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Human source identification by using a human-associated *Escherichia coli* genetic marker in the Mae Klong River, Thailand

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ABSTRACT

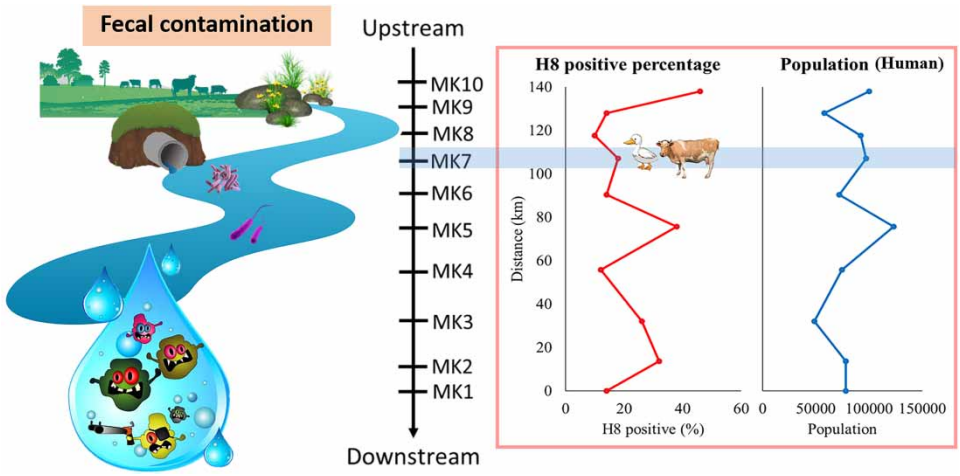
Contamination caused by microbial fecal pollution in water bodies is a serious problem in many countries, especially in low- and middle-income countries. Therefore, fecal source tracking is an important method used to understand the source of fecal contamination and to decrease the hazard of waterborne diseases that occurs in the environment. In this study, a human-associated genetic marker for *Escherichia coli* (H8) was used to investigate the source of fecal contamination in the Mae Klong River, Thailand. Real-time PCR was performed with this marker for 500 *E. coli* isolates collected from 10 sampling sites along the river, including MK10 (upstream) to MK1 (downstream). The results showed that the proportions of H8-positive isolates were 46, 14, 10, 18, 14, 38, 12, 26, 32, and 14% at MK10–MK1, respectively. All positive proportions were significantly different between the locations ($p < 0.001$). The higher occurrence of *E. coli* with H8 marker detection indicated that domestic wastewater was largely discharged without proper treatment, which is attributable to the high population and the absence of proper sewage treatment in those areas.

Key words: *E. coli*, fecal source tracking, H8 marker, human genetic marker, Mae Klong River, Thailand

HIGHLIGHTS

- The H8 marker was applied to investigate the human source of fecal pollution in the Mae Klong River, Thailand.
- High domestic wastewater discharge owing to a high population resulted in higher concentrations of *E. coli* with the H8 marker detection.
- In addition to GIS data, fecal source-tracking methods and other information were needed to identify fecal pollution sources.

GRAPHICAL ABSTRACT



INTRODUCTION

Fecal contamination of receiving water from point and non-point sources causes waterborne diseases. This is a major concern in many countries, especially in low- and middle-income countries (Pandey *et al.* 2014). A high level of fecal bacteria in environmental water is worrisome, and identifying the sources of bacteria is crucial for mitigating fecal pollution and preventing waterborne diseases (Gomi *et al.* 2014). Pathogens are transmitted to humans via poorly managed water and sanitation systems. Moreover, waterborne bacteria can be associated with diarrhea, which is caused by the fecal–oral transmission of pathogens through direct human contact with fecal matter or indirect ingestion through food, fingers, and environmental water contaminated with feces (Penakalapati *et al.* 2017).

