15]. Pathogenic bacteria in sediments may become resuspended in the water due to human recreational or construction activities, altered water courses of flow rates or from natural turbulence [16]. As a result, the pathogenic bacteria can then be transmitted by the water and contaminate aquatic animals in parallel and consequently be transmitted to humans via water usage, such as drinking, recreational activities, irrigation, and shellfish harvesting [17,18].

Hazards Bacteria is associated with the consumption of aquatic animals. It is a major concern in the seafood industry and for public health agencies. Bivalve mollusks show great indication for environmental variability investigation in marine and coastal ecosystems. Bivalve mollusks are sessile and often the dominate species to play a role in the biodegradable biomass in benthic communities. Bacteria is contaminated in the tissue of bivalve mollusks from surrounding waters and sediments during the filter-feeding process and the mollusks are admitted as the reservoir for various bacterial pathogens [19]. In this study area, *Polymesoda bengalensis* was chosen to be the local model of species of mollusk to be found in the sediment of the Khwai canal in mangroves, Thepha District and consumed by people in the community. *P. bengalensis* can be found to inhabit mangrove areas in Southeast Asia [20]. In Thailand, *P. bengalensis* is found in small areas of Songkhla Province and some parts of the eastern region in Chanthaburi Province. Therefore, the study of bacterial contamination in bivalves that is found in the study area will indicate levels of bacterial contamination in aquatic animals in the area.

In general, EIA and EHIA studies relate to results about the environmental effects after a project or incidence. This is the earlier data that can collected from day 0 before any near project or city invasion in the specific area has happened an affected to mangrove environment.

This study is focused on investigating the relative levels of 5 pathogenic bacterial groups: 1) *Escherichia coli*/total coliform bacteria, 2) *Salmonella* spp., 3) *Vibrio* spp., 4) *Bacillus* spp. and 5) *Clostridium* spp. from sediments and bivalve mollusks (*P. bengalensis*) in the surface water of the Thepa District. In addition, the relationship between the relative population levels of these bacteria and the physical-chemical parameters were examined and compiled. These results can be baseline data to guide selection of bacterial indicators for further environmental and health assessment monitoring of the surface water in the mangrove area in the southern part of Thailand.

Materials and methods

Study area

The study area was located on the east coast of southern Thailand (GOT coast) in Songkhla Province. Sediment samples were collected from the surface water sediment at 6 stations along the Thepha District canal and riverine (**Figure 1**). In addition, the bivalve mollusks (*P. bengalensis*) were collected at 3 sites on the Khwai canal (station 4). All stations were located and marked by GPS coordinates for future reference.

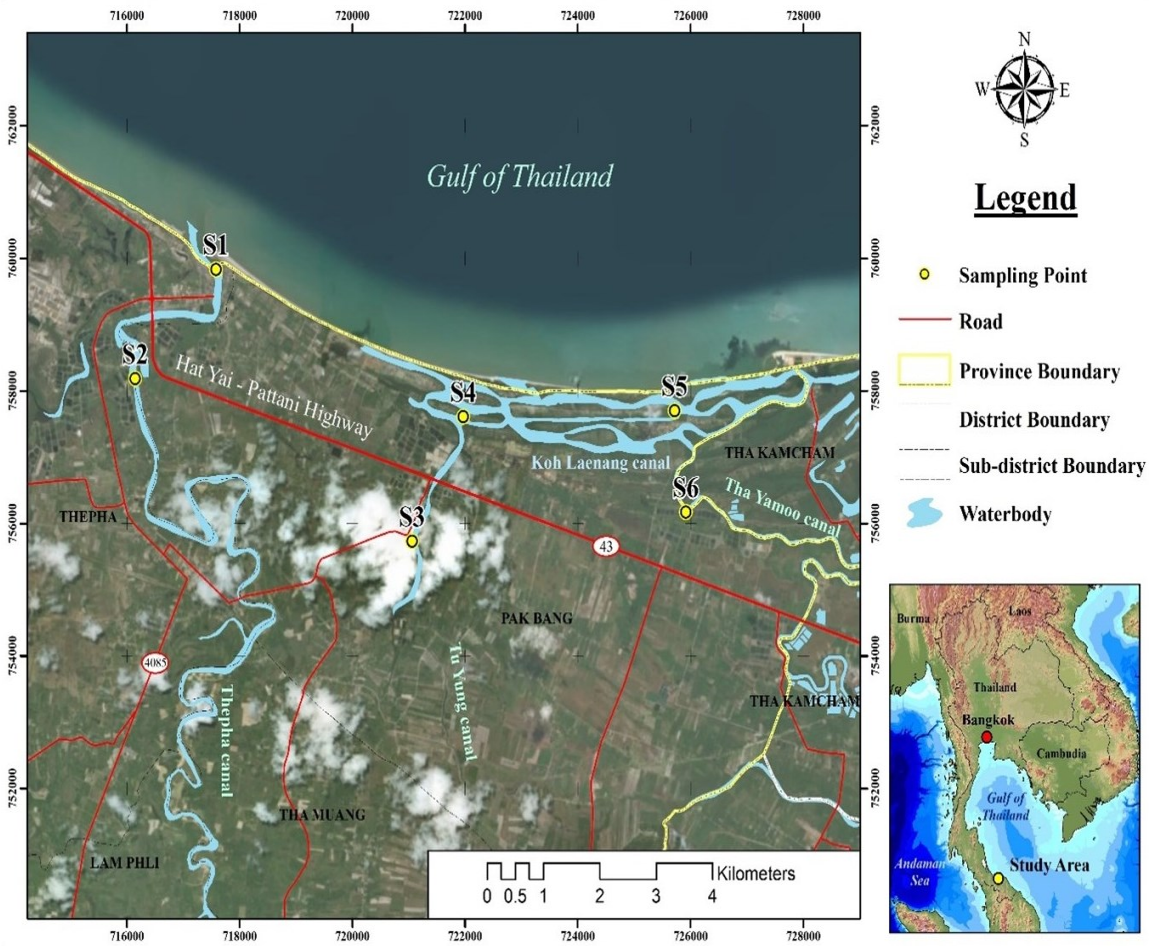

Figure 1 Location of sample collection of surface water in Songkhla province.

