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RESEARCH ARTICLE

Environmental Microbiology

Faecal indicator bacteria and microbial source tracking as tools for testing water quality and sources of contamination of Rawan-Oya tributary of the Mahaweli River, in Sri Lanka

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Abstract: Faecal indicator bacteria (FIB) are used to assess water quality, indicating potential contamination from faeces, while microbial source tracking (MST) identifies the specific source of that contamination. These are essential tools for assessing the level and source of faecal contamination in water, and this study evaluated faecal contamination in the Rawan-Oya tributary of the Mahaweli River, a crucial drinking water source in Sri Lanka, using these tools. Water samples were collected from nine sites representing forested (n = 19), agricultural (n = 38), rural (n = 38), semi-urban (n = 38), and urban (n = 38) areas. These samples were analysed for FIBs including total coliforms (TC), faecal coliforms (FC), *Escherichia coli*, and faecal streptococci (FS) using membrane filtration method. DNA markers were used in order to identify human and animal faecal contaminant sources. Results showed an increase in FIB concentrations from forested to urban areas, indicating increased numbers in urban areas compared to those in forested areas. TC concentration in water ranged from 2.7 × 10^2 to 1.6 × 10^5 CFU/100 mL. FC counts increased from 16 to 3.2×10^4 CFU/100 mL, and FS counts fluctuated between 5.4×10^2 and 6.2×10^3 CFU/100 mL. All samples tested positive for the universal FIB DNA marker. Microbial source tracking analysis using human- and cattle-specific *Bacteroides* markers indicated the highest levels of human faecal contamination in urban sites, while significant cattle faeces contamination was observed in both agricultural and urban areas. The microbial quality of water did not meet the drinking water standards (FC and TC, 0 CFU/100 mL) in any of the sites tested. However, the forested area met the recreational water standards (FC ≤ 235 CFU/100 mL). This study identifies the potential of using FIB and MST as a means of confirming the specific sources and locations of faecal contamination in water bodies, which is crucial for developing targeted water management strategies and for alerting local communities and authorities about the status of drinking water quality and factors that contribute towards the deterioration of the quality of water bodies.

Keywords: Faecal indicator bacteria, microbial source tracking, PCR, water quality, *Bacteroides*

INTRODUCTION

Faecal contamination from anthropogenic and zoogenic activities is a primary source of microbial pollution in natural waters; therefore, microbiological parameters are crucial in determining the quality of surface water. Faecal indicator bacteria (FIB) are used to assess the microbial quality and faecal contamination levels of water (Devane et al., 2020; Kostyla et al., 2015). They reveal the presence of intestinal pathogens, helping to prevent the spread of water-borne diseases. It has been shown that the presence of pathogens correlates well with the presence of faecal contamination (Leclerc et al., 2001), and as a result, modern drinking water testing relies on faecal bacteria as indicators of both faecal contamination and the possible presence of disease-causing organisms.

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Faecal indicator bacteria are consistently found in the intestines and faeces of humans and animals, and their survival characteristics are similar to those of intestinal pathogens. They are easy to isolate and enumerate, making them effective indicators of pollution levels. Typically, FIB are non-pathogenic and do not multiply significantly once they leave the body and enter the water, making them reliable for assessing microbial contamination in aquatic environments (Fujioka et al., 2015; Goshu et al., 2021).

The most commonly used FIB includes total coliforms (TC), faecal coliforms (FC), *Escherichia coli* (*E. coli*), and faecal streptococci (FS). Total coliforms are widely distributed in nature and can be found in the intestines of both homeothermic and poikilothermic animals, as well as in the soil and animal manure. Faecal coliforms, a subgroup of TC, are more specific to faecal material from homeothermic animals, with *E. coli* being the most common member (Edberg et al., 2000; Devane et al., 2020). Faecal streptococci, which include enterococci and non-enterococci species, are considered superior to coliforms as pollution indicators due to their ability to grow at elevated temperatures and in bile broth, closely mimicking many pathogens (Boehm & Sassoubre, 2014; Raji et al., 2015; Devane et al., 2020). They are considered to be better in some cases due to their ability to survive under environmental stress.

Traditional methods for monitoring FIB contamination often fail to distinguish between human and animal sources (Toledo-Hernandez et al., 2013). The ratio of FC to FS can provide some indication of the source, but novel molecular-based microbial source tracking (MST) methods are more reliable (Hagedorn et al., 2011). These methods, which often utilize PCR assays targeting bacterial genomic DNA, do not require the culture of microbes, making them time- and cost-effective (Ebentier et al., 2013; Kapoor et al., 2015; Goshu et al., 2021). They are used to identify the sources of faecal contamination, such as human, animal, and bird waste (Hagedorn et al., 2011). Host-specific markers such as *Bacteroides* spp., *Faecalibacterium* spp., *E. coli* toxin genes, or human-associated viruses can be used to detect the source of faecal contamination (Bower et al., 2005). PCR assays targeting members of the order *Bacteroidales* are the most widely used faecal source identifiers in water (Ballesté & Blanch, 2010; Ahmed et al., 2016). These bacteria are obligate anaerobes and cannot multiply in the environment outside the host (Zheng et al., 2009; Zhang et al., 2020). Microbial source tracking (MST) is crucial for controlling water pollution, especially in environments affected by both point and non-point sources of faecal pollution (Garcia-Armisen & Servais, 2007; Frick et al., 2020; Goshu et al., 2021).

Faecal contamination of surface waters can vary with time of day, rainfall patterns, and other environmental factors (Kostyla et al., 2015). Coliform bacteria are prevalent in streams, particularly following rainfall when surface runoff is significant. However, stormwater can sometimes dilute coliform counts, especially if bacteria enter the stream primarily through point sources (Augustyn et al., 2016). During dry weather, coliform counts are more likely to be related to point source pollution, such as wastewater discharges. Moreover, FIB is interrelated with the physical and chemical parameters of the water (Augustyn et al., 2016; Bisimwa et al., 2022).

In developing countries, including Sri Lanka, freshwater resources are significantly affected by faecal pollution due to inadequate sanitary conditions (Kumar et al., 2019). Water quality deterioration from faecal contamination is a significant issue, with several studies revealing high levels of FIB contamination in major rivers like the Kelani (Kumar et al., 2020), Mahaweli (Liyanage et al., 2021), and Pussalla-Oya (Rajapakshe et al., 2008). However, most studies have focused on the main rivers rather than their tributaries. This study aims to fill that gap by examining FIB levels and tracking pollution sources in the Rawan-Oya tributary of the Mahaweli River, the primary source that provides drinking and household water for the nearby community, and a freshwater source that sustains local ecosystems and agricultural fields.