Fecal indicator bacteria have been selected to monitor fecal pollution in environmental water because of their high levels in sewage and feces and because they are easy to measure (Griffith *et al.* 2009). In addition, *Escherichia coli* has been investigated with respect to water quality and has been used as an indicator organism of fecal pollution in freshwater for decades (Anderson *et al.* 2005); however, the limited information does not provide original data from wastewater owing to the nature of this species (found in cold and warm-blooded animals and human feces) (McLellan 2004; Field & Samadpour 2007). Fecal source tracking has been important in understanding the source of fecal contamination for better water quality management and to decrease the hazard of waterborne diseases in the environment. Along with other microbial source-tracking markers, a human-associated genetic marker for *E. coli* H8 has recently been developed and used in Japan, Australia, and Bangladesh (Gomi *et al.* 2014; Warish *et al.* 2015; Harada *et al.* 2018). The H8 marker has significantly higher sensitivity and specificity (>90%), which may consequently lead to minimum false-positive and false-negative results. It has been effectively used for evaluating environmental water samples (Warish *et al.* 2015; Senkbeil *et al.* 2019). The performance and effectiveness of the marker was also tested with the fecal and wastewater samples in Thailand (Nopprapun *et al.* 2020). However, the marker has not been applied widely in other areas especially in surface water where other factors could be different.

The Mae Klong River is situated in western Thailand. The lower reaches of the river receive water from irrigation canal systems, industries, and domestic wastewater. Water from the river is intensively used to produce tap water by the people in the western part (Khalil *et al.* 2018), and the river has now been polluted by fecal matter of which the source has been unknown. Therefore, this study aimed to apply the H8 marker to the Mae Klong River and understand the sources of fecal contamination in the river by using it in association with land use data in the watershed.

MATERIALS AND METHODS

Sampling sites and sample collection

Water samples were collected from the Mae Klong River during dry weather flow as fecal contamination in each location might not emanate from runoff but could be from the direct discharge of sewage. There were 10 sampling sites throughout the Mae Klong River – MK1 (downstream) to MK10 (upstream). Samples were taken on two separate days (March 13 and

18) based on the similar weather conditions and tide level. The samples on the first day were taken from MK1 to MK5 (Samut Songkhram to Ratchaburi province), whereas the samples on the second day were taken from MK6 to MK10 (Ratchaburi to Kanchanaburi province) to analyze fecal contamination sources. The grab sample of volume 1,000 mL was collected in a sterilized bottle from the middle portion of the river flow. The following water quality parameters were evaluated for all samples: electrical conductivity (EC), temperature, dissolved oxygen (DO), pH, turbidity, and total organic carbon (TOC). The samples were transported in a cooling box to the laboratory, kept in the dark at 4 °C, and processed within 12 h. Detailed information of each sampling site and its location is shown in Table 1 and Figure 1.

Escherichia coli isolation

A total of 100 mL of river water samples was filtered using a sterilized funnel and disposable filtration devices (0.45 μm, white MCE membrane, Merck, Germany) for each sample. Five replicates were considered for each of the sampling sites. The filter papers were put on HiCrome Chromogenic Coliform Agar (HiMedia, Mumbai, India) for the detection of *E. coli*. After incubation for 22 h at 37 °C, blue colonies indicating *E. coli* on the filters were counted to determine the *E. coli* concentration at each sampling site of the Mae Klong River. *E. coli* isolates were used for source tracking with a maximum of 10 isolates from each replicated plate and a total of 50 isolates from five replicated plates. Each colony was picked using sterilized toothpicks and transferred into a 96-well plate filled with 50 μL MilliQ water. The 96-well plates were stored at −20 °C for a maximum of 24 h before PCR analysis.

H8 marker analysis

SYBR green-based real-time PCR assays were performed on each *E. coli* isolate with the H8 marker, following Gomi *et al.* (2014) and Harada *et al.* (2018). For the real-time PCR assays, the total 15 μL PCR mixture was composed of 7.5 μL of QuantiFast SYBR green PCR (QIAGEN, Germany), 0.3 μL each of forward and reverse primer, 2 μL of *E. coli* samples, and 4.9 μL of MilliQ water. All PCR reactions were performed in a 96-well plate for real-time PCR with a CFX96 Touch real-time PCR Detection System (BIO RAD, Singapore). Positive (DNA from control strains) and negative (sterile water) controls were included for each PCR assay. The real-time PCR conditions were set at 95 °C × 5 min + (95 °C × 10 s + 60 °C × 30 s) × 40 cycles + melting curve analysis. The H8 genetic primer sets used for SYBR green-based PCR assays are shown in the primer and target DNA sequences in Table 2.

Data of land use and livestock numbers and population

Land use data were provided by the Land Department Development in Thailand (Land Department Development 2015). Moreover, the population of livestock in the research area was obtained from the Department of Livestock Development in Thailand (Department of Livestock Development 2017) and the Department of Provincial Administration in Thailand (Department of Provincial Administration 2018), respectively. These land use data were processed using ArcGIS 10.1.