Station 1 (S1) is located in the mouth of the Thepha canal (717582 E, 759835 N) that drains into the GOT, and S1 was 3.18 km away from Sub-district Administration Organization Thepha (sub-district Thepha) in distance.

Station 2 (S2) is located in the Thepha canal (716145 E, 758186 N) some 1.25 km from sub-district Thepha. Thepha canal connects to Thepha River to the mouth of the GOT.

Station 3 (S3) is located in Tu Yung canal (721061 E, 755735 N) some 5.6 km from sub-district Thepha. Tu Yung canal is natural surface water that is affected by the tide from the GOT. In the monsoon season this canal receives overflowing water from Thepha river. This station is close to a shrimp farm.

Station 4 (S4) is located in the Khwai canal (721968 E, 757611 N) some 6.73 km from sub-district Thepha. The Khwai canal receives water from Tu Yung canal and water flows to the Koh Laenang canal. Normally, the water in this station is saltwater because there are tidal forces of the sea from the mouth of the Koh Laenang canal, and in the monsoon season the Khwai canal will receive water from the overflowing of Thepha canal that turns into fresh water. This station is close to a tiger shrimp farm.

Station 5 (S5) is located in Koh Laenang canal (725719 E, 757702 N) some 9.69 km from sub-district Thepha. The Koh Laenang canal connects with the Khwai canal and Tu Yung canals to the West of Thepha District, and with the Tha Yamoo canal to the East.

Station 6 (S6) is located in the Tha Yamoo canal (725916 E, 756174 N) some 10.36 km from sub-district Thepha. The Tha Yamoo canal is downstream in the Khokpho District, Pattani Province and an upstream of the Koh Laenang canal and river mouth of the GOT at Pattani Province.

Sediment and bivalve mollusks sampling

Six sediment samples were collected from each station during each day of December (monsoon), April (pre-monsoon) and July (monsoon). The sediment samples were collected using a grab sample and a portion of the top 15 - 30 cm of sediment, which was transferred into 50 mL sterilized bottles. All samples

were stored in ice and transported to the laboratory for further analysis within 48 h. Physical and chemical parameters such as pH, salinity, dissolved oxygen (DO) and temperature were measured**.**

Three replicates of *P. bengalensis* were collected from the Khwai canal, by 1×1 m^2 quadrat methods. *P. bengalensis* was taken during April (pre-monsoon) and July (monsoon) and samples were stored in an ice box.

Bacteria selective process

Soil sediments and *P. bengalensis* samples (1 g of each sample) and mollusk solution (1 mL of each sample) were transferred into an enrich-medium and selective medium [21] for bacteria identification by nucleotide sequence analysis and biochemical tests.

Collection of each 3 replicates of soil and sediment sample in each area were pulled. Then, 1 g of sediment from each area was inoculated in selective medium to limit bacteria species prior before screening process. After transfer 3 times, bacteria count was performed and pure isolates were re-streaked on agar plate. Biochemical test was performed. Bacteria investigation in *Polymesoda bengalensis* was achieved by collected 1 mL of solution of *P. bengalensis* mixture in steriled distilled water. Preparation of *P. bengalensis* mixture was performed by kept freshy *P. bengalensis* samples and grined it with steriled instruments. The 1 g of grinded *P. bengalensis* was mixed in 25 mL steriled distilled water and mixing 3 h by belly dancer.

Bacteria species identification was executed by standard protocol for species identification following standard methods of Bacteriological Analytical Manual. *Escherichia coli* and total coliforms identification and counting were done by 3M Petrifilm^{TM} *E. coli*/ Coliform Count Plate following AOAC Official Method 991.14. The 1 g of sample was added to 9 mL of 0.85 % steriled normal saline solution. Serial dilution was performed until countable dilution reach to 50 - 200 colony per spread. pH was adjusted to be 6.6 - 7.2 then transferred 1 mL dilution on petrifilm. Incubation of petrifilm was done at 37 °C for 18 - 24 h. Total coliform results can be determined by blue and red colonies dot on petrifilm. Total *E. coli* (CFU/g) is counted by incubate petrifilm further 46 - 48 h. Blue dots of bacteria colonies with gas bubble can be defined to be *E. coli* (CFU/g).

Salmonella spp. were enumerated using the Rappapaort-Vassiliadis (RV) broth as an enrichment media and Salmonella Shigella (SS) agar as the selective media [22] following Bacteriological Analytical Manual, chapter 5, *Salmonella*. Enormous *Salmonella* spp. From samples were done by add 1 g of sample into 10 mL of Lactose medium (Himedia, India) at pH 6.8 ± 0.2. Incubation was done at 35 ± 2 °C for 18 - 24 h. Selective medium for *Salmonella* species in this experiment is Rappapaort-Vassiliadis broth (RV) with incubation at 42 ± 2 °C at 24 ± 2 h. Salmonella Shigella Agar (SS agar) was applied for *Salmonella* identification at 35 ± 2 °C at 18 - 24 h. Crystal clear colony with black dot in orange agar are identified prior as *Salmonella* spp. Five pure isolates were selected for further fine species identification by molecular techniques.

