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Title

Metagenomic Profiles of Bacterial communities and environmental factors associated with proliferation of malaria vector mosquitoes within the Kenyan Coast

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Abstract

Background

Anopheles mosquitoes are the main malaria vector and as malaria cases decline in Sub Saharan Africa, there is a growing realisation that new interventions need to be added to complement the existing control strategies. To date, vector control is the most effective way to prevent malaria. Since the malaria parasite is maintained by mosquitoes which oviposit, feed and rest in the outdoor environment, there is an urgent need to focus on the control of oviposition sites seeking malaria vectors. In this regard, a detailed understanding of their larval ecology is necessary. In this study, the bacterial community structure and their interactions with

physicochemical factors in relation to oviposition site selection in mosquito larval habitats was characterised in Kwale County, where malaria is endemic.

Materials and methods

The physical characteristics of each site with regard to type, permanence, substrate type of the habitat, depth, size and type of vegetation and water physicochemical parameters of the habitats were determined and recorded. Water samples were also collected from the sites for total genomic DNA extraction in order to characterise the bacterial communities in the breeding sites.

Results and Discussion

Physicochemical parameters assessed were not different between the positive and negative sites throughout the dry and rainy seasons (R^2 = 0.1180, df = 1, P = 0.106). We found that 63.15% of the positive sites had *Anopheles gambiae* larvae only, 5.20% had *Culex* sp. larvae only, and 31.65% had both *Anopheles gambiae* and *Culex* sp. larvae. Proteobacteria (48.16 %) was the most common phyla recovered in all samples followed by Bacteroidota (32.91%) and then Actinobacteriota (10.94%). It was also observed that the presence or absence of mosquito larvae in a potential proliferation site was not related to the bacterial community structure in the site (R^2 = 0.18157, df = 1, P = 0.353) but was positively correlated with bacterial richness and evenness (F = 2.928, df = 8, P = 0.032). There were 16 most commonly identified bacterial genera that were significantly abundant in the positive sites than in the negative sites. Although bacterial abundance was higher in the positive sites than in the negative sites, these differences were not associated with the physicochemical parameters that were evaluated (Kruskal-Wallis chi-squared = 6, df = 6, P = 0.4232).

Conclusion

Generally, the presence of *Anopheles* mosquito larvae was found to be positively correlated with bacterial richness and evenness, and negatively correlated with Electrical conductivity, total dissolved solids, salinity and ammonia. Mosquito oviposition and subsequent proliferation are possible in environmental waters with a variety of physicochemical properties and bacterial community compositions but the mosquitoes prefer mostly clean and unpolluted water. The findings of this study may have implications for predicting the potential of environmental water samples to become proliferation sites.

Data summary

The authors confirm that all supporting data, code and protocols have been provided within the article. All sequence data generated in this project was deposited in the National Center for Biotechnology Information (NCBI BioProject ID: PRJNA953183; BioSample accession numbers: SAMN34109148, SAMN34109149, SAMN34109150, SAMN34109151, SAMN34109152, SAMN34109153, SAMN34109154).

INTRODUCTION

Most studies have implicated *Anopheles* mosquitoes as the leading vectors of malaria parasites in Sub-Saharan Africa (1). They are found both in urban and rural areas but with high populations in relatively wet regions near significantly large and permanent water bodies such as lakes and oceans (2,3). In regions where the vector population is high, malaria is endemic. These mosquitoes commonly oviposit in small, sunlit, semi-permanent and turbid water bodies

like animal footprints, the edges of boreholes, puddles on the roadside formed by tires of vehicles and tracks, irrigation canals and other artificial water sources (4).