Table 1 | Location details of each sampling site

River water sampling sites	Provinces	Districts	Location	
			Latitude	Longitude
MK10	Kanchanaburi	Mueang Kanchanaburi	14.00369	99.53522
MK9		Tha Muang	13.95302	99.62713
MK8		Tha Maka	13.94975	99.74892
MK7	Ratchaburi	Ban Pong	13.79929	99.86965
MK6		Photharam	13.66837	99.81740
MK5		Mueang Ratchaburi	13.55214	99.82865
MK4		Damnoen Saduak	13.49169	99.92582
MK3	Samut Songkhram	Amphawa	13.42013	99.95972
MK2		Mueang Samut Songkhram	13.38641	99.99947
MK1		Mueang Samut Songkhram	13.35498	100.00553

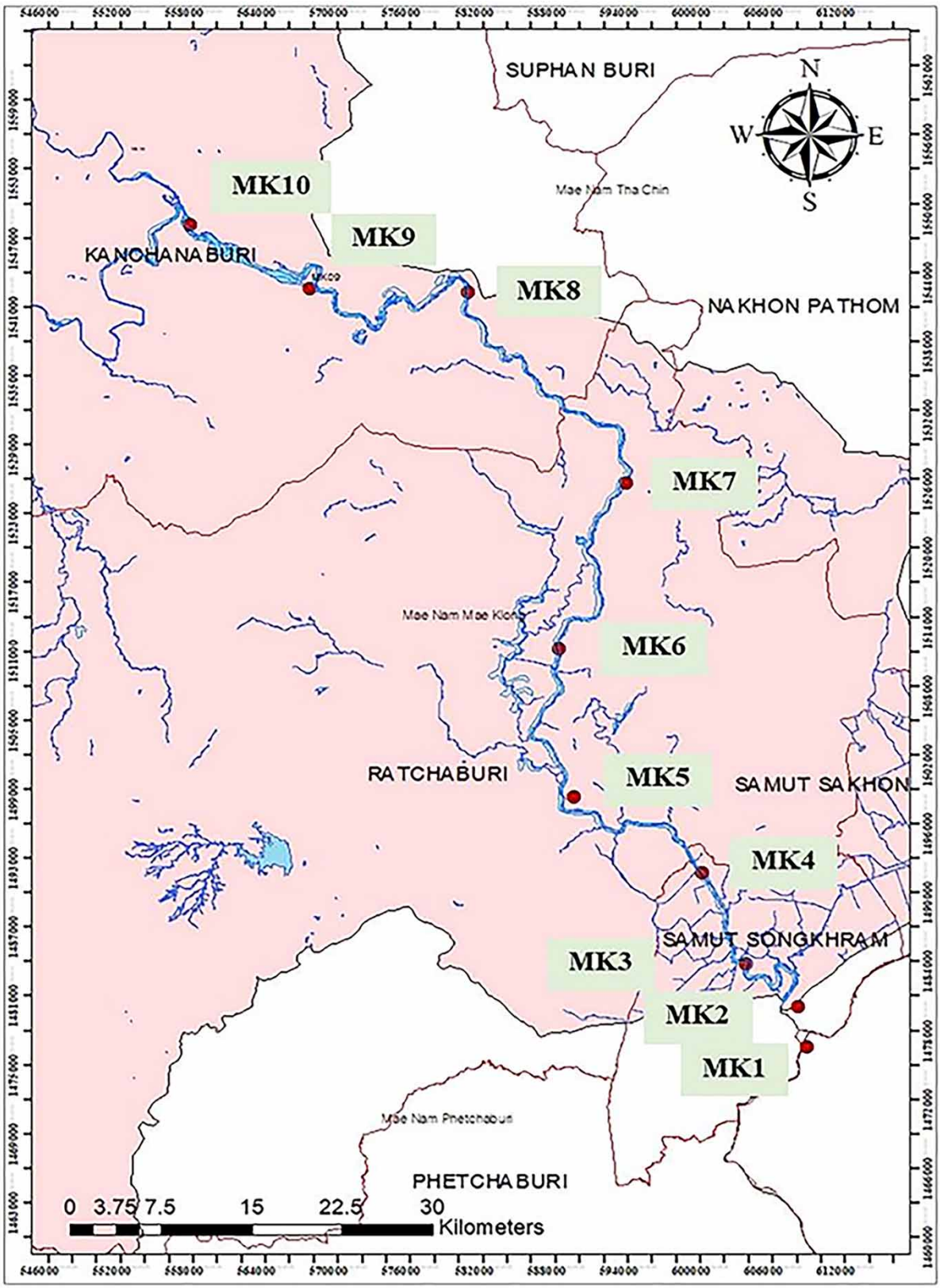


Figure 1 | Sampling sites along the Mae Klong River.