Vibrio spp. were isolated by the most probable number (MPN) method using a phosphate buffered saline (PBS) broth and alkaline peptone water broth for dilution and enrichment, and the thiosulfate citrate bile sucrose (TCBS) agar as selective media [23,24]. The 1 g of sediment sample was solubilized in 9 mL Phosphate Buffered Saline (PBS). Serial dilution was set in dilution of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}, respectively. Alkaline Peptone water (APW) was applied for stimulate *Vibrio* spp. at 35 ± 2 °C for 18 - 24 h. Thiosulfate Citrate Bile Sucrose (TCBS) agar was adapted for species selection at 35 ± 2 °C for 18 - 24 h. Green and yellow colonies were prior identified as *Vibrio* spp. Select 5 isolates to identified further.

Bacillus spp. were selected and isolated by applying *Bacillus cereus* analysis in foods following Plate Count Method (PCM) following APHA 2001 [23]. Peptone water was using for bacteria dilution and enrichment. Serial dilution was performed at 10^{-1}, 10^{-2} and 10^{-3}. Mannitol egg yolk polymyxin (MYP) agar was applied for detection and enumeration of *Bacillus* spp. 0.3 mL of dilution at 10^{-1}, 10^{-2} and 10^{-3} were spreaded on MYP agar, incubate at 30 - 32 °C 20 - 24 h. Red colonies with clear zone arounded were identified prior as *Bacillus* spp. The yellow one was identified as *B. subtilis*.

Clostridium spp. were isolated following *Clostridium perfringens* protocol in food APHA 2001 using Tryptose Sulfite Cycloserine (TSC) agar and enumerated using the PCM as reported [23]. The 1 g of sample was inoculated in Peptone solution. Serial dilution of samples at 10^{-1}, 10^{-2} and 10^{-3} were poured into selective medium of TSC agar plate. Top on with TSC agar layer and incubate under anaerobic condition (Anaerobic jar with reagent) at 35 - 37 °C for 18 - 24 h. Black colonies on selective agar were identified prior as *Clostridium* spp.

Figure 2 Bacterial colonies on selective medium and API 20E biochemical identification kit *Bacillus* spp. (Left) *Vibrio* spp. (Right)

Bacteria species identification by API kit (BioMérieux, France)

Each group of isolated bacteria were cultured until 18 - 24 h before biochemical identification by API kit (BioMérieux, France). *Vibrio* spp. and *Salmonella* spp. were tested by API 20E kit identification. Tested isolates were suspended in 0.85 % steriled sodium chloride solution 5 mL and pipetted to the strip test. Citrate utilization: CIT, acetoin production by Voges Proskauer: VP, gelatin hydrolysis (GEL) test were performed both in strip and reaction tube. Arginine dihydrolase (ADH), Lysine decarboxylase (LDC), Ornithine decarboxylase (ODC), Urease (URE) and H_2S production (H_2S) were tested following manual recommendation. Anaerobic condition in test was induced by mineral oil. Incubation was done at 36 °C for 18 - 24 h. *Bacillus* spp. were analysed by API 50CHB kit identification and API 20E kit identification. The 50 CHB kit identification can be applied with bacterial colony directly by mixing in solution and test 120 µL solution on strip. Incubation of strip was applied at 37 °C for 24 ± 2 h. To identified *Clostridium* spp., API 20A kit identification was practiced. Bacterial colonies were blended to solution and tested in API 20 A strip. gelatin (GEL) hydrolysis was tried on test cup in kit and Indole formation (IND) was verified with mineral oil. Incubation was done at 36 ± 2 °C 24 ± 2 h under anaerobic condition (Bactron, Sheldon, USA). After incubation period, results were demonstrated by Apiweb^{TM} software.

Bacteria species identification by *16SrDNA* sequence

Bacterial isolates from each media were identified by biochemical tests using the API 20E kit for identification of *Vibrio* spp. and *Salmonella* spp. API 50CHB/20E for *Bacillus* spp.and API 20A for *Clostridium* spp. Confirmation of unclear isolates was resolved by sequence analysis of the partial 16S ribosomal RNA (16S rRNA) gene fragment using 97 % nucleotide identity cut off to annotate sequences in GenBank. PCR amplified using the forward primer was 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and the reverse primer was 1492R (5′-GGTTACCTTGTTACGACTT-3′) [25]. The PCR reaction mixtures contained 1 *x i-taq* buffer, 0.25 mmol/L of each dNTP, 0.25 µmol/L of each primer and 1.25 U of Phusion DNA polymerase (New England Biolabs Inc., USA) in a total volume of 50 mL. The reaction was performed at 95 °C for 3 min, followed by 25 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min and then finally at 72 °C for 10 min. PCR amplicons were checked by 0.8 % agarose gel analysis and sequenced by Pacific Science (Bangkok, Thailand). The obtained sequences, as molecular operational taxonomic units (MOTU), were searched against the GenBank database using BLASTn and ascribed to species level using a 97 % nucleotide identity cutoff against species annotated sequences in GenBank.

Environmental parameter observation

The nutrient levels in each sediment sample were analyzed for the nitrogen (N), phosphorus (P) and organic matter (OM) by the ISO 5983-2 (2009) method [26], AOAC 962.02 (2016) method [27] and standard test methods for moisture, ash, and organic matter (ASTM D2974-87, 1993) [28], respectively by Overseas Merchandise Inspection Co. Ltd (Bangkok, Thailand) and the organic carbon (OC) by the combustion method using a total organic carbon (TOC) analyzer (Center of excellence on hazardous substance management; Chulalongkorn University).