The main objective of this study is to examine TC, FC, and FS contamination along the Rawan-Oya tributary. Specific objectives include identifying differences in TC, FC, and FS levels across nine sampling sites representing forested, agricultural, rural, semi-urban, and urban habitats, as well as between dry and wet seasons. Additionally, the study aims to track faecal contamination sources using both culture-dependent FC-to-FS ratio methods and culture-independent genotypic MST methods. These observations will provide valuable insights for developing water quality management strategies for the Rawan-Oya tributary (Kolawole et al., 2011).

MATERIALS AND METHODS

Study site

The Rawan-Oya is a perennial stream and one of the major water sources of the Polgolla reservoir located in the Kandy District of the Central Province of Sri Lanka. Rawan-Oya begins from the Hunnasgiriya mountain (1,400 m), located in the Campbell's Lane Forest Reserve. The upper watershed area of the stream is covered with evergreen montane and sub-montane forest

cover. Thereafter, the stream flows through secondary forests, home gardens, agricultural lands, semi-urban and urban landscapes, and joins the Mahaweli River through the Polgolla reservoir at Polgolla, Kandy (longitude 80º 39 – 42' E and latitude 7º 19 – 23'N). Rawan-Oya is a 13.5 km long stream with a stream watershed area of 31.07 km^2. The mean annual daily temperature ranges between 22 – 27 °C, and the mean annual precipitation exceeds 2,000 mm in the watershed area of the tributary. The catchment boundaries and land-use types along the stream were identified using QGIS desktop 2.18.3 software and Google Maps (2017). Sample collection was conducted at nine sites along the stream, ranging from a pristine, forested upstream area to the downstream site near the end of the tributary (S1 to S9; Table 1), indicating the presence of human activities adjacent to the waterway (Figure 1).

Table 1: Description of the nine sampling sites along the Rawan-Oya tributary of Mahaweli River

Site ID	Land use classification	Coordinates (Latitude, Longitude)	Site description and anthropogenic activities
S1	Forested (Pristine reference site)	7°22'31"N, 80°42'20"E	Headwaters originating from Hunasgiriya Mountain (1400 m) within Campbell's Lane Forest Reserve. Covered by evergreen montane and submontane forest; minimal anthropogenic impact.
S2	Agricultural	7°21'23"N, 80°41'47"E	Dominated by paddy fields in the Udugoda village area. Potential source of microbial load from agricultural runoff.
S3	Rural	7°20'53"N, 80°41'31"E	Low population density. Landscape features include steep terrain, forest gardens, dense forest, and a waterfall.
S4	Semi-Urban	7°20'35"N, 80°41'05"E	Moderately populated residential area (Pitiyegedara). Characterized by moderate human density and some agricultural practices.
S5	Urban	7°20'41"N, 80°40'39"E	Highly residential and commercial area in Wattegama Town. Expected to receive high microbial input from domestic wastewater and commercial activities.
S6	Urban	7°19'41"N 80°40'26"E	Highly residential and commercial area in Madawala Town. High population density implies significant discharge of domestic and commercial waste.
S7	Rural	7°20'01"N, 80°39'28"E	Low population density with forest gardens as the predominant land use in the Megammana-Polgolla area.
S8	Agricultural	7°19'50"N, 80°39'18"E	Agricultural practices, including paddy fields, vegetable plantations, and animal husbandry (Polgolla area). High potential for contamination from animal and agricultural waste.
S9	Semi-Urban	7°19'35"N, 80°39'08"E	Moderately populated residential and commercial area in Polgolla, located close to the Polgolla Dam. Represents the final cumulative microbial load before the reservoir/dam area.

Sample collection

Water samples were collected monthly from June 2020 to March 2022 to evaluate the FIB contamination pattern during both dry and rainy seasons and to implement molecular-based tracking. Samples were collected into pre-sterilised glass sampling bottles (1000 mL) from a depth of about 20–30 cm below the water surface, against the direction of water flow. All samples were placed in an insulated box filled with ice packs and transported to the laboratory, and kept in a refrigerator at 4 °C. The analysis commenced within 24 h of collecting the first sample.

Tests for faecal indicator bacteria: total coliforms (TC), faecal coliforms (FC), and faecal streptococci (FS)

Enumeration of TC and FC was performed using the membrane filtration (MF) method, as described by APHA (1992) and Oshiro (2002). For each sample, several dilutions of the water samples were made, and duplicate plates were inoculated with each dilution to determine the number of specific bacteria present in the sample. Water samples from the forested area were filtered without dilution, and all other samples were subjected to

10^{-1}, 10^{-2}, and 10^{-3} dilutions, appropriate for each type of FIB to obtain a countable number of 20 to 200 colonies (Nnadozie & Harcourt, 2016). Samples were diluted with sterilised distilled water to a final volume of 100 ml. Each diluted sample was filtered through a sterilised 47 mm diameter cellulose nitrate membrane filter with a pore size of 0.45 µm (Sartorius, Germany). The filtration apparatus was sterilised using UV radiation before filtering each sample to prevent cross-contamination.

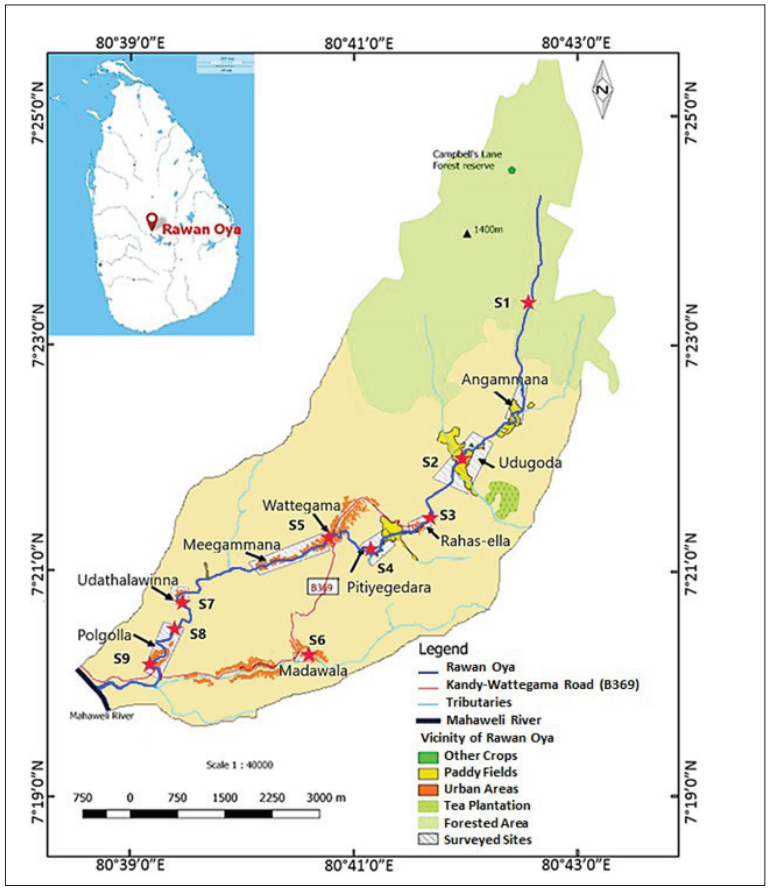

Figure 1: The location of the study site, Rawan-Oya tributary, the distribution of different land use patterns along the tributary, and the selected sampling sites (S1- Forested area, S2- Agricultural area, S3- Rural area, S4- Semi-urban area, S5 and S6- Urban area, S7- Rural area, S8- Agricultural area, and S9- Semi-urban area)