Table 2 | Primer sets for SYBR green-based PCR assays

Gene	Name	Primer sequence	Target DNA sequence	Product size	Source
H8	H8-F H8-R	ACAGTCAGCGAGATTCTTC GAACGTCAGCACCACCAA	ACAGTCAGCGAGATTCTTC TTGGTGGTGCTGACGTTC	177 bp	Gomi *et al.* (2014)

Statistical testing

Statistical analyses were conducted using RStudio (R 3.4.0), a programming language for statistical analysis and data science. Proportion tests were used to assess the positive proportion between the locations. The significant differences among the sampling sites were tested using the chi-square test. The *p*-value was set at a significance level of $p < 0.001$.

RESULTS AND DISCUSSION

Fecal pollution sources along the Mae Klong River

The results of the water quality parameter tests, particularly EC, TOC, and DO, revealed a gradual decline in water quality along the river flow. This finding highlights the pollution caused by organic and inorganic substances, which are discharged from the upstream point, and non-point sources accumulate in the downstream region as shown in Supplementary Material, Figure S1. Particularly, for *E. coli* concentration, the results show a slightly increased polluted trend from upstream to downstream, with the exception of MK10, MK7, and MK5, which may be largely affected by domestic and animal waste contamination, and MK1, which is affected by seawater mixing. From MK10 to MK1, the geometric mean of *E. coli* concentration was 349, 60, 55, 455, 95, 218, 92, 149, 375, and 134 CFU/100 mL, respectively, as shown in Table 3. Only in the case of MK9, MK8, MK6, and MK4, the *E. coli* concentrations were below the maximum allowable limit (126 CFU/100 mL) set by USEPA (2012). This finding indicates that the Mae Klong River water is polluted by human and animal waste at different places and is not suitable for recreational or irrigation purposes. Pathogenic microbes present in the water may spread through contact or consumption, thereby adversely affecting human health. The *E. coli* concentration indicated that fecal microorganisms comprised a high level of contamination, especially at MK10, MK7, MK5, and MK2, they were too numerous to count using the standard plate count method. The results of the PCR assay with the H8 marker showed that of 500 *E. coli* isolates, 112 (22%) were H8 positive for all sampling sites. Gomi *et al.* (2014) reported that 27% of 549 *E. coli* isolates were H8 positive for the river water in Japan; Warish *et al.* (2015) reported 26% of H8-positive isolates from 307 *E. coli* isolates from environmental water in Australia. The H8-positive proportion was in a similar range to that in these previous studies.

The transitions of H8-positive percentages along the river were 46, 14, 10, 18, 14, 38, 12, 26, 32, and 14% at MK10 (upstream) to MK1 (downstream), respectively, as shown in Figure 2. The positive proportions along the Mae Kong River were significantly different among the locations ($p < 0.001$). These results indicated that human-associated *E. coli* highly

Table 3 | Comparison of *Escherichia coli*, H8-positive percentage, and urban area percentages along the Mae Klong River

Sampling sites	*E. coli* (CFU/100 mL)	H8-positive (%)	Urban area (%)
MK10	350 (TNTC)	46	22
MK9	61	14	31
MK8	55	10	34
MK7	455 (TNTC)	18	43
MK6	95	14	33
MK5	219 (TNTC)	38	32
MK4	93	12	21
MK3	149	26	28
MK2	378 (TNTC)	32	29
MK1	135	14	18

Note: The average *E. coli* concentration was determined from five replicates. TNTC indicates 'too numerous to count', when the number of *E. coli* colonies exceeded 200 per plate.

contributed to the *E. coli* concentration at MK10, MK5, and MK2, which are Mueang districts in Kanchanaburi province, Ratchaburi, and Samut Songkhram province, respectively. This suggests that the fecal pollution at these three sites was caused by human sources more so than at other sites.