Results and discussion

Analysis of bacterial groups in sediment samples

Directed dilution to extinction-resuscitation in sediments

The quantitative abundance and variations in the different groups of bacteria in the sediment samples are presented in **Table 1**. The number of *E. coli* and total coliforms were higher during the monsoon than in pre-monsoon periods. These results are consistent with those reported by Borade *et al.* [29]. The levels of *E. coli* and total coliforms at station 5 were 4.2×10^4 colony forming unit (CFU)/g and 3.0×10^6 CFU/g, respectively, during the monsoon season and were lowest at station 3 (< 10 CFU/g) in pre-monsoon periods. The total coliforms level was below the maximum permissible level of water quality standard [30] at stations 1, 3 and 5 during the first monsoon, but was above this standard (20,000 MPN/100 mL) and therefore unacceptable at all other stations and seasons. However, several reports have discussed that the number of bacteria in sediment is much higher than in the ground water from the same area. The numbers of pathogenic bacteria from sediments were 10 to 1000fold higher than in the water samples [13]. Other microbes in intertidal mangrove sediment were investigated to observe the turnover rate of microbial communities and pathogens for underwater sediment quality assessment.

Table 1 Average values of bacterial population in sediment samples at different stations during 3 seasons at Thepha District in December - July.

Sample site	Type of bacteria	Season			Water quality standard (MPN/100 mL)
		Monsoon 1 (December)	Pre-monsoon (April)	Monsoon 2 (July)	
Station 1	*E. coli* (CFU/g)	< 10	2.0×10^2	10^2	
	Total coliforms (CFU/g)	< 10	8.0×10^3	10^5	20,000
	Salmonella spp. (CFU/g)	ND	ND	ND	
	Vibrio spp. (MPN/g)	< 3	> 1,100	> 1,100	
	Bacillus spp. (CFU/g)	4.3×10^2	9.3×10^7	7.4×10^7	
	Clostridium spp. (CFU/g)	9.2×10^2	5.6×10^4	6.1×10^2	
Station 2	*E. coli* (CFU/g)	6.1×10^3	< 10	< 10	
	Total coliforms (CFU/g)	1.5×10^4	3.4×10^4	4.5×10^4	20,000
	Salmonella spp. (CFU/g)	ND	ND	ND	
	Vibrio spp. (MPN/g)	> 1,100	> 1,100	> 1,100	
	Bacillus spp. (CFU/g)	9.8×10^5	1.2×10^7	1.2×10^6	
	Clostridium spp. (CFU/g)	4.4×10^3	2.3×10^3	2.6×10^4	
Station 3	*E. coli* (CFU/g)	< 10	< 10	3.3×10^2	
	Total coliforms (CFU/g)	< 10	< 10	1.2×10^4	20,000
	Salmonella spp. (CFU/g)	ND	ND	ND	
	Vibrio spp. (MPN/g)	< 3	> 1,100	> 1,100	
	Bacillus spp. (CFU/g)	3.4×10^3	6.3×10^6	9.7×10^5	
	Clostridium spp. (CFU/g)	< 10	3.5×10^3	3.4×10^2	
Station 4	*E. coli* (CFU/g)	9.0×10^2	1.4×10^3	5.0×10^2	
	Total coliforms (CFU/g)	5.9×10^3	1.2×10^4	1.7×10^5	20,000

Sample site	Type of bacteria	Season			Water quality standard (MPN/100 mL)
		Monsoon 1 (December)	Pre-monsoon (April)	Monsoon 2 (July)	
	Salmonella spp. (CFU/g)	340	ND	ND	
	Vibrio spp. (MPN/g)	460	> 1,100	> 1,100	
	Bacillus spp. (CFU/g)	1.2×10^5	1.9×10^6	1.4×10^6	
	Clostridium spp. (CFU/g)	1.4×10^3	2.4×10^2	9.5×10^2	
Station 5	*E. coli* (CFU/g)	5.8×10^3	< 10	4.2×10^4	
	Total coliforms (CFU/g)	4.4×10^4	7.6×10^4	3.0×10^6	20,000
	Salmonella spp. ((CFU/g)	ND	ND	ND	
	Vibrio spp. (MPN/g)	< 3	150	1,100	
	Bacillus spp. (CFU/g)	5.8×10^4	1.9×10^7	1.0×10^7	
	Clostridium spp. (CFU/g)	2.5×10^2	3.7×10^4	6.7×10^4	
station 6	*E. coli* (CFU/g)	2.2×10^3	6.5×10^3	< 10	
	Total coliforms (CFU/g)	2.6×10^3	7.0×10^4	1.3×10^4	20,000
	Salmonella spp. (CFU/g)	40	ND	ND	
	Vibrio spp. (MPN/g)	460	> 1,100	> 1,100	
	Bacillus spp. (CFU/g)	9.4×10^2	9.7×10^4	1.2×10^5	
	Clostridium spp. (CFU/g)	< 10	20	3.4×10^2	

