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Library-independent source tracking of fecal contamination in selected stations and tributaries of Laguna Lake, Philippines

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ABSTRACT

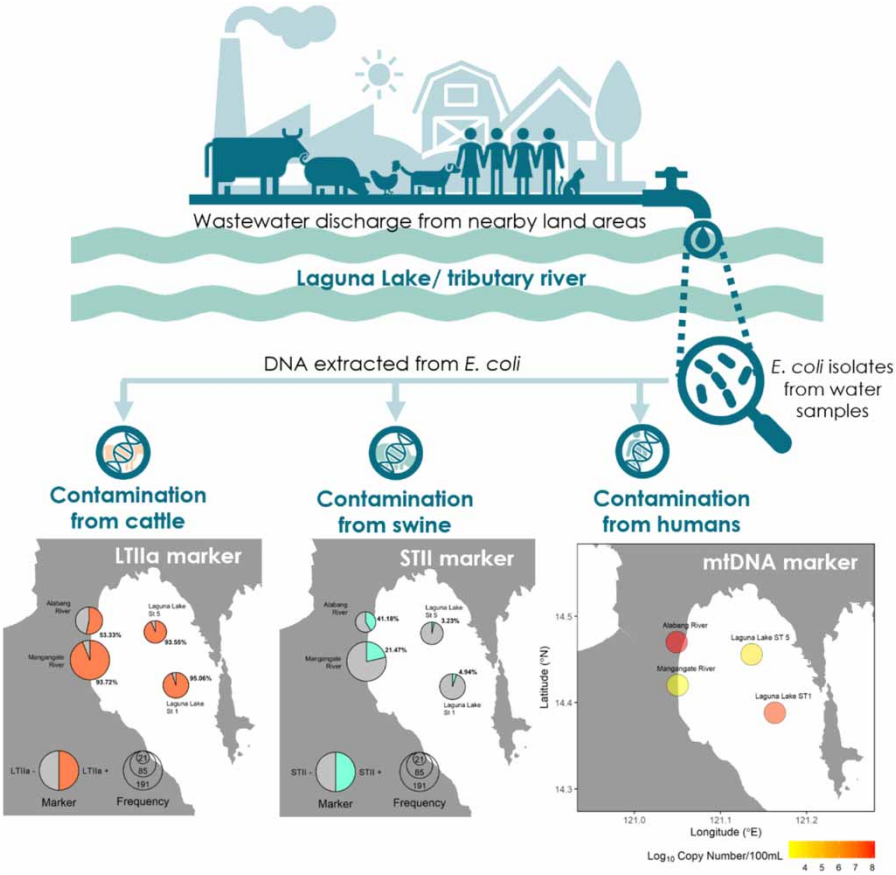
Laguna Lake is the largest inland freshwater body in the Philippines. Although it is classified to be usable for agricultural and recreational purposes by the country's Department of Environment and Natural Resources (DENR), studies looking at lake ecology revealed severe fecal contamination which contributes to the deterioration of water quality. Determining the sources of fecal contamination is necessary for lake protection and management. This study utilized a library-independent method of microbial source tracking (LIM-MST) to identify sources of fecal contamination in selected Laguna Lake stations and tributaries. Genetic markers of the host-associated *Escherichia coli*, heat-labile toxin (*LTIIA*) and heat-stable II (*STII*), were used to identify cattle and swine fecal contaminations, respectively. Meanwhile, human mitochondrial DNA (mtDNA) was used to identify human fecal contamination. Results identified the presence of agricultural and human fecal contamination in Laguna Lake Stations 1 and 5, Mangangate River, and Alabang River. The selected sites are known to be surrounded by residential and industrial complexes, and most of their discharges find their way into the lake. The identification of the specific sources of fecal contamination will guide management practices that aim to regulate the discharges in order to improve the water quality of Laguna Lake.

Key words: *Escherichia coli*, Laguna Lake, microbial source tracking, mitochondrial DNA, Philippines

HIGHLIGHTS

- The *Escherichia coli LTIIa* and *STII* genes, as well as human NADH mtDNA, are useful biomarkers for detecting fecal contamination in Laguna Lake.
- Fecal contamination from agricultural and human origins can be detected in some lake stations and rivers near Laguna Lake.
- As indicated by the high detection of *LTIIa* gene, fecal contamination in the sampling sites was mostly of cattle origin.