Land use along the Mae Klong River

Built-up land, including villages, cities, towns, commercial and industrial land, communication, and utility and institutional land, are located along the river (Land Department Development 2015). The percentages of built-up land surrounding the sampling sites were 22, 31, 34, 43, 33, 32, 21, 28, 29, and 18% at MK10–MK1, respectively. Table 3 shows the comparison of the average concentration of *E. coli*, H8-positive percentages, and urban area percentages along the Mae Klong River. The *E. coli* concentrations were averaged from five replicates for each sampling site. Supplementary Material, Table S1 can be referred for complete data.

The percentage of urban area results was not significantly different, and the river water seemed to be highly polluted through the water flow. In addition, we could not identify human pollution sources from upstream to downstream and these were not comparable with H8-positive percentages ($p = 0.687$) and *E. coli* ($p = 0.416$). Gentry-Shields *et al.* (2012) reported that land use changes have been significantly associated with higher concentrations of human sources of fecal indicator microorganisms, in addition to affecting the water quality. These results indicated that in addition to GIS data, fecal source-tracking methods and other information were needed to identify fecal pollution sources.

Association between fecal pollution sources along the Mae Klong River and population

E. coli comprises gram-negative bacteria that can be found in the intestines of a variety of warm-blooded animals and humans (Belanger *et al.* 2011). These bacteria can represent animal and human fecal pollution from water discharge, which can affect environmental water (Ishii & Sadowsky 2008). According to Figure 2, a higher *E. coli* concentration resulted in higher H8-positive percentages in the Mae Klong River, except at MK7. This relationship indicated that fecal pollution mainly increases owing to the human-originated fecal matter. In contrast, MK7 showed a different relationship between the *E. coli* concentration and percentage of the H8 marker. Whereas the *E. coli* concentration was high, the H8-positive proportion was low. This implies that there is a possible discharge source of fecal matter of non-human origin causing the increase in the *E. coli* concentration in this area. This possibility can be supported by the number of livestock in Kanchanaburi (2014), Ratchaburi (2015), and Samut Songkhram (2012) provinces, as shown in Figure 3. It was found that dairy cattle, buffaloes, and ducks were the most bred species at Ban Pong district (MK7) in Ratchaburi province. This large number of livestock and resultant animal feces could be a possible reason for the higher *E. coli* concentration at MK7.

Figure 4 shows the H8-positive percentages with the populations in each district along the Mae Klong River. The results showed that the population of the three provinces, Kanchanaburi, Ratchaburi, and Samut Songkhram, where MK10, MK5, and MK2 of the Mueang district are respectively located, were high. This population trend visually corresponds to the trend in the H8-positive proportion. The Spearman's rank correlation between H8-positive percentage and the total population at each sampling site was 0.63 (p-value = 0.06) for sampling sites in Kachanaburi and Ratchaburi provinces, where the effect of salt concentration was not observed. The higher positive proportion of a human-associated *E. coli* marker seems to be associated with anthropogenic activities in more populated areas. Though MK2 and MK1 are located in the same district, MK1 is in the coastal area with higher salt concentration, resulting in reduced *E. coli* growth (Omotoyinbo & Omotoyinbo

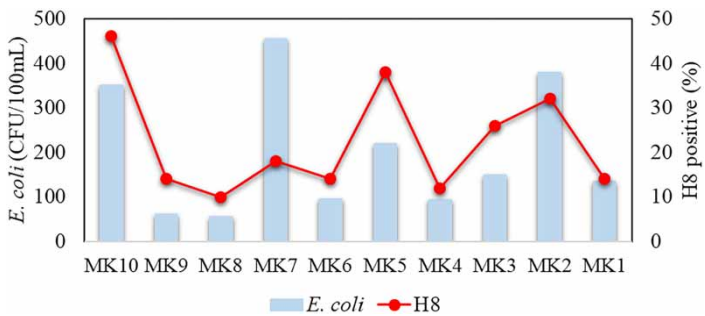


Figure 2 | *Escherichia coli* concentration and H8-positive percentage along the Mae Klong River.