Note: All the values are taken as an average of 3 readings, ND = not detected

The second group of pathogenic bacteria used as an environmental bioindicator in this study was *Salmonella* spp. Notably, *Salmonella* spp. could be detected and isolated only at the station 4 and 6 during the monsoon period. Thus, *Salmonella* spp. are usually found at high numbers during the rainy season and after flooding. These results are similar to those reported by Haley *et al.* [31] and Massinai *et al.* [32]. The *Salmonella* spp. in this study was identified as *Salmonella enterica* from API bio-chemical test kit and *16S rRNA* partial sequence analysis (**Table 2**). *Salmonella enterica* is one of the most important leading causes of waterborne disease in humans and should always be isolated from contaminated water. Although, *Salmonella* spp. was not found at the other stations, there were other pathogenic bacteria such as *E. coli*, *Cronobacter sakazakii*, *Citrobacter fruendii* and *Klebsiella pneumoniae* (**Table 2**). In comparison to a previous study [33], they observed *Staphylococcus aureus* and *Salmonella* species in crabmeat and mangrove sediment in Paraiba riverine. The results indicated that the number of bacteria varies and there is no *Staphylococcus* or *Salmonella* present in crabmeat also. *Vibrio* spp. were detected at the highest number during the second monsoon and decreased rapidly to the lowest number during first monsoon. Station 2 had the highest levels of *Vibrio* spp. with levels above 1,100 MPN/g in all 3 samples periods. Moreover, the highest overall number and the most diverse number of *Vibrio* species were recorded during the summer, which was somewhat similar to that reported by Böer *et al.* [34] and Di *et al.* [35]. In these previous studies, pathogenic *Vibrio* bacteria in the German North Sea and nearby the Southern coast of South Korea were reported, respectively. In addition, the *Vibrio* spp. isolates were reported to be comprised of *V. parahaemolyticus*, *V. alginolyticus, V. diazotrophicus* and *V. Fluvialis*. These bacteria are frequently found in areas where waterborne diseases are spread. In this study, the *Vibrio* spp. isolates were identified as indicated by a API biochemical test kit and *16S rRNA* partial sequence analysis revealed the species of *V. alginolyticus*, *V. parahaemolyticus*, *V. diazotrophicus* and *V. fluvialis* and found other species from isolation were *Aeromonas hydrophila* and *Shewanella algae* (**Table 2**). In an earlier report, *Shewanella* species also belonged to phylum Proteobateria, which can cause disease in several saltwater fish. The presence of *Shewanella* can be an important sign indicating waterborn diseases or toxins spreading in water resources. As previously described, the genus *Shewanella* has the ability related to the bioremediation process. It can reduce various electron acceptors which are toxic substances or heavy metals.

The total *Bacillus* spp. count ranged between 4.3×10^2 to 9.3×10^7 CFU/g of sediment. The highest and lowest number of *Bacillus* spp. was found at station 1, which is benthic estuary located near a local fisherman village during the pre-monsoon and monsoon 2017 periods, respectively. However, the lowest average number of *Bacillus* spp. was observed during the monsoon 2017 period, and the highest was in the

pre-monsoon period, which is also the highest temperature range in this coastal area. This observation of high *Bacillus* spp. levels is at a high temperature rising as previous results of Azmi and Chatterjee [36], which observed *Bacillus* spp. in coastal soils of Digha, West Bengal, India. There are several clues indicating that this coastal area is decadent and *Bacillus* species was selected to be a pathogenic indicator bacteria/indicator organisms (FIOs). The species of *Bacillus* spp. found in this survey, as indicated by the API biochemical test kit and *16S rRNA* partial sequence analysis revealed the species of *B. cereus*, *B. subtilis*, *B. pumilus*, *B. mycoides* and *B. licheniforms* (**Table 2**). Further investigation should be mentioned with quantity of soil sediment and the amount of organic matter in benthic soil sediment presence.

The level of *Clostridium* spp. was the highest at station 5 during the pre-monsoon period 6.7×10^4 CFU/g and the lowest at station 6 during the first monsoon period (< 10 CFU/g). However, the level of *Clostridium* spp. did not change according to the season. The most common pathogenic *Clostridium* spp. that were identified in samples were *C. perfingens* and *C. bifermentans* (**Table 2**), which are well known bacteria in food contamination.

The results revealed that the highest bacterial numbers were generally found in the pre-monsoon season (April), which accords with temperatures for bacterial growth and the low rainfall and hence absence washout. These observations support the general data description of bacterial species in tropical regions that tend to rise rapidly in the region during the summer season. Furthermore, the presence of other different pathogenic bacterial groups could be observed such as *Aeromonas hydrophila*, *Citrobacter fruendii* and *Klebsiella pneumoniae* in this mangrove area (**Table 2**). Therefore, bacterial accumulation/eutrophication from pollution is one of the important factors that is sensitive to environmental changes. It should be of concern that a higher level than that in standards can indicate a potential of health risk/pathogenic problem in human who live closed to the observed area in a conservative mangrove forest. Microbial communities exhibited in benthic or mangrove sediment are diverse because of the characteristic of collected samples. In the study of Chen *et al.* [1], microbial communities in marine sediments were also investigated by functional metagenome techniques. It also can be concluded that intertidal sediment is extremely diverse. However, culture-dependent techniques are a significant tool to identify viable and culturable (VBC) bacteria in the environment. There are also viable-and-non-culturable (VBNC) bacteria in sediment which needed further intensive focus.

Table 2 Bacterial identification in sediments by API bio-chemical test kit and 16S rRNA partial analysis.

Type of bacteria	Sample site	Seasons					
		Monsoon 1 (December)		**Pre-monsoon (April)**		**Monsoon 2 (July)**	
		Species	%*	**Species**	%*	**Species**	%*
Salmonella spp.	Station 1	*Klebsiella oxytoca*	40	*C. sakazakii*	40	*C. sakazakii*	40
		Raoultella platicoca	20	*R. ornithinolytica*	40	*E. coli*	40
		Raoultella ornithinolytica	20	*C. fruendii*	20	*R. ornithinolytica*	20
		E. coli	20				
	Station 2	*C. sakazakii*	20	*C. sakazakii*	40	*E. cloacae*	100
		R. ornithinolytica	60	*R. ornithinolytica*	40		
		Enterobacter cloacae	20	*E. cloacae*	20		
	Station 3	*R. ornithinolytica*	80	ND	ND	*E. cloacae*	60
		C. sakazakii	20			*V. parahaemolyticus*	40
	Station 4	*S. enterica*	100	ND	ND	*K. pneumoniae*	60
						E. coli	40
	Station 5	*E. coli*	40	ND	ND	*E. coli*	40
		R. ornithinolytica	60			*Aeromonas sobria*	40
						R. ornithinolytica	20
	Station 6	*S. enterica*	100	*E. coli*	100	*E. coli*	100
Vibrio spp.	Station 1	ND		*V. diazotrophicus*	60	*V. parahaemolyticus*	80
				V. alginolyticus	40	*V. alginolyticus*	20
	Station 2	*V. diazotrophicus*	40	*V. diazotrophicus*	80	*V. diazotrophicus*	100
		V. alginolyticus	60	*V. alginolyticus*	20		
	Station 3	ND	ND	*V. diazotrophicus*	40	*V. parahaemolyticus*	40
				V. alginolyticus	60	*V. diazotrophicus*	40
						V. fluvialis	20
	Station 4	*V. alginolyticus*	40	*V. alginolyticus*	40	*V. alginolyticus*	20