The choice of egg-laying sites by female *Anopheles* mosquitoes depends on the biotic and abiotic factors present in specific aquatic habitats. This, in turn, affects the abundance and distribution of their larvae (5), leading to varying levels of vector distribution and abundance in a specific region which in turn affects the spatio-temporal patterns of vector distribution and abundance within a given region (6). Despite a lack of understanding of the key factors affecting the proliferation sites and the driving forces behind oviposition site preference, even for the most prominent malaria vectors (7), it has been suggested that mosquitoes lay their eggs in locations that provide optimal conditions for larval survival and growth, thus increasing the chances of success for their species (8).

To date, vector control remains the most effective way to prevent malaria (9,10). Most vector control strategies have targeted the indoor host seeking behaviour of the mosquitoes which has succeeded to a large extent (11,12). Despite this remarkable success, elimination of malaria remains a big challenge since the malaria parasite is maintained by mosquitoes which oviposit, feed and rest in the outdoor environment(13). Because of this setback together with the emergence of highly drug-resistant malaria parasites (14), there is an urgent need to focus on the management and control of oviposition sites seeking malaria vectors (15). Furthermore, in malaria endemic countries like Kenya, efficient intervention and preventive protocols should be guided by knowledge of the abundance, distribution and characteristics of the proliferation sites of these vectors if malaria were to be effectively eliminated (16).

In order to effectively control mosquitoes, a comprehensive understanding of their larval ecology is essential. This includes examining the interplay between biotic and abiotic factors in breeding habitats, such as the types and preferences of breeding sites, the distribution and abundance of those sites, and the biological and physico-chemical conditions present (5). Research has suggested that proper management of mosquito breeding habitats in sub-Saharan Africa could help reduce vector populations and curb malaria transmission (17). By analysing the choice of oviposition sites and its impact on the distribution and abundance of malaria vector mosquitoes, we may be able to explain differences in malaria transmission intensity across different regions (18). This information is valuable in the creation of integrated control strategies for Anopheles mosquitoes and health education programs at the community level, aimed at lowering mosquito populations and reducing the risk of human-vector contact.

At present, the knowledge about the impact of proliferation site distribution, biotic and abiotic factors on the distribution and density of malaria vectors in Kenya is scarce and inadequate to explain the patterns of adult mosquito distribution and abundance with certainty. This makes it difficult to implement effective malaria vector control strategies through the management of the larval forms (19–22). In response to this gap in knowledge, this study aims to characterise the total bacterial community structure and their interactions with physico-chemical ecological factors in mosquito breeding habitats in LungaLunga along the Kenyan coast, where malaria is widespread.

MATERIALS AND METHODS

Study area

The mosquito breeding sites were sampled along three major roads in Lunga Lunga Sub-county, Kwale County, located along the South Coast of Kenya (as shown in figure 1). The selection of the sampling sites was based on the presence of larval habitats and their accessibility during the rainy season. Samples were collected along the Ramisi-Lunga Lunga road between Kanana junction (coordinates: -4.539395, 39.366467) and the Umba river in Lunga Lunga town (coordinates: -4.554633, 39.125867), the road between Lunga Lunga town (coordinates: -4.554633, 39.125867) and Ngozi Girls Secondary School in Jego village (coordinates: -4.590374, 39.158956), and the road between Jego Village and Kanana junction on Lunga Lunga-Ramisi road (coordinates: -4.539395, 39.366467).

The region experiences two rainy seasons each year, between March to June and from October to November, with significant variations each year. For example, during the time of the study, there was no rainfall in October but the rainy season started towards the end of November until the end of December. Most of the residents in the region rely on small-scale farming and fishing to make a living. The area has a high prevalence of malaria among its local residents. Three species of malaria vectors, including *An. arabiensis*, *An. gambiae s.s*., and *An. funestus*, have been previously identified in the region (23,24).