Membrane filters were aseptically placed on pre-sterilised Petri dishes with M-Endo agar LES (HiMedia, India) and M-FC agar (HiMedia, India). Plates with M-Endo agar and M-FC agar were incubated for 24 – 48 h at 35 ± 0.5 °C and at 44.5 ± 0.2 °C for the detection of TC and FC, respectively. Sterilised distilled water and standard culture of coliform (*Escherichia coli*: ATCC 25922) were used as negative and positive controls, respectively, in the detection of coliforms. Colonies with a typical red colour and a green metallic sheen on the M-Endo medium were counted as TC, while colonies with a typical blue colour on the M-FC medium were counted as FC. The concentrations of TC, FC, and FS were expressed as colony-forming units (CFU) per 100 mL. The CFU count from the membrane filter was divided by the volume of the original (undiluted) sample represented by the filtered volume to calculate the concentration. Given that the volume filtered for the diluted samples was 100 mL and the final concentration is expressed per 100 mL.

A confirmation test for coliforms was performed by inoculating colonies into brilliant green bile (BGB) (Oxoid, UK) broth tubes and incubated at 37 °C for 24 – 48 h. The formation of gas in the Durham tubes was considered a positive test for confirmed TC. Similarly, colonies isolated on M-FC medium were confirmed by inoculating into BGB broth tubes and incubated at 44.5 ºC for 24 – 48 h. The formation of gas in the Durham tubes was considered a positive FC test. Two or three drops from the positive BGB tubes were added to tryptone water tubes. Following incubation at 44.5 ºC for 24 h, 0.2 – 0.3 ml of Kovac's reagent was added to the culture. The immediate appearance of a red/pink colour ring in the upper layer was recorded as a positive indole reaction, confirming the presence of *E. coli.*

For the detection and enumeration of FS, an MF procedure similar to that used for the coliform test was followed with the filtration step. Subsequently, membrane filters were aseptically placed on pre-sterilised petri dishes with KF Streptococcus agar (Oxoid, UK). The plates were incubated for 48 h at 35 ± 0.5 °C. Sterilised distilled water and a standard culture of *Enterococcus faecalis* (ATCC 24212) were used as negative and positive controls, respectively, in the detection of FS. Pink to dark red colonies with yellow halos on the KF streptococcal medium were counted as FS. For FS confirmation, positive colonies were sub-cultured into tubes containing brain heart infusion (BHI) broth (Sinton et al., 1993). The formation of gas in the Durham tube and a cloudy broth at 37 ºC in BHI broth confirmed the presence of FS. A loop-full of inocula from the BHI broth were streaked on bile esculine agar (BEA) slants and incubated at 37 ºC for 24 h. The black colour formation of the slant confirmed the presence of FS.

Inference of faecal contamination source by the FC/FS ratio method

The ratio of FC to FS counts in water samples, from each of the sampling sites, was used to distinguish between faecal pollution of human and animal origin, as described previously.

The standard FC/FS ratios used were as follows (Table 2, Coyne & Howell, 1994):

The FC/FS ratio was calculated with the results obtained from the FC and FS tests.

Table 2: The standard FC/FS ratios for different sources of contamination

Source of contamination	FC/FS ratio
Human faeces	greater than 4.0.
Faeces of domesticated animals	between 0.1 and 4.0,
Faeces of wild animals	less than 0.1

Library independent molecular-based microbial source tracking tests

Faecal sample collection for microbial source tracking marker evaluation

To test the sensitivity and specificity of the MST markers used for the experiment, fresh faecal samples were collected from humans, cattle, and dogs from the same geographical area as the water sample collection. Faecal samples were collected in sterile containers, avoiding contact with soil and grass as much as possible. Samples were placed in an insulated box filled with ice packs, transported to the laboratory, and kept in a refrigerator at 4 °C. Each sample was processed individually by mixing 1 g of faecal material with 4.0 ml of nuclease-free water. The resulting slurry was stored at 4 °C for a minimum of 2 h and up to a maximum of 24 h prior to DNA extraction.

Water and faecal sample preparation and DNA extraction

The water samples were filtered in the laboratory using the filtration apparatus with a vacuum pump within 24 h of collecting the first sample. For the filtration, sterilised 47 mm diameter cellulose nitrate membrane filters with a 0.45 µm pore size (Sartorius, Germany) were used. Then, the membrane filters were transferred into sterile 15 mL plastic conical tubes using flame-sterilised tweezers. The tubes containing membrane filters were stored at -20 ºC until DNA extraction. In addition, DNA extracted from membrane filters and faecal slurry samples were prepared using the ReliaPrep™ g DNA Tissue Miniprep System (Promega, USA) following the manufacturer's instructions with minor modifications. For the PCR assay, 12 µL of the DNA product from each sample extraction was used. Four aliquots of 3 µL were combined with each forward and reverse primer, master mix (GO Taq Green

Master Mix, Promega, USA), and nuclease-free water to produce a final volume of 25 µL. Each sample was primed with a Bac32F, HF183F, and CF193F forward primer and a Bac708R reverse primer and an ED-1F forward, and an ED-1R reverse primer to amplify universal and human- and cattle-specific *Bacteroides* markers and a dog-specific *Faecalibacterium* marker, respectively. The specificities of the MST primers used for this study are depicted in Table 3. Thereafter, PCR amplifications were performed in an Applied Biosystems-Veriti Thermal Cycler under the following cycling conditions: one initial denaturation step at 95 °C, 1 min at the optimum annealing temperature (55 °C for Ba32F, HF183F, and CF193F and 64 °C for ED-1F), and 1 min at 72 °C. PCR products were visualised on a 1.5% agarose gel with ethidium bromide using a 50 bp DNA ladder (GeneRuler 50 bp DNA Ladder, Thermo Scientific, USA). The gel was visualised using the UV gel doc system (BioPrint, Vilber, France).