Type of bacteria	Sample site	Seasons					
		Monsoon 1 (December)		**Pre-monsoon (April)**		**Monsoon 2 (July)**	
		Species	%*	**Species**	%*	**Species**	%*
		V. parahaemolyticus	40	*V. parahaemolyticus*	60	*V. parahaemolyticus*	80
		A. hydrophila	20				
	Station 5	ND	ND	*V. diazotrophicus*	40	*V. parahaemolyticus*	100
				S. algae	40		
				A. hydrophila	20		
	Station 6	*V. alginolyticus*	100	*V. alginolyticus*	60	*V. alginolyticus*	20
				V. diazotrophicus	40	*V. diazotrophicus*	20
						V. fluvialis	10
Bacillus spp.	Station 1	*B. cereus*	60	*B. cereus*	60	*B. cereus*	60
		B. subtilis	40	*B. subtilis*	20	*B. subtilis*	20
				B. licheniforms	20	*B. licheniforms*	20
	Station 2	*B. cereus*	100	*B. cereus*	40	*B. cereus*	60
				B. mycoides	20	*B. subtilis*	40
				B. subtilis	40		
	Station 3	*B. cereus*	60	*B. cereus*	80	*B. cereus*	20
		B. pumilus	20	*B. subtilis*	20	*B. subtilis*	40
		B. subtilis	20			*B. pumilus*	20
						B. mycoides	20
	Station 4	*B. cereus*	60	*B. cereus*	60	*B. cereus*	60
		B. mycoides	40	*B. subtilis*	40	*B. subtilis*	40
	Station 5	*B. cereus*	60	*B. cereus*	80	*B. cereus*	20
		B. subtilis	20	*B. subtilis*	20	*B. subtilis*	20
		B. pumilus	20			*B. pumilus*	40
						B. mycoides	20
	Station 6	*B. cereus*	60	*B. cereus*	20	*B. cereus*	89
		B. pumilus	40	*B. subtilis*	80	*B. pumilus*	20
Clostridium spp.	Station 1	*C. perfingens*	100	*C. perfingens*	100	ND	ND
	Station 2	ND	ND	ND	ND	ND	ND
	Station 3	ND	ND	ND	ND	*C. perfingens*	100
	Station 4	*C. cadaveris*	20	*C. cadaveris*	20	*C. benerinckii*	40
		C. botulinum	40	*C. benerinckii*	20	*C. perfingens*	60
		C. perfingens	40	*C. perfingens*	60		
	Station 5	*C. perfingens*	100	*C. perfingens*	100	*C. perfingens*	50
						C. bifermentans	50
	Station 6	ND	ND	ND	ND	ND	ND

Note: ND = not detected

Bivalve mollusk (*P. bengalensis*)

In addition to the abundance of microbes in riverine sediment, we also investigated bacterial species in bivalve mollusks collected from the studied area. Quantitative abundance and variations of different groups of bacteria in bivalve mollusks (*P. bengalensis*) samples at the Khwai canal are presented in **Table 3**. The 6 samples of *P. bengalensis* in the pre-monsoon period have a higher bacteria contamination than the monsoon period because of the flooding during previous year. This incident can elute organic substance from other areas and is related to bacteria or pathogen spreading. In addition, it is a period when there is a low level of water passing through the canal. Bacteria and other organic nutrients can cause bacteria contamination in the mollusks that live in the surface area of riverine sediment as well. The 6 samples of *P. bengalensis* in the pre-monsoon period have higher total coliform contamination than the monsoon 2 period, with an average amount of 8.8×10^4 and 6×10^3 CFU/mL, respectively. Moreover, the presence of *B. cereus* contamination was much higher than in monsoon 2 period, with an average amount of 6.7×10^4 and 40 CFU/mL, respectively. Besides, *Clostridium* spp. contamination in the sample from monsoon 2018 season had 15 CFU/mL. After inspection, bacteria contamination in *P. bengalensis* was not more than that in the benchmark. Regarding microbiological requirements, bivalve mollusks fresh body consumption are under the standard value in fresh seafood [37]. Additionally, other species of 5 groups bacteria have been

found in *P. bengalensis*, including *Aeromonas allosaccharophila*, *S. algae, Enterococcus* spp. and *Rahnella aquatilis*, which is a rare gram negative rod-shape pathogenic bacteria in humans (**Table 3**). In summary, the current standard of bacteriological examination does not cover other bacterial groups that affect consumer's health. Also, due to the contamination of several pathogenic bacterial found in *P. bengalensis*, consumers should avoid consuming raw *P. bengalensis* such as raw oysters or Thai style spicy salad.

Comparing the number of bacteria between *P. bengalensis* and sediment (**Table 3**) found that there is no coherence in the *E. coli*, *Salmonella* spp. and *Vibrio* spp. Remarkably, all identified bacteria are found in estuary mangrove sediment but not in *P. bengalensis*. There is a biological mechanism that prevents pathogenic bacteria contamination in mollusks, even though mollusks have the behavior of drilling a hole in mangrove sediment. However, there was consistency in the *Bacillus* spp. and *Clostridium* spp. *Bacillus* cereus was found in sediments and *P. bengalensis* in both seasons. *B. cereus* is a contagious food that is commonly found in food. In addition, *Clostridium* spp. was found in *P. bengalensis* only in the second monsoon period, which shows high contamination found in *P. bengalensis* during second monsoon period season.