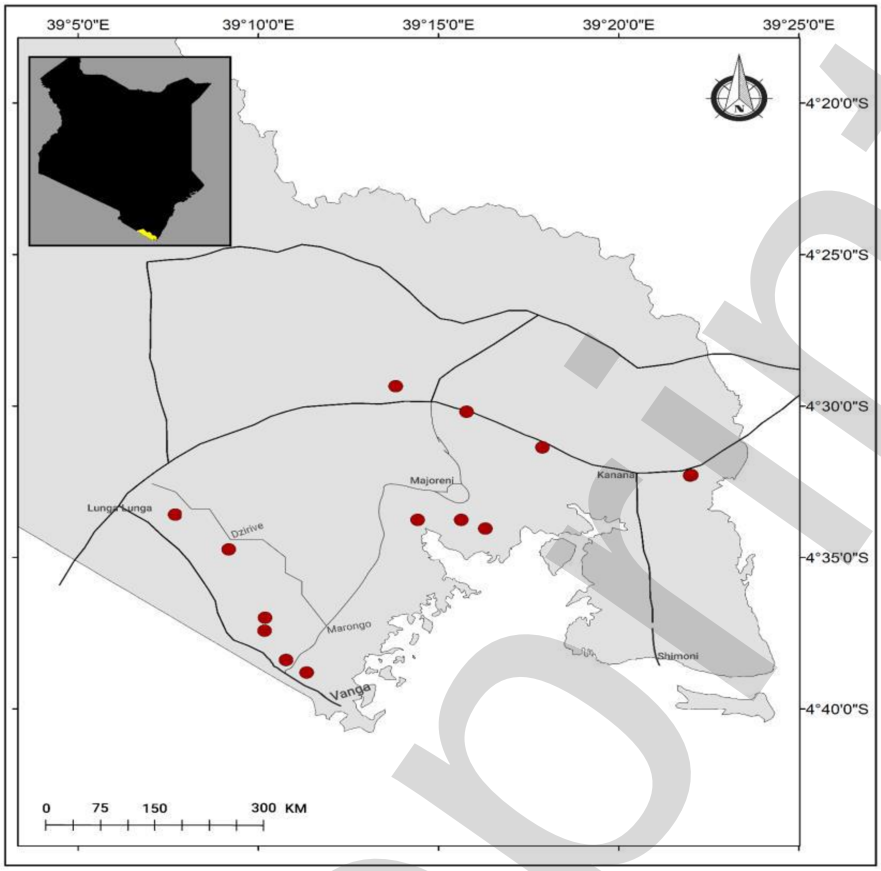


Figure 1: *Sampling sites along 3 major roads within Lungalunga Sub-county in Kwale county.*

Study design and sample size

We conducted a cross-sectional study where 35 proliferation sites were sampled according to the formula as described by Naing and others (25);

$$n = \frac{Z^2 pqD}{d^2}$$

whereby n = required sample size, Z = standard normal variate which is 1.96, p = anticipated probability at 99%, q = failure (1-p), D = design effect of control given a value of 2, and d = allowable error (0.05).

Sample collection

Identification of proliferation sites

Before the actual sample collection, each sampling site was accurately located using a GPS device (Garmin, Gpsmap 64, Garmin International Inc., Switzerland). The physical characteristics of the sites, including their natural or artificial nature, permanence, substrate

type, depth, size, and vegetation, were recorded. Each potential mosquito breeding site was first visually inspected for the presence of larvae, and if larvae were not detected, a minimum of ten dips were made using a standard 350 ml dipper (BioQuip products, Rancho Dominguez, USA) to confirm the absence of larvae. A site was considered positive if at least one larva was found, and negative if no larvae were detected. The samples were collected from the selected mosquito breeding sites during both the dry and rainy seasons between June 2021 and April 2022. The sample collection was done between 7.00 am and 6.00 pm.

Water samples collection

From each selected site, a single 500 mL and two 250 mL of water samples were collected using sterile plastic and glass bottles, respectively. The sampling bottles were first rinsed with the site water, which was carefully discarded before the sample was collected. Three controls were also included in the sample collection process. Nuclease-free water (500 mL) was used as a control by opening and uncapping the bottle during sampling. The 500 mL water samples were set aside for metagenome analysis, while the two 250 mL water samples were split as follows: one for nutrient analysis (nitrates, nitrites, ammonium, and phosphates) and the other for the determination of Biological Oxygen Demand (BOD). The sample for the BOD determination was wrapped in aluminium foil to keep out light and prevent photosynthetic activity, which could alter the concentration of oxygen in the bottles. Samples for nutrient analysis were kept at ambient temperature, while those for metagenome analysis and BOD determination were preserved in a cooler box with ice packs and transported to the laboratory immediately for processing.

Mosquito proliferation sites water quality

Physico-chemical parameters, including water conductivity, temperature, total dissolved solids (TDS), dissolved oxygen (DO), pH, hardness (calcium and magnesium ions), and salinity, were measured in situ at each selected site using a YSI Professional Plus (Pro Plus) multi-parameter water metre (manufactured by YSI Inc., located in Yellow Springs, Ohio, USA). Three measurements were taken for each parameter. Turbidity was measured using a pre-calibrated AQUAfast AQ3010 turbidity metre (manufactured by Thermo Fisher Scientific, USA) following the manufacturer's instructions.

The biological oxygen demand (BOD) of all the collected water samples was determined using the ManTech PC-BODTM analyzer (located at Highway 6 North Guelph, Ontario N1H 6J2 Canada), which provides automated BOD analysis technology. The nutrients (nitrates, nitrites, ammonium, and phosphates) in the samples were analysed using the QuAAtro AutoAnalyzer (manufactured by SEAL Analytical, located at Porvair Sciences Clywedog Road South, Wrexham Industrial Estate, Wrexham, United Kingdom), which employs a continuous segmented flow analysis (CFA/SFA) technique.

Mosquito larvae collection

The collection of mosquito larvae from the selected sites was performed using standard 350 ml larval dippers (BioQuip products, Rancho Dominguez, USA). To ensure adequate collection of larvae, several dips were made at each positive site, and all collected larvae were placed into 2-

litre plastic containers. After collection, the larvae were immediately transported to the laboratory for further analysis and examination. The use of standard larval dippers and plastic containers ensured that the larvae were collected and transported in a safe and secure manner, minimising the risk of contamination and preserving their viability for further analysis.

Morphological characterization of mosquito larvae

The collected mosquito larvae were filtered and placed in shallow plastic trays containing tap water. To provide proper nutrition and growth conditions for the larvae, 200 mg of powdery tetramin baby fish feed was added to the trays every morning. The water in the trays was changed every three days to ensure a clean and healthy environment for the larvae. Once the larvae pupated, the pupae were collected using a 5 ml plastic dropper and transferred to 500 ml plastic cups for the adult mosquitoes to emerge. The cups were covered with a fine cotton net and secured with a rubber band, with a small opening created in the centre for aspirating the emerging adult mosquitoes. This opening was covered with a piece of cotton wool to prevent any mosquitoes from escaping.Once the adult mosquitoes emerged, they were aspirated into 15 ml sterile vials using a standard mouth aspirator (Model 412) and stored in a refrigerator at 4°C to allow the mosquitoes to die. The morphological features of the adult mosquitoes were then observed under a dissecting light microscope and identified based on morphological characters described in previously published keys (26).

Metagenomics

Sample preparation and total genomic DNA extraction

The thirty-five water samples collected were grouped into seven final samples based on the proximity of the sites and the presence or absence of mosquito larvae. Samples that were collected from sites where mosquito larvae were observed were labelled as F1, F2, F4, F5, and F7, while those without larvae were labelled as F3 and F6. The samples were collected from different regions along the river, with F1 and F2 obtained from sites located between Kanana Junction and River Umba in Lunga Lunga, F3 and F4 from sites located between River Umba and Ngozi Girls Secondary School in Jego village, and F5, F6, and F7 collected from sites between Jego village and Kanana Junction.

The preparation of the water samples for the extraction of total genomic DNA was performed as described before (27). To prepare