GRAPHICAL ABSTRACT



INTRODUCTION

Microbial source tracking (MST) emerged in the late 20th century with the aim of identifying the sources of fecal contamination in the environment, especially in water sources (Stoeckel & Harwood 2007). It was developed mainly to address the different health risks of microbial contamination in water sources. In view of this, Guan & Holley (2003) reviewed the movements of different fecal contaminants from water sources to fresh produce in relation to disease outbreaks.

Several MST studies investigated different fecal indicator bacteria (FIB) and their possible sources (Khatib *et al.* 2002; Stoeckel & Harwood 2007; Harwood *et al.* 2014; Schriewer *et al.* 2015). MST consists of two methods: library-dependent method (LDM) and library-independent method (LIM). The former uses culture-based techniques that focus more on the identification and correlation of FIB with their specific sources in the environment. *Escherichia coli* and *Enterococcus* sp. are the common FIB used in LDM (Stoeckel & Harwood 2007). Through recent advancements in molecular biology, the use of gene markers in tracking the source of contamination has become useful. As a result, LIM became more focused on the development of markers specific to the host sources, thus eliminating the use of culture-based techniques (Harwood *et al.* 2014).

Several studies emphasized the importance of determining the sources of microbial contamination. Schriewer *et al.* (2015) examined the presence of *Bacteriodales* genetic markers in India through quantitative polymerase chain reaction (qPCR) and found that there was prevalent human fecal contamination which could pose a major public health risk. Another study found that *LTIIa* and *STII* toxin genes of *E. coli* can be associated with fecal contamination of cows and water buffalo, and pigs, respectively (Khatib *et al.* 2002, 2003). In addition, Krolik *et al.* (2014) investigated different public drinking well waters in Ontario, Canada. The use of human and bovine markers revealed a higher degree of human fecal contamination compared with bovine contamination. Also, it is now known that human mitochondrial DNA (mtDNA) is being used as one of the most promising biomarkers of MST to identify human fecal contamination. Studies on human mtDNA were based on the idea that humans shed cellular materials, including mitochondria, in their feces (He *et al.* 2016).

In the Philippines, there is generally a lack of scientific information on the use of MST in identifying sources of microbial contamination. One of the preliminary studies involving MST in the country was by Labrador *et al.* (2020), in which the best marker combination for the LDM of MST in Laguna Lake was identified. So far, most of the studies focused on the identification and quantification of FIB present in an environment. Garcia *et al.* (2015) detected the fecal contamination in irrigation water and checked the microbial quality of vegetables in selected urban farms in Metro Manila. Moe *et al.* (1991) reported bacterial indicators of drinking water quality and identified risks for waterborne diarrheal diseases in the Philippines.

At present, one of the major environmental concerns in the Philippines is the regulation and rehabilitation of the different bodies of water, one of which is Laguna Lake. The lake is situated east of Metro Manila between the provinces of Laguna (south) and Rizal (north). It plays an important role as a site of aquaculture for fish production, a source of hydroelectric power and water for drinking and irrigation, and a place for recreation (Rivera *et al.* 2002). However, this important water resource has been greatly affected by developmental pressures like population growth, rapid industrialization, and resource allocation. Considering the economic and cultural importance of the lake, the Laguna Lake Development Authority (LLDA), the sole government agency that has jurisdiction over its management, is regularly monitoring the lake's water quality. However, the agency's water quality monitoring is only focused on the enumeration of total fecal coliforms for microbial quality measurement of biological oxygen demand, dissolved oxygen, and other physico-chemical parameters. Given the need to maintain the water quality of the lake, it is important to identify the specific sources of fecal contamination. There are different sets of gene markers that can be used for this purpose. For example, the *LTIIa* gene and *STII* genes of *E. coli* are used to confirm cow and pig fecal contamination (Khatib *et al.* 2002, 2003), respectively, while the human mtDNA is used to confirm human fecal contamination (Caldwell & Levine 2009).

The general objective of this study was to identify the different sources of fecal contamination in selected stations of Laguna Lake. Specifically, it aimed to identify the sources of fecal contamination using the LIM (1) through the detection of host-associated (cattle and swine) markers in *E. coli* isolates and (2) through direct screening of water samples for human mitochondrial genes to determine possible sources of human fecal contamination. LIM screening was done to speed up the process of source tracking and to avoid sampling bias in building up a host-origin library. The results of this study may help the concerned agencies to properly regulate wastewater discharges to Laguna Lake.

MATERIALS AND METHODS

Collection of water samples

Water samples were collected from two sites within Laguna Lake (Station 1 and Station 5) and two rivers that feed into the lake, Mangangate River and Alabang River (Figure 1).

Water samples (500 mL) were collected in duplicates using sterile wide-mouth Nalgene® bottles. Sampling was done once during the months of May, July, September, and November 2018. There was no sample collected from Alabang River in November 2018 due to its closure to give way to construction-related activities. The water samples were transported in coolers for immediate processing in the laboratory.

Isolation and identification of *E. coli*

For the detection and enumeration of *E. coli* in the samples, 10-fold serial dilutions up to 10^{-5} were performed on all water samples using 0.9% saline solution as the diluent. These were filtered through the GN-6 Metricel membrane filter (47 mm diameter, 0.45 μM pore size; Pall Corp., USA), and the last three dilutions (10^{-3} to 10^{-5}) were plated on modified membrane thermotolerant *E. coli* (mTEC) agar. The plates were incubated at 35–37 °C for 2 h and then at 44.5 °C for 18–24 h. The growth of purple to deep violet colonies after incubation was considered presumptive *E. coli*. These colonies were streaked on eosin methylene blue (EMB) agar and then incubated again overnight at 35–37 °C. Colonies exhibiting green metallic sheen were identified as *E. coli*.

The molecular identification of *E. coli* isolates was confirmed through PCR. DNA extraction of the positive *E. coli* cultures from the EMB agar was done using a boil-lysis method (De Medici *et al.* 2003). The primer pairs were adapted from Takahashi *et al.* (2009). These primers target the *uidA* gene of the organism and can detect even the pathogenic *E. coli* 0157:H7. The 20 μL of PCR mixture consisted of Promega GoTaq® Green PCR Mastermix, 0.10 μM of forward and reverse primers, nuclease-free water, and 2 μL of DNA template, where each set of samples was assayed in duplicate. Negative, positive, and non-template controls were included in each run. PCR thermocycling conditions were adapted from the study of Vital *et al.* (2014), with an initial denaturation of 98 °C for 2 min, followed by 35 cycles at 98 °C for 30 s, 63 °C for 1 min, and 72 °C for 1 min and a final extension of 72 °C for 5 min.

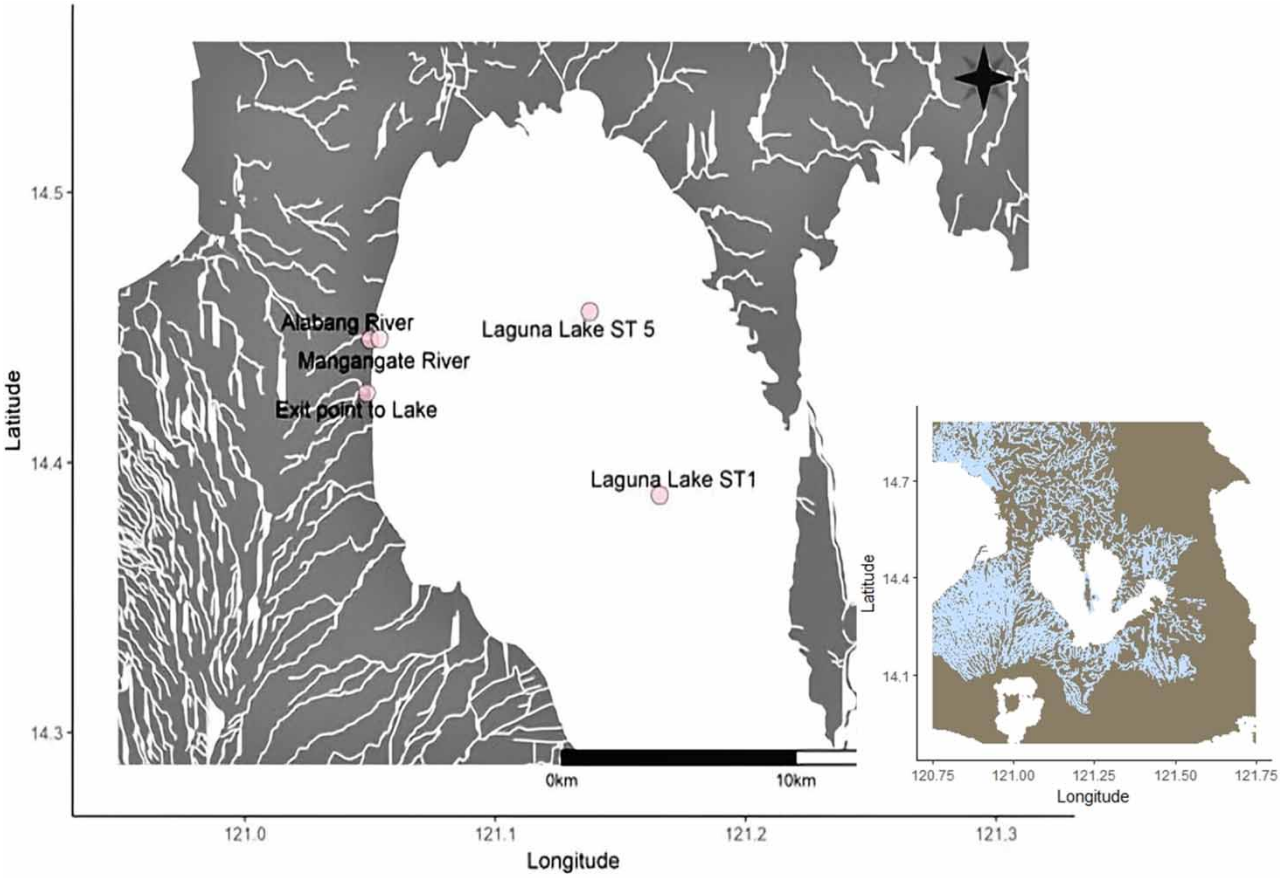


Figure 1 | Sampling sites in Laguna Lake and its tributaries.

MST using *E. coli* toxin genes and human *NADH* mitochondrial markers

All confirmed *E. coli* isolates were screened for the presence of the heat-labile toxin IIA (*LTIIa*) gene (Khatib *et al.* 2002) and the heat-stable II (*STII*) gene (Khatib *et al.* 2003) as biomarkers for cattle and swine fecal contaminations, respectively. The 20 μL of PCR mixture consisted of Promega GoTaq® Green PCR Mastermix, 0.10 μM of forward and reverse primers, nuclease-free water, and 5 μL of DNA template, where each set of samples was assayed in duplicates. Moreover, the PCR conditions for *LTIIa* and *STII* genes were as follows: 95 °C for 1 min, followed by 30 cycles at 95 °C for 30 s, 61 °C for 30 s (*LTIIa* gene) or 47 °C for 30 s (*STII* gene), 72 °C for 30 s, and a final extension of 6 min at 72 °C (Khatib *et al.* 2002, 2003). DNA extracted from the fecal material of cattle and swine served as positive controls in the PCR run.

The presence of *LTIIa*- and *STII*-positive *E. coli* was computed using the formula: total number of *LTIIa*- or *STII*-positive *E. coli* divided by the total number of *E. coli* isolates multiplied by 100.

For the detection of human fecal contamination, human mtDNA was used as a biomarker. Water samples, with a volume of 1 L each, were collected from sampling sites and were filtered using the GN-6 Metricel membrane filter (47 mm diameter, 0.45 μM pore size; Pall Corp., USA). The filter was then directly subjected to DNA extraction using the PureLink™ Microbiome DNA Purification Kit, with a yield volume of 50 μL DNA sample. Afterwards, qPCR analysis was performed with the reaction mixture consisting of 20 μL of PowerUp SYBR Green® PCR Mastermix, 0.10 μM of forward and reverse primers, nuclease-free water, and 2 μL of DNA template. Primer sequences and amplification protocols were adopted from Caldwell & Levine (2009). Human fecal genomic DNA served as positive control. Table 1 shows the various primers utilized in this study.

Amplification curves were observed in all samples, and DNA copy number was calculated to determine the amount of human fecal contamination present in each sampling site. DNA copy number was calculated by the machine using the absolute quantification assay of qPCR where the concentration of the unknown samples is tested against the standard curve of the known concentration of positive control (i.e., human fecal DNA). Comparing the threshold cycle values of the unknown samples to

Table 1 | Primer sequences used in this study

Gene	Primer sequences	Reference
Heat-labile toxin (*LTIIa*)	Forward: 5′-GGGTGTGCATTTCAGCGAC-3′ Reverse: 5′-TGGTATATTCCGGGTGGACG-3′	Khatib *et al.* (2002)
Heat-stable toxin (*STII*)	Forward: 5′-TGCCTATGCATCTACACAAT-3′ Reverse: 5′-TAGAGATGGTACTGCTGGAAG-3′	Khatib *et al.* (2003)
Mitochondrial NADH dehydrogenase	Forward: 5′-CAGCAGCCATTCAAGCAATGC-3′ Reverse: 5′-GGTGGAGACCTAATTGGGCTGATTAG-3′	Caldwell *et al.* (2007)
uidA	Forward: 5′-GCA AGG TGC ACG GGA ATA TT-3′ Reverse: 5′-CAG GTG ATC GGA CGC GT-3′	Takahashi *et al.* (2009)

this standard curve allows the quantification of the target DNA template. DNA copy number is calculated using the amount of DNA in nanogram multiplied by Avogadro's constant, divided by the length of DNA, a conversion factor of (1×10^9), and the average mass of 1 bp of dsDNA which is 650 g. (This is based on the premise that the average weight of a single base pair is 650 Da. Hence, an average mass of 1 bp dsDNA is 650 g.) The value of the DNA copy number may infer the extent of human fecal contamination in the sample. All DNA copy number values were then log-transformed to provide normality and homoscedasticity.

Ethics approval

The protocol for handling human samples was reviewed and approved by the University of the Philippines Manila Research Ethics Board (UPMREB-2018-356-01).

RESULTS

E. coli detected in Laguna Lake sampling stations

A total of 424 *E. coli* were isolated from water samples obtained from four sampling stations in Laguna Lake. The greatest number of isolates was recovered from Mangangate River ($n = 191$), followed by Alabang River ($n = 90$), Laguna Lake Station 1 ($n = 81$), and Laguna Lake Station 5 ($n = 62$).

Detection of *LTIIa* and *STII* genes in *E. coli* isolates

LTIIa and *STII* genes, which are markers for cattle and swine, respectively, were detected from *E. coli* isolated from different sampling sites (Table 2). As shown in Figure 2, there is 93.72% detection of *LTIIa* gene in isolates from Mangangate, 53.33% in Alabang, 95.06% in Lake Station 1, and 93.55% in Lake Station 5. On the other hand, lower detection of *STII* genes was observed. Among the *E. coli* isolates from Mangangate, there is only 2.09% with *STII*, while only 23.33% in Alabang isolates. For the lake stations, only 4.94% of samples from Station 1 have *STII*, while only 3.23% in Station 5. Few of the isolates also did not show the presence of either one of each gene (Alabang = 23.33%, Mangangate = 4.19%, and Station 5 = 3.22%).

Detection of host-specific human *NADH* mitochondrial genes in water samples

A total of 15 environmental DNA samples were obtained from each selected site. Among the sampling sites, Alabang and Laguna Lake Station 1 have the highest DNA copy number (Figure 3). The DNA copy number values indicate the presence of human *NADH* mitochondrial gene in the environmental sites.

DISCUSSION

Detection of *E. coli* in Laguna Lake sampling stations

Among all sampling stations, Mangangate River has the highest number of *E. coli* isolated. This information concurs with the data of LLDA on fecal coliform count of the lake stations and river tributaries (http://www.llda.gov.ph/). Alabang River has the second most number of *E. coli* species. This river is not part of the major tributaries of Laguna Lake; however, it merges downstream with Mangangate River, thus contributing to the source of contamination in the lake. Low levels of *E. coli* detected in lake stations also concur with the fecal coliform data which is less than 100 MPN/100 mL (http://www.llda.gov.ph/).

Table 2 | Frequency of samples that tested positive and negative for *LTIIa* and *STII*, respectively

Site	Sampling month	*LTIIa*		*STII*	
		+	−	+	−
Alabang River	May	28	2	2	28
	July	15	15	10	0
	September	5	25	9	1
	November[a]	ND	ND	ND	ND
Mangangate River	May	20	0	15	20
	July	20	0	20	25
	September	51	1	3	25
	November	88	11	3	88
Laguna Lake Station 1	May	23	0	1	23
	July	29	1	0	30
	September	12	3	2	11
	November	13	0	0	12
Laguna Lake Station 5	May	26	2	2	20
	July	22	1	0	20
	September	8	1	0	20
	November	2	0	0	2
424 *E. coli* isolates screened					

All samples were obtained in 2018.

[a]ND, no data; samples were not obtained during this time because of the construction activities resulting in road closures.

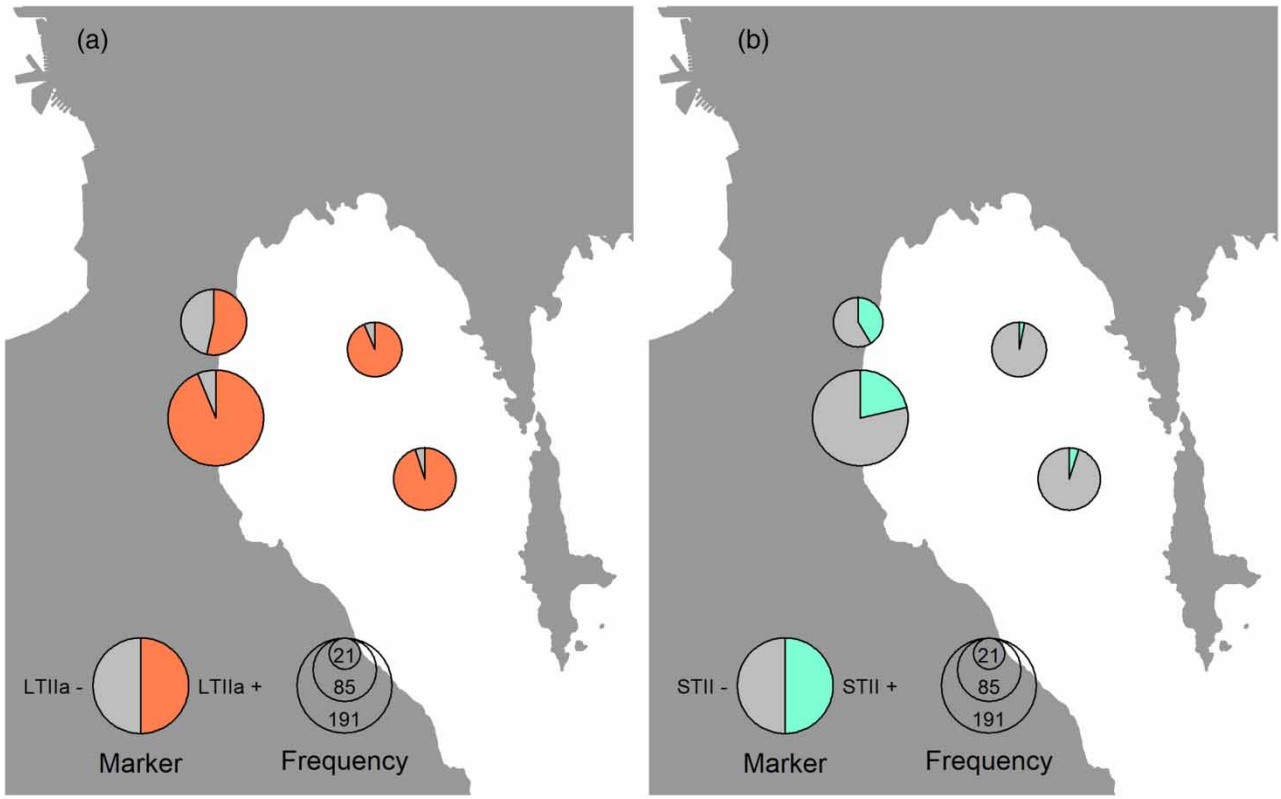


Figure 2 | Presence of *LTIIa* (a) and *STII* (b) genes among *E. coli* isolated from different sampling sites: the orange shade indicates *LTIIa*-positive and the green shade indicates *STII*-positive. The size of the circle is proportional to the frequency of samples that were collected at a particular site, the values of which are shown in the legend.

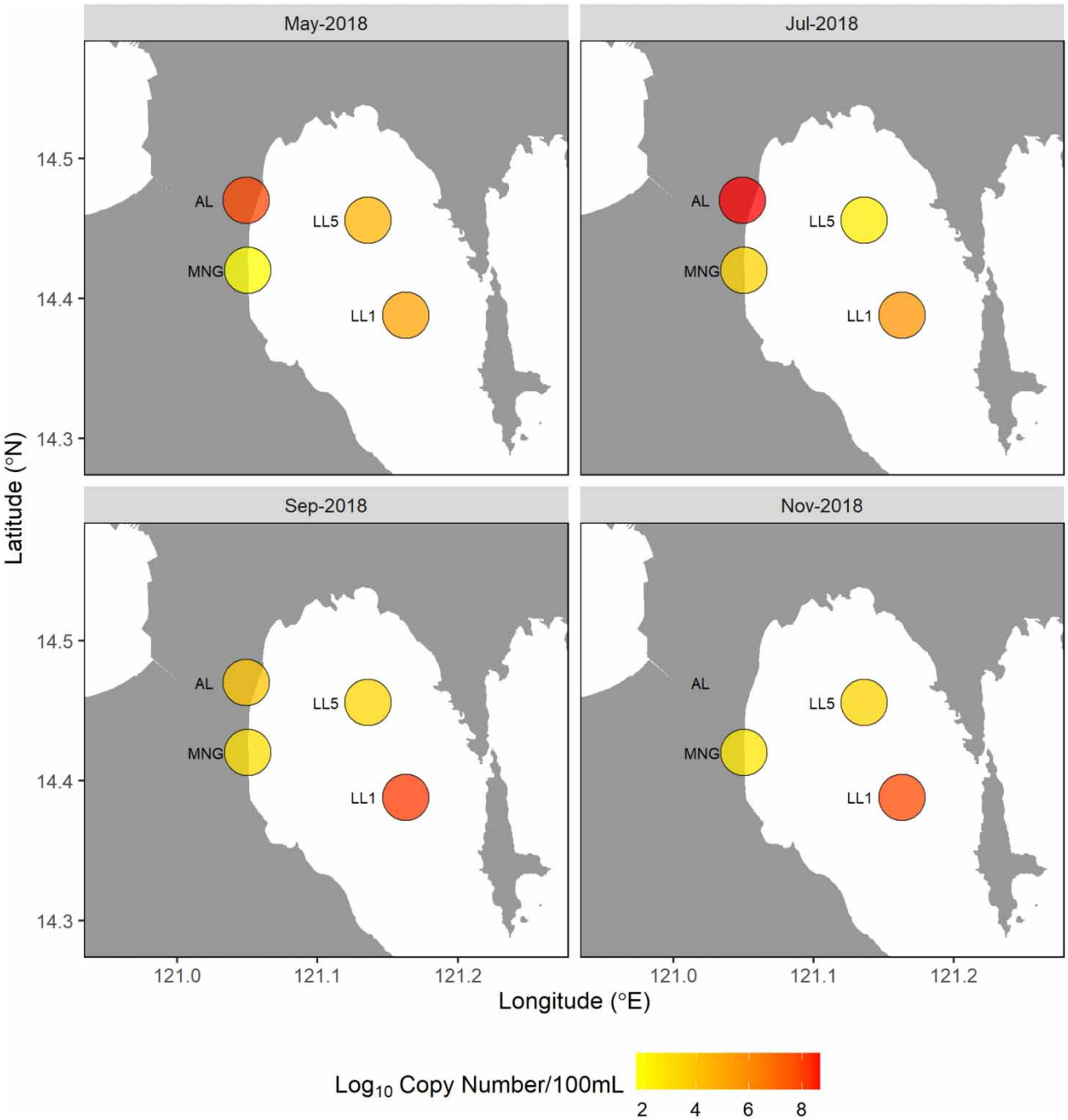


Figure 3 | Log_{10} values of mtDNA copies per 100 mL isolated from the different sites for each sampling period (LL1, Laguna Lake Station 1; LL5, Laguna Lake Station 5; MNG, Mangangate River; AL, Alabang). No data were obtained from Alabang in November 2018 due to road closure.

LTIIa and *STII* genes as biomarkers for fecal contamination from agricultural sources

The PCR-based assay demonstrated the utility of heat-labile toxin IIa (*LTIIa*) and heat-stable toxin II (*STII*) from enterotoxigenic *E. coli* as biomarkers for cattle and swine fecal contaminations, respectively. Based on the results, fecal contamination in the sampling sites was mostly of cattle origin as indicated by the higher detection of the *LTIIa* gene compared with the *STII* gene among all sampling stations. The detection of these genes indicates that contamination in the water samples may be coming from cows and pigs even though Mangangate and Alabang are located in highly urbanized areas. Since *E. coli* are natural inhabitants of the intestines of animals, it is possible that their presence is associated with agricultural and industrial

effluents such as those coming from livestock backyard farms, cattle and swine abattoirs, and meat manufacturing companies that find their way to the river systems.

The *E. coli LTIIa* and *STII* toxin genes are both useful biomarkers for MST since they are source-specific. They are sensitive and geographically widespread. Sequence analyses of *LTIIa* and *STII* showed that the two markers were 100% specific to cattle and pig, respectively (Harwood *et al.* 2014). This explains why there was no concurrent detection of genes in one *E. coli* isolate. Either *LTIIa* or *STII* gene is present in one isolate, confirming the utility of these gene markers for specifically indicating cattle or swine fecal contamination.

NADH mtDNA as a biomarker for human fecal contamination

One of mtDNA's major advantages among others is that it is geographically stable. The host mtDNA is not easily influenced by diet, compared with FIB, which are part of the host's gut microbiota (Graves *et al.* 2007). Several studies proved the effectiveness of mtDNA in MST. In the study of Caldwell *et al.* (2007), multiplex real-time PCR amplifying fecal mtDNA exhibited the identification of human mtDNA with no cross-reaction between human and non-human sources. Although mtDNA may not be as abundant in the environment as other microbial DNA markers like the *Bacteriodales* 16S rRNA gene marker, this marker is a good candidate for the identification of human fecal contamination in the environment. Another study showed the application of mtDNA in source tracking in shellfish harvesting waters wherein mtDNA was used to detect human fecal contamination from single fecal sources in experimental microcosms and shellfish samples (Baker-Austin *et al.* 2010).

In this study, human mtDNA was successfully detected from all target sampling sites through the qPCR assay. The highest DNA copy number was detected in Alabang River and Laguna Lake Station 1 (Figure 3). Overall, this finding indicates that there are sources of human fecal contamination in all sites. Both Alabang and Mangangate Rivers traverse directly along residential areas and industries which may directly discharge their wastes into the river. Moreover, the increased prevalence of human mtDNA in the lake stations can be due to various factors such as the presence of fishermen in the area and the other river tributaries that discharge their waste in the lake. The lake stations are being surrounded by various household infrastructures, farms, and small-scale agriculture and manufacturing industries. Unregulated waste discharged into these rivers may have eventually found its way into the lake, thus the detection of mtDNA in all sampling sites. This is alarming since the lake water is utilized for irrigation and cultivation of small backyard farms for vegetables and fishes as well as a source of potable water. Though there are considerable advantages in using mtDNA as a source tracking tool, it is also important to note that mtDNA could also come from other non-fecal sources such as from human skin and other tissues of the body. It was reported that healthy human blood samples containing mononuclear cells have around 1,000 mtDNA copies/cell (Gourlain *et al.* 2003) and normal endometrial cells have around 158–2625 mtDNA copies/cell (Wang *et al.* 2005). Hence, there is an ongoing debate on this marker and other fecal indicator organisms based on host relevance, viability, and source reliability.

Different studies were conducted to check for the ecological status of the lake. One study, that of Sia Su & Cervantes (2008), pointed out that the lake is currently in a disturbed state and this is supported by pieces of evidence, indicating that the pollution present in the lake affects the growth of agricultural animals. As a result, lower harvests in the open waters ensue because the food chain is adversely affected by pollution and perennially high water turbidity and sedimentation rate in the lake.

At present, Laguna Lake is continuously meeting the needs of numerous sectors of society particularly as a source of freshwater supply. Hence, a study that identifies the sources of contamination would help in addressing the problem of pollution in this specific body of water. With this, local government units, as well as the larger governing body, may include in their policies different ways to regulate various discharges in the lake and its tributaries.

CONCLUSION

The use of two library-independent MST platforms, one focusing on the microbial species as a biomarker (i.e., *LTIIa* gene and *STII* gene of *E. coli* species) and the other using human mtDNA biomarker *NADH* gene, revealed that fecal contamination in Laguna Lake comes from agricultural (cow and pig) and human sources. The biomarkers used are host-specific and do not require large amounts of water samples for detection, making them good markers for MST. The findings of this study can be used as a basis for drafting regulations in managing the land use of areas surrounding the lake and in designing water treatment strategies.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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