The results of this study show that preventative measures are needed to ensure the safety of bivalve mollusks consumption. Therefore, water quality should be monitored at the bivalve mollusks culture area and bacteria in the mollusks after harvesting or cooking and before consuming bivalve mollusks that are harvested, especially during the monsoon season to ensure safety for the consumption of bivalve mussels in Thepha District, Songkhla Province.

Table 3 Bacterial identification in bivalve mollusk (P. bengalensis) and sediments at station 4 Khwai canal, Thepha District.

Type of bacteria	Seasons											
	Pre-monsoon (April)						Monsoon (July)					
	sediments			bivalve mollusk			sediments			bivalve mollusk		
	number	species	%*	number	species	%*	number	species	%*	number	species	%*
E. coli	1.4×10^3	*E. coli*	ND	ND	ND	ND	5.0×10^2	*E. coli*	ND	ND	ND	ND
Total coliforms	1.2×10^4	Total coliforms	ND	8.8×10^4	Total coliforms	ND	1.7×10^5	Total coliforms	ND	4.1×10^4	Total coliforms	ND
Salmonella spp.	ND	ND	60 40	ND	ND	ND	ND	*K. pneumoniae* *E. coli*	60 40	ND	ND	ND
Vibrio spp.	> 1,100	*V. parahaemolyticus* *V. alginolyticus*	60 40	ND	*A. allosaccharophila* *S. algae*	40 60	> 1,100	*V. alginolyticus* *V. parahaemolyticus*	20 80	ND	*Enterococcus* spp. *A. allosaccharophila*	20 80
Bacillus spp.	1.9×10^6	*B. cereus* *B. subtilis*	60 40	6.7×10^4	*B. cereus* *R. aquatilis* *Enterococcus faecalis*	40 40 20	1.4×10^6	*B. cereus* *B. subtilis*	60 40	40	*B. cereus* *E. faecalis*	80 20
Clostridium spp.	2.4×10^2	*C. perfingens* *C. cadaveris* *C. benerinckii*	60 20 20	ND	ND	ND	9.5×10^2	*C. benerinckii* *C. perfingens*	40 60	15	*C. bifermentans* *Clostridium* spp.	60 40

Note: All the values are taken as an average of 3 readings, ND = not detected, * = occurrence (%)
E. coli (CFU/g), TC-Total Coliform (CFU/g), *Salmonella* spp. (CFU/g), *Vibrio* spp. (MPN/g), *Bacillus* spp. (CFU/g), *Clostridium* spp. (CFU/g)

Environmental related parameter observation

The temperature range varied from 25.9 to 34.3 °C, with the maximum point during the pre-monsoon season. The DO varied from 4.4 to 8.0 mg/L, while the salinity varied from 0.0 to 31.4 ppt. and was higher during the pre-monsoon when compared to the salinity in the monsoon 2017 and monsoon 2018 periods, except for at station 5, which had the highest salinity level during the monsoon 2. This may reflect the organic carbon and organic matter and other factors that accumulate in this area. The salinity was lowest during the first monsoon period because of the dilution affect from rainfall. The pH varied from 5.3 to 6.7 and was highest at station 1.

The nitrogen, phosphorous, organic carbon and organic matter concentrations in the sediments had a maximum value of 0.065, 0.11, 3.858 and 7.36 %, respectively, and were highest in the pre-monsoon period at station 4. Station 4 is located very close to the shrimp estuary in a local fishing-village, which could account for the elevated nutrient levels and the higher number of bacteria in every season, as well as for the higher diversity of bacterial species found at this site.

Statistical analysis of bacterial species correlated with environmental parameters

The correlation among the bacterial population and physical and chemical factors were presented in **Table 4** and **Figure 3**. The correlation matrix exhibits a very strong positive relation of temperature with *Vibrio* spp. ($p < 0.05$; $r^2 = 0.580$) and *Bacillus* spp. ($p < 0.01$; $r^2 = 0.622$), total coliform and *Clostridium* spp. are also positive relations. This situation was linked to the occasion of exponential bacterial population rate and the activities in the summer. The correlation matrix showed a very good positive relationship of salinity concentration with the bacteria counts (*E. coli*, *Vibrio* spp. and *Clostridium* spp.) except *Salmonella* spp. and there was a strong positive correlation with Total coliforms ($p < 0.05$; $r^2 = 0.483$). pH was a strong positive correlate with *Vibrio* spp. ($p < 0.05$; $r^2 = 0.583$) and other bacterial population. A negative correlation was observed for pH with *Salmonella* spp. In addition, *Bacillus* spp. had a strong positive relation with *Vibrio* spp. ($p < 0.01$; $r^2 = 0.627$). and *Clostridium* spp. ($p < 0.01$; $r^2 = 0.673$).

The high variability number of bacterial indicators in each season from underwater soil sediments of surface water in Thepha District may be due to the variation of environmental conditions such as temperature, dissolved oxygen, salinity, pH and nutrients.

Table 4 Physical-chemical factors and nutrients in different stations of Thepha District.