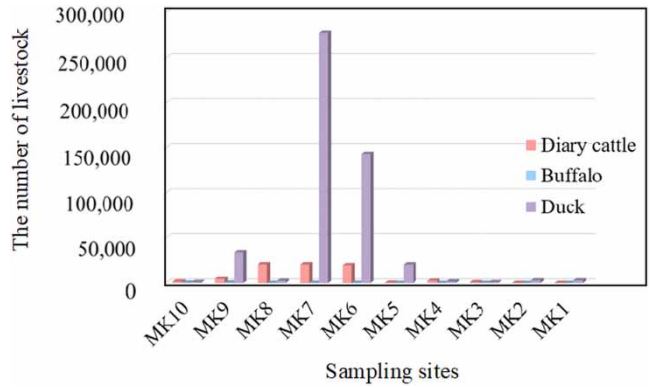


Figure 3 | The number of dairy cattle, buffaloes, and ducks in each district along the Mae Klong River. The data originated from the Kanchanaburi Provincial Livestock Office (2014), Ratchaburi Provincial Livestock Office (2015), and Samut Songkhram Provincial Livestock Office (2012).

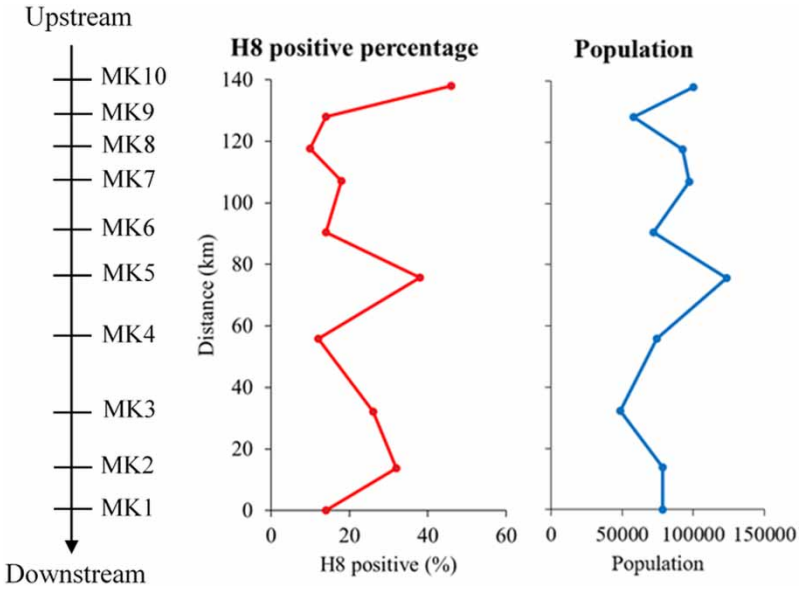


Figure 4 | H8-positive percentage with the population in each district along the Mae Klong River. The population data in each district along sampling sites originated from the Department of Provincial Administration (2018).

2017), as shown in Figure 2. Previous studies also reported the high concentration of fecal indicator bacteria associated with high populations along environmental water (DiDonato *et al.* 2009; Rowny & Stewart 2012). The results of this study were consistent with those of Tanaka (2018). The sampling site with a significant relationship between *E. coli* concentration and H8 positivity can be used to identify fecal contamination from humans, whereas at the sampling site with an insignificant relationship between *E. coli* concentration and H8 marker. The human-associated *E. coli* genetic marker data can be extremely beneficial for environmental organizations or agencies in classifying the source of contamination and focusing on the most efficient and appropriate method for contamination cleanup.

CONCLUSION

The H8 marker was applied to investigate human sources of fecal pollution in the Mae Klong River, Thailand. River water samples were collected during dry weather flow, indicating that fecal contamination might originate from the direct discharge of sewage. Our results indicated the significance of fecal source tracking. *E. coli* concentrations alone was insufficient to identify the source of contamination. The highest *E. coli* concentration at MK7 was found to have a different relationship

with the percent positivity of the H8 marker. According to the number of livestock, it was found that dairy cattle, buffalo, and duck comprised the greatest number of animals at MK7. Furthermore, population data in each district along sampling sites showed that MK10, MK5, and MK2 had high population numbers, which resulted in a similar trend with that of the H8-positive percentage in upstream, middle stream, and downstream areas. Therefore, this study showed that higher prevalences and concentrations of *E. coli* along the Mae Klong River with the H8 marker detection indicate that domestic wastewater was largely discharged owing to a high population and anthropogenic activity in those areas. Moreover, the H8 marker is recommended as a suitable method of fecal source tracking to identify human sources of fecal pollution and for proper water quality management.

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AUTHOR DISCLOSURE STATEMENTS

No competing financial interests exist.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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