Table 3: Primers used for microbial source tracking and their respective sequences, annealing temperatures, and times

Target	Primer	Sequence (5′–3′)	Annealing temperature (°C)	Annealing time(s)
Universal	Bac32F	AAC-GCT-AGC-TAC-AGG-CTT	54	60
	Bac708R	CAA-TCG-GAG-TTC-TTC-GTG		
Human	HF183F	ATC-ATG-AGT-TCA-CAT-GTC-CG	55	60
	Bac708R	CAA-TCG-GAG-TTC-TTC-GTG		
Cattle	CF193F	TAT-GAA-AGC-TCC-GGC-C	55	30
	Bac708R	CAA-TCG-GAG-TTC-TTC-GTG		
Dog	ED-1F	CAGGCGGACTCTTAAGTC	64	60
	ED-1R	GCGATCGGAGTTCTTCAT		

(Source: Somnark et al., 2018; Zhang et al., 2020)

Faecal indicator bacteria and microbial Source tracking

The specificity and sensitivity of the MST markers were tested using faecal DNA extracts from local animals and humans. Sensitivity (r) and specificity (s) are defined as $r = a/(a + c)\times100$ and $s = d/(b + d)\times100$, where a is when a faecal DNA sample is positive for the PCR marker of its own target (true positive); b is when a faecal DNA sample is positive for a PCR marker of another target (false positive); c is when a faecal DNA sample is negative for a PCR marker of its own target (false negative); d is when a faecal DNA sample is negative for a PCR marker of another target (true negative) (Balleste et al., 2020).

Statistical analysis

A generalised linear model with a negative binomial distribution was used to determine whether the FIB counts varied by site. A post-hoc multiple comparison of this model was performed to determine whether the FIB counts differed between the dry and wet weather conditions. A standard Pearson correlation analysis was performed to check the correlation between the three FIB groups tested. The enumerated bacterial data were $\log_{10}$-transformed to attain a normal distribution. The $\log_{10}$ transformed colony counts (log CFU/100 mL) were used in the Pearson correlation analysis. The hierarchical cluster analysis (CA) was implemented by means of Ward's method, using squared Euclidean distances as a measure of similarity to emulate the underlying patterns of variation of FIB in relation to anthropogenic activities and land use patterns of the study sites (Emad & Eethar, 2012). A chi-square statistical test was performed to determine whether there were significant differences in MST markers between the sampling sites and across seasons. The statistical analyses were performed using Microsoft Excel (2013), PAST version 4.03 (Hammer et al., 2001), and R statistical software version 4.0.3 (R Core Team, 2021), using package MASS (Venables & Ripley, 2002) for negative binomial models and package multcomp (Hothorn et al., 2008) for multiple comparison tests.

RESULTS AND DISCUSSION

Distribution and concentration of faecal indicator bacteria along Rawan-Oya

The TC and FC were identified and enumerated based on colony morphology, with the concentration of the specific type of FIB expressed in CFU per 100 mL of water sample.

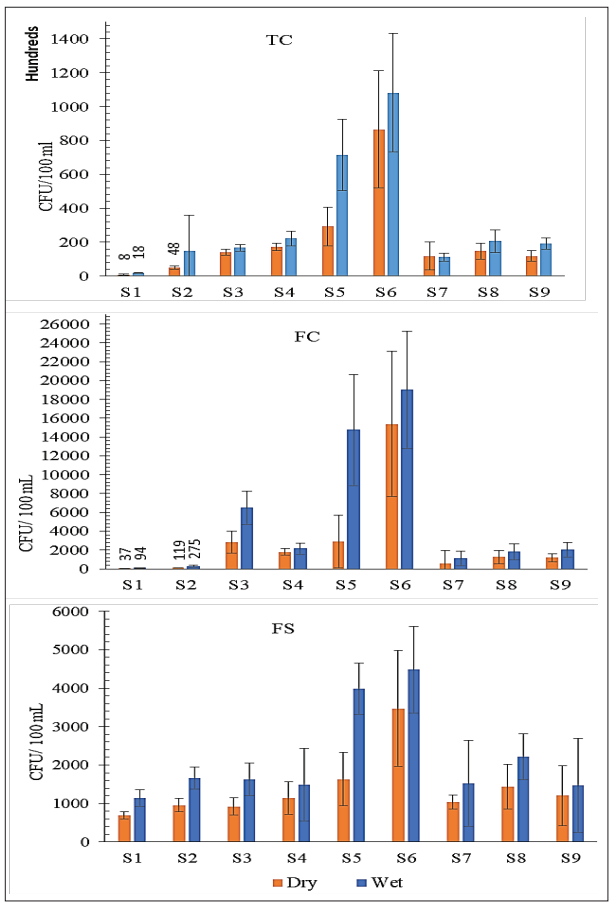

Figure 2: The geometric means of total coliform (TC), faecal coliform (FC) and faecal streptococcus (FS) concentrations (CFU/100 mL) at nine sites along the Rawan-Oya tributary of the Mahaweli River during dry and wet seasons from June 2020 to March 2022. (S1- Forested area, S2- Agricultural area, S3- Rural area, S4- Semi-urban area, S5 and S6- Urban area, S7- Rural area, S8- Agricultural area, and S9- Semi-urban area).

Results showed significant variability in TC, FC, and FS counts spatially and temporally. The TC concentration in stream water ranged from 2.7×10^2 to 1.6×10^5 CFU/100 mL, FC counts varied from 16 to 3.2×10^4 CFU/100 mL, and FS concentrations fluctuated between 5.4×10^2 to 6.2×10^3 CFU/100 mL throughout the study period between the nine sampling sites along the stream.

Figure 2 illustrates the geometric means of TC, FC, and FS concentrations (CFU/100 mL) at nine sites along the tributary during dry and wet seasons. The lowest geometric means of TC (1.2×10^3 CFU/100 mL), FC (6.1×10^1 CFU/100 mL), and FS (8.3×10^2 CFU/100 mL) were observed in the forested area (S1). Specifically, at site S1, the lowest TC (2.7×10^2 CFU/100 mL) and FC (1.6×10^1 CFU/100 mL) values were recorded during February 2021, the driest month with the least precipitation (0.1 mm), while the lowest FS concentration (5.4×10^2 CFU/100 mL) was observed in June 2020. The highest geometric mean of TC (9.7×10^4 CFU/100 mL), FC (1.7×10^4 CFU/100 mL), and FS (3.9×10^3 CFU/100 mL) counts were recorded at site S6 located in a highly urban area. The highest TC (1.6×10^5 CFU/100 mL) and FC (3.2×10^4 CFU/100 mL) concentrations were recorded in the urban area in April 2021, following a 5.6 mm rainfall event after a dry period. The peak of the FS (6.2×10^3 CFU/100 mL) concentration was observed in February 2022, also a dry period with 0.1 mm of precipitation. There was an increase in TC, FC, and FS counts during the wet season compared to the dry season. Even with less rainfall (5 – 6 mm) after a dry season, the FIB levels increased rapidly (Figure 3).

Figure 4 presents a map of the Rawan-Oya tributary illustrating the distribution patterns of TC, FC, and FS in the nine sampling sites along the stream.

The presumptive identification of isolates from the membrane filtration method was confirmed using standard biochemical assays to determine the reliability of the identification, with the following results: Total coliforms showed 100% positive results for the BGB confirmation test at 37 °C for 24 – 48 h. The FC demonstrated 98% positivity for the BGB test at 44.5 °C for 24 – 48 h. The Indole test confirmed 95% positive results for *E. coli*. Faecal Streptococci showed 100% positive results for the BEA slant test.

The generalized linear model showed no significant differences in TC, FC, and FS concentrations in water samples between wet and dry seasons. (GLM, $p > 0.05$).

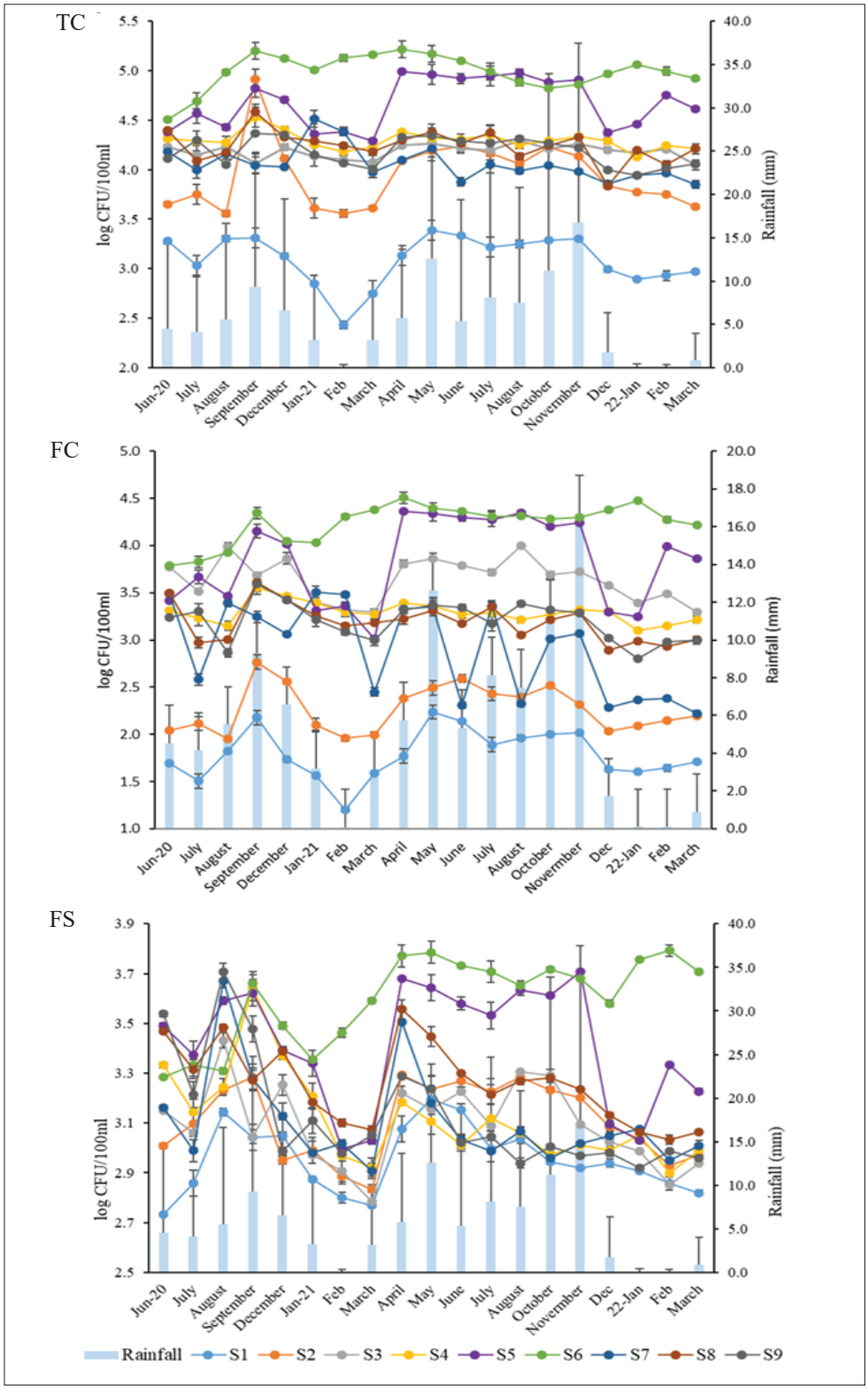

Figure 3: Total coliform (TC), Faecal coliform (FC), and Faecal Streptococcus (FS) levels measured at nine sites along the Rawan-Oya Tributary of the Mahaweli River from June 2020 to March 2022 against monthly rainfall data (Sampling sites: S1- Forested area, S2- Agricultural area, S3- Rural area, S4- Semi-urban area, S5 and S6- Urban area, S7- Rural area, S8- Agricultural area, and S9- Semi-urban area).

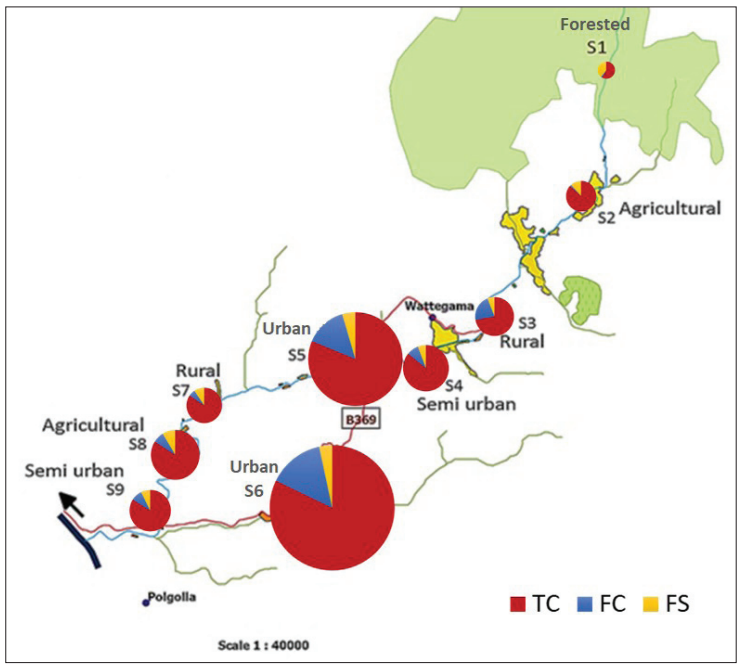

Figure 4: Map of the Rawan-Oya tributary of the Mahaweli River illustrating the distribution patterns of total coliform (TC), faecal coliform (FC) and faecal streptococci (FS) at nine sampling sites from June 2020 to March 2022 (S1- Forested area, S2- Agricultural area, S3- Rural area, S4- Semi-urban area, S5 and S6- Urban area, S7- Rural area, S8- Agricultural area, and S9- Semi-urban area).

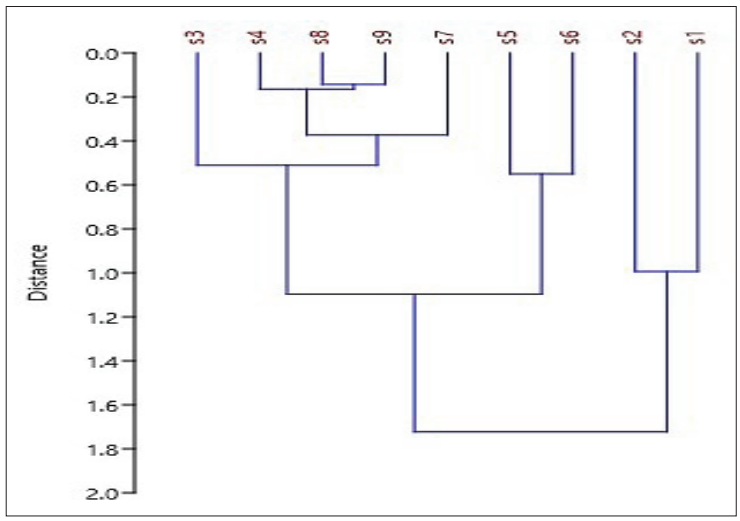

Figure 5: Hierarchical clustering of sampling sites of the Rawan-Oya tributary of the Mahaweli River based on the distribution of FIB levels. (Sampling areas: S1- Forested area, S2- Agricultural area, S3- Rural area, S4- Semi-urban area, S5 and S6- Urban area, S7- Rural area, S8- Agricultural area, and S9- Semi-urban area).

A significantly higher concentration of TC was found at site S6 (GLM, z = 2.16, p = 0.03) compared to other sites during both wet and dry seasons. Faecal coliform levels fluctuated significantly between all sites (GLM, p < 0.05), except between sites S1, S2, and S7 (GLM, z = 0.78, p = 0.4 and GLM, z = 1.69, p = 0.09). However, FS concentrations did not vary significantly between sites or during the wet and dry seasons (GLM, p > 0.05).

The hierarchical cluster analysis showed similarities in FIB distribution among the study sites with three main clusters. Cluster 1 consisted of sites S1 and S2, with S1 being the most upstream site in a forested area with minimal human activity, and S2 located approximately 1.5 km downstream of S1, characterized by predominantly agricultural land use. Cluster 2 consisted of S5 and S6 in urban habitats with high population densities. Cluster 3 comprised sites S3, S4, S7, S8 and S9, surrounded by rural, semi-urban and agricultural land-use types (Figure 5).

A Pearson correlation coefficient was computed to assess the linear relationship between FIB concentrations. There was a strong positive correlation between the TC and FC distributions (r = 0.92, df = 169, $p < 0.001$). The Pearson correlation between TC and FS was 0.71, and the p-value was less than 0.001. Moreover, a moderately positive correlation was observed between FC and FS counts along the stream (r = 0.68, df = 169, $p < 0.001$) (Figure 6).

Inference of faecal source based on FC/FS ratio method

According to the results of the FC/FS standard ratios, the primary source of faecal contamination in the forested area (S1) was primarily attributed to wild animals. Sites S2, S4, S7, S8, and S9 showed faecal pollution from domestic animals. Site S3 and S5 showed pollution from domestic animals only during the dry season; however, during the wet season, it was determined to be human-origin faecal contamination. Site S6 had the highest FIB counts, and the FC/FS ratio showed that the main source of contamination at this site during both seasons was human faecal matter (Figure 7).

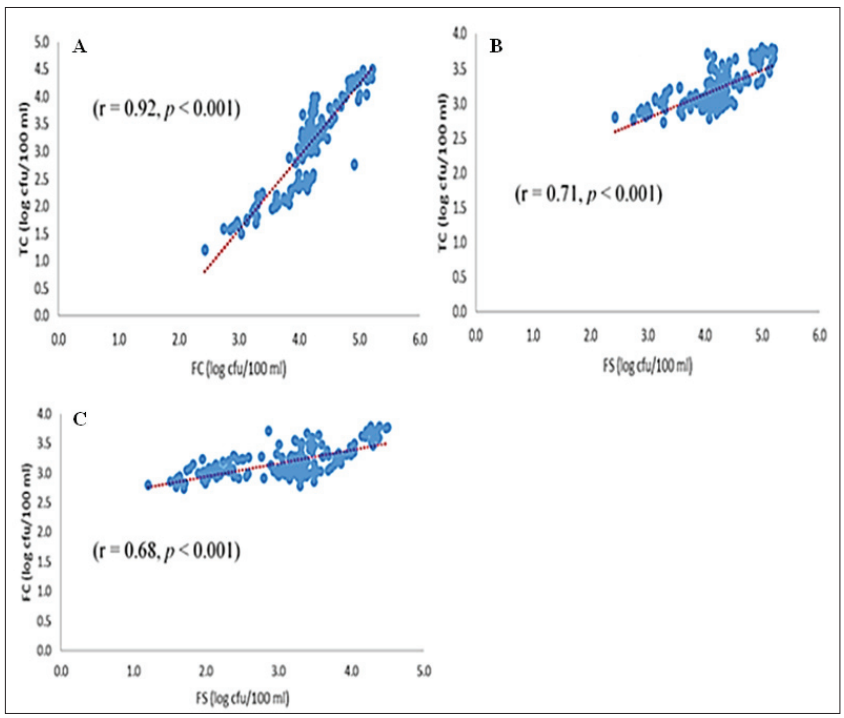

Figure 6: Correlations among faecal indicator bacteria in the Rawan-Oya tributary, showing the relationships between A. Total coliforms (TC) and faecal coliforms (FC); B. Total coliforms and faecal streptococcus (FS); and C. Faecal coliforms and faecal streptococcus, from June 2020 to March 2022.

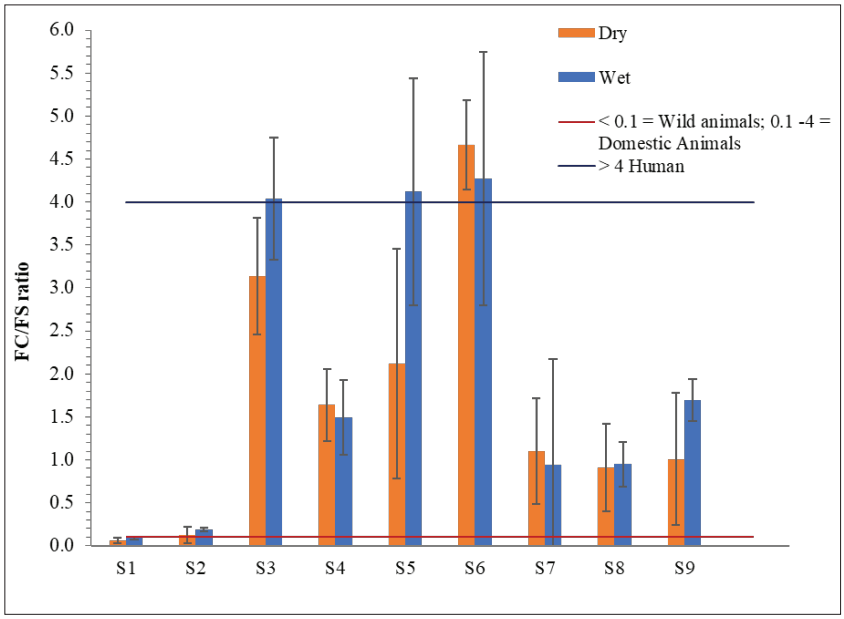

Figure 7: FC/FS ratio of nine sites along the Rawan-Oya tributary of the Mahaweli river during dry and wet seasons (Sampling sites: S1- Forested area, S2- Agricultural area, S3- Rural area, S4- Semi-urban area, S5 and S6- Urban area, S7- Rural area, S8- Agricultural area, and S9- Semi-urban area).

Site S6 is in a highly urban area where all drainage channels and wastewater outlets from houses, meat shops and other buildings directly flow into the stream. Moreover, dumping solid waste into the river and in the vicinity was common in this area. However, the most important factor for this high faecal contamination is likely the public toilets located on the riverbank at this site.

Faecal contamination source identification with microbial source tracking (molecular method)

In this study, Bac32 was used as a universal marker, while human and cattle faecal contaminations were detected through the use of human-specific (HF183) and cattle-specific (CF193) *Bacteroides* markers. Dog faecal contamination was traced using the dog-specific *Faecalibacterium* (Ed-1) marker. Results were reported as positive or negative for each host-specific marker, with DNA fragment confidence being assessed through a comparison with a DNA ladder. The accuracy of the DNA fragment was determined by its location in relation to the ladder, with an acceptable range of ± 10% of the "true" base-specific value (Universal *Bacteroides* = 676, cow 193 = 515, human 183 = 525, dog = 145) (Somnark et al., 2018; Zhang, 2020). Sensitivity analysis further revealed that the universal marker (Bac32) and the human marker exhibited 100% sensitivity, while the cattle and dog markers showed 90% and 70% sensitivity, respectively. Specificities for all markers were 90% or higher.

All samples tested positive for the universal *Bacteroides* marker, confirming faecal contamination in all samples. The distribution patterns of MST markers at the nine sites along the stream are illustrated in Figure 8.

There were significant differences in the distribution of human ($\chi^2 = 68.69$; df = 8; $p < 0.0001$), cattle ($\chi^2 = 62.47$; df = 8; $p < 0.0001$), and dog-specific ($\chi^2 = 50.12$; df = 8; $p < 0.0001$) markers between the nine sampling sites. Water samples collected from the forested area (S1) did not contain human-specific markers. The agricultural areas, sites S2 and S8, were positive for the HF183 marker at 26% and 52%, respectively. A rural site (S3) with high recreational activities showed human faecal contamination in 84% of the samples, while in the other rural site (S7), 47% of the samples were positive for human contamination. Two semi-urban sites (S4 and S9) contained human-specific markers in 58% and 63% of the samples, respectively. All the samples (100%) collected from the urban sites (S5 and S6) were positive for the HF183 assay. The presence of cattle-specific markers was

also low (21%) at the forested site. The CF193 marker was prominent in agricultural areas (S2 = 95% and S8 = 100%) and the urban site S6 (100%). Subsequent sites to the agricultural areas (S3 and S9) contained 68% and 78% of the cattle-specific marker. When considering the dog-specific marker distribution, only 10% of the samples from the forested area contained the ED-1 marker. It was noticeably high in urban areas (S6 = 89%, S5 = 84%) and in semi-urban sites (S4 = 84%). In agricultural areas, about 30% of samples were positive.

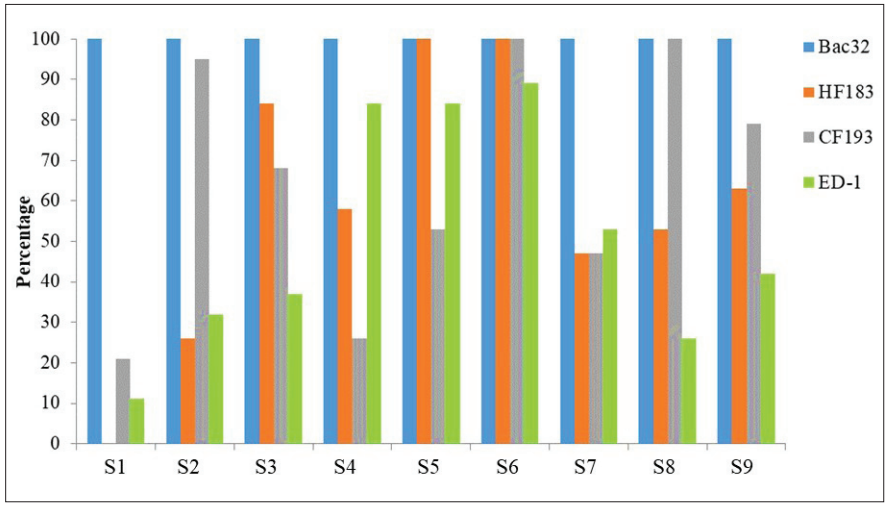

Figure 8: Percentage of positive results for the MST markers at nine sites along the Rawan-Oya tributary: Bac32- Universal *Bacteroides* marker, HF183- Human-specific *Bacteroides* marker, CF193- Cattle specific *Bacteroides* marker, Ed-1- Dog-specific *Faecalibacterium* marker (Sampling sites: S1- Forested area, S2- Agricultural area, S3- Rural area, S4- Semi-urban area, S5 and S6- Urban area, S7- Rural area, S8- Agricultural area, and S9- Semi-urban area).

Variations of human-specific (χ^2 = 29.18; df = 1; $p < 0.0001$) and dog-specific (χ^2 = 4.23; df = 1; p = 0.039) markers were significantly higher during the wet season compared to the dry season. However, there were no significant differences in the presence of cattle-specific markers between seasons (χ^2 = 2.15; df = 1; p = 0.14). Surface runoff due to rainfall is a major transporter of faeces into streams (Kostyla et al., 2015). The Rawan-Oya tributary exhibited diverse patterns of faecal contamination along the waterway. Moreover, the results obtained from the MST procedure were more specific and informative than those from the FC/FS ratio method. The type of faecal contamination at all sites, except sites S1 and S2, was significantly compatible between the MST and ratio methods (χ^2; $p < 0.05$).

DISCUSSION

The consistently high concentrations of FIB (TC, FC, and FS) observed across all sampling sites indicate widespread and significant faecal contamination of the Rawan-Oya tributary, originating from anthropogenic and/or mammalian sources. The observed variation in FIB concentrations along the tributary can be attributed to changing land use patterns (from forested to agricultural and urbanized zones), anthropogenic activities encountered along the course, as well as natural influences such as the presence of wildlife, precipitation, and surface runoff. Increasing human activity and population density along the catchment of the Rawan-Oya tributary has been reported (Kapukotuwa et al., 2023), which is consistent with similar findings reported for other Sri Lankan catchments (Rajapakshe et al., 2008), supporting the hypothesis that land use is a major contributor to the microbiological quality of the water.

According to the regulatory standards, the Rawan-Oya stream water at all locations tested was not suitable for direct consumption, as both TC and FC levels significantly exceeded the Sri Lanka Standard (SLS, 2013) limits for potable water. Furthermore, except for the upstream forested area, the water quality at all other

study sites exceeded the permissible microbial limits for recreational use (FC: 235 CFU/100 mL; FS: 70 CFU/100 mL) set by the WHO and EPA (USEPA, 2012; WHO, 2021). This is a critical public health issue, particularly considering the local context, as Kapukotuwa et al. (2023) showed a substantial portion of the population in the areas (~75%) utilizes this water for bathing, washing clothes, and other household activities. The fact that some households (28.3%) receive chlorinated water derived from this source (Kapukotuwa et al., 2023), particularly in sites S1, S2, and S5, underscores the necessity of robust treatment and source-specific contamination control. The bacteriological quality of the Mahaweli River also exhibits similarly poor microbial quality (Liyanage et al., 2021), indicating that the tributary contributes to larger systemic water quality issues.

Seasonal changes in FIB content are known to be influenced by precipitation-driven runoff, temperature fluctuations, hydrological conditions, and land-use or point-source inputs. These factors often determine whether FIB levels rise during wet seasons, due to stormwater and sewage overflows, or decline in dry seasons when dilution and bacterial die-off dominate. In the present study, sampling was conducted over 18 months (June 2020 – March 2022) to capture contamination patterns across seasons; however, no significant differences in overall FIB counts were observed between the dry and wet seasons in the Rawan-Oya tributary. The absence of seasonal variation in Rawan-Oya can be attributed to continuous contamination sources, such as urban sewage, agricultural runoff, and inadequate sanitation infrastructure, which sustain persistently high bacterial loads year-round, thereby masking the influence of rainfall or hydrological seasonality. Previous studies have shown that when FIB levels remain consistently elevated in polluted river systems, seasonal fluctuations are often absent due to chronic contamination inputs (Muñoz-Delgado et al., 2025). Furthermore, seasonal drivers such as precipitation, runoff, and point-source discharges do not always produce clear FIB patterns, with variability observed across sites and years (Wilson et al., 2022). Similar findings have been reported in other river systems globally, where no significant seasonal flux in FIB concentrations was detected (Bojarczuk et al., 2018), aligning with the present study's observation of persistent contamination from diffuse sources even after centralized point-source controls were implemented (Bhat & Danek, 2012). Nevertheless, other studies have documented increases in FIB levels during wet seasons due to precipitation and stormwater inputs, with dry-season conditions favoring dilution and bacterial die-off (Dila et al., 2018). Conversely, strong positive correlations between FIB and precipitation have been reported in certain systems (Sinclair et al., 2009), highlighting the variability in seasonal responses across different hydrological and contamination contexts.

Significant differences in human, cattle, and dog-specific markers were observed across nine sites in the Rawan-Oya tributary. Human contamination was absent in the forested site (S1) but highest in urban areas (S5, S6: 100%) and a rural recreational site (S3: 84%). The absence of human-specific markers in the forested site (S1) indicates minimal anthropogenic activity. In contrast, urban sites (S5, S6) showed 100% positivity, consistent with untreated sewage discharges, leaking septic systems, and poor sanitation infrastructure. The high prevalence at the rural recreational site (S3) suggests direct inputs from visitors and inadequate waste management. Cattle markers were most prominent in agricultural sites S2 (95%) and S8 (100%), as well as in urban site S6 (100%), reflecting the proximity of livestock and manure runoff. The presence of cattle markers in urban S6 likely results from mixed land use, including peri-urban farming and improper disposal of animal waste. The lack of seasonal variation in cattle markers suggests that continuous inputs from livestock operations occur, independent of rainfall. Dog markers peaked in urban (S5, S6: >80%) and semi-urban sites (S4: 84%), highlighting the role of domestic pets and stray dog populations. Poor waste disposal practices and high densities of companion animals contribute to persistent contamination. Seasonal increases in dog markers during wet periods are likely due to stormwater mobilizing fecal deposits from streets and open areas. Human and dog markers increased significantly during wet seasons, consistent with rainfall-driven runoff and sewage overflows transporting fecal material into streams. Conversely, cattle markers remained stable across seasons, reflecting year-round agricultural inputs that mask hydrological variability. Similar distribution patterns are reported in studies using comparable genotypic analyses, such as the Dargle River in Dublin, where human markers peaked in urban areas and ruminant markers in agricultural regions (Balleste et al., 2010).

To identify host sources of faecal pollution, both the conventional FC/FS ratio and PCR-based MST methods were applied. The FC/FS ratio offered a rapid, preliminary indication of contamination sources (wildlife in forested areas, human in urban sites, and domestic animals elsewhere) based on established human-to-animal ratios (Jagals et al., 1995). However, its reliability is limited by environmental factors such as temperature, pH, and organic matter (Bisimwa et al., 2022). In contrast, the library- and culture-independent PCR-based MST method provided definitive source attribution, revealing

diverse and spatially variable contamination patterns strongly aligned with local anthropogenic activities; MST thus offered scientifically robust evidence beyond general contamination levels.

As Bussi et al. (2017) point out, the specificity of the data derived from this study is crucial for effective resource management. The site-specific source identification provided by the MST analysis offers local authorities essential, actionable data to inform targeted source control and implement long-term water planning and management schemes. For instance, the detection of human markers in urban zones justifies focused investment in sanitation infrastructure, while the detection of ruminant markers in agricultural areas necessitates the implementation of best management practices for livestock waste. The findings are therefore important for developing effective strategies for water quality management and ensuring the safety of drinking water supplied by the Rawan-Oya tributary, as well as serving as a model for managing other vital water resources in Sri Lanka facing similar faecal pollution threats.

CONCLUSIONS

The study confirmed significant faecal contamination in the Rawan-Oya tributary, a critical freshwater source for nearby communities. Contamination levels, measured by FIB counts, increased progressively from the upstream forested areas to the downstream urban areas, rendering the water unsafe for both drinking (SLS, 2013) and recreational use (WHO, 2021). Only the forested area met acceptable microbial quality standards for recreational use.

This research represents a pioneering effort in Sri Lanka by successfully implementing the MST method to accurately identify and differentiate specific sources of pollution. These insights offer local authorities essential, actionable data to inform targeted water management strategies. Ultimately, the study not only quantifies the extent of pollution but also charts a clear path towards protecting public health. The findings are crucial for developing effective strategies for water quality management and the long-term protection of public health, particularly in relation to the Rawan-Oya tributary and other vital water resources in Sri Lanka.

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