Parameter	Monsoon (December)						Pre-monsoon (April)						Monsoon (July)					
	S1	S2	S3	S4	S5	S6	S1	S2	S3	S4	S5	S6	S1	S2	S3	S4	S5	S6
Temperature	25.9	27.4	25.9	27.6	25.8	28.0	33.6	32.6	31.3	34.3	30.9	32.1	29.9	30.0	31.1	30.9	30.8	30.1
DO	6.4	4.7	6.6	6.3	4.7	4.9	5.9	5.5	4.5	6.0	4.4	5.0	4.6	4.7	8.0	3.9	5.5	3.9
Salinity	0.012	0	0.018	0.019	0.101	0.024	5.340	4.940	28.800	24.600	28.200	20.900	3.650	0.750	5.200	15.600	31.400	2.800
pH	6.1	6.1	5.7	5.3	5.8	5.9	6.7	5.4	5.8	6.0	6.2	6.8	6.8	6.6	6.4	6.2	6.5	6.4
N (%)	0.018	0.024	0	0.032	0.019	0.058	0.058	0.011	0.048	0.065	0.063	0.016	0.046	0.025	0.033	0.061	0.047	0.008
P (%)	0.08	0.08	0.09	0.07	0.08	0.04	0.06	0.06	0.07	0.06	0.09	0.06	0.06	0.10	0.09	0.11	0.06	0.06
OC (%)	1.224	0.677	0.141	2.806	0.933	3.443	0.715	0.731	2.742	3.858	0.881	3.113	0.338	0.388	1.129	1.432	1.756	1.129
OM (%)	5.33	2.09	2.21	7.36	3.67	2.72	4.53	3.19	6.43	3.84	5.46	3.06	4.35	2.64	3.72	5.56	5.25	2.75

Note: Temp-Temperature (˚C), DO-Dissolved Oxygen (mg/L), Salinity (ppt.), N-Nitrogen (%), P-Phosphorous (%), OC-Organic Carbon (%), OM-Organic Matter

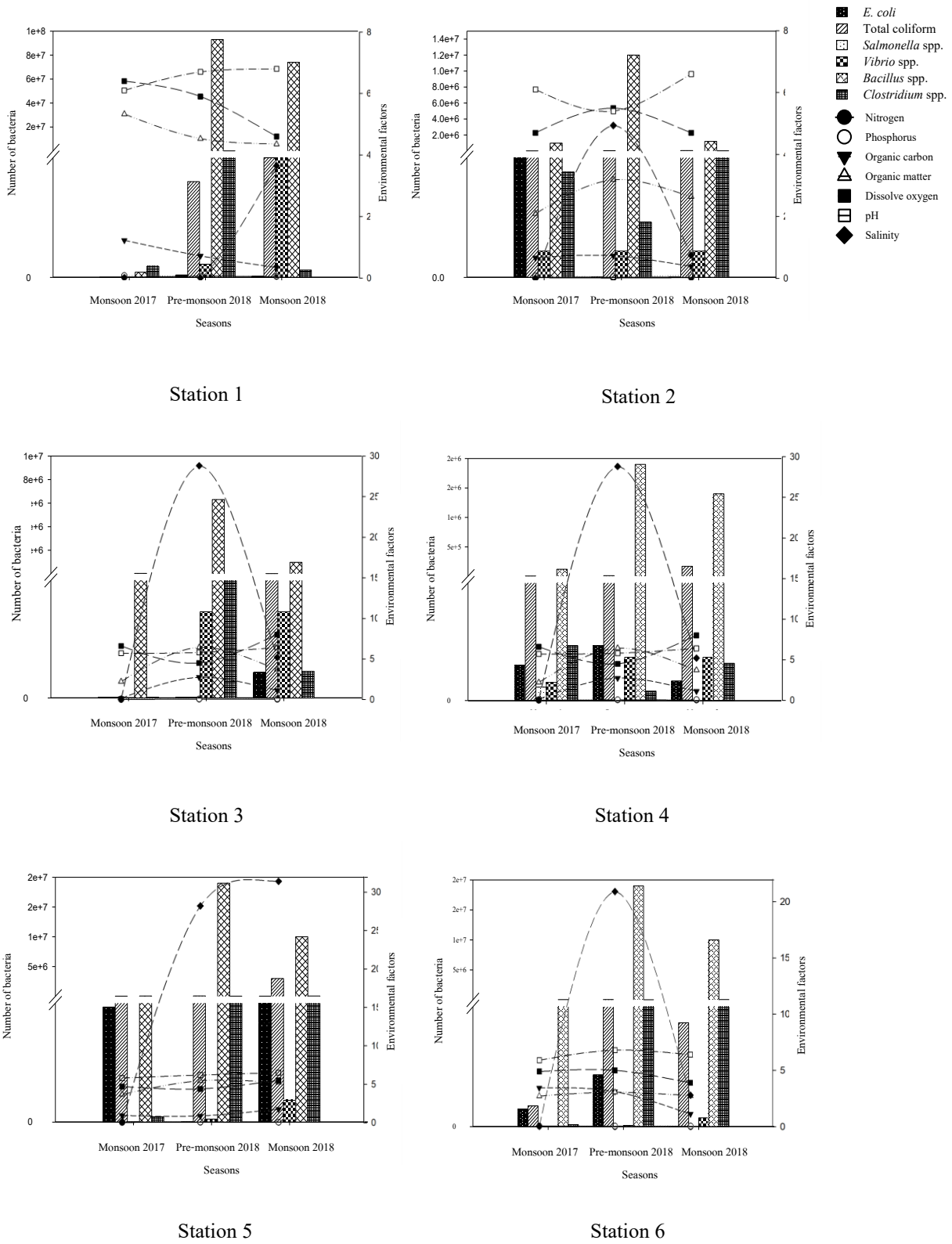

Figure 3 Relationship between number of bacteria and environmental parameters at different seasons.

Conclusions

The bacterial groups as potential bioindicators showed seasonal and local variation in population size in the Thepha district (study site), with a higher number of bacteria found in the area nearby the estuary and human community. Indicator bacteria, such as *E. coli*, total coliform, *Salmonella* spp. and *Vibrio* spp. showed a clear seasonal variation. Besides, total coliform, *Bacillus* spp. and *Clostridium* spp. found contamination in *P. bengalensis* that is a local bivalve mollusk in this area study. Bacterial population size and distribution form a baseline data of the Thepha area that can act as a guideline to assess and monitor the surface water environmental and health impacts. This data may be useful for the environmental management of the surface water area to prevent water pollution and water borne diseases, especially with any disturbance of water courses and sediment from future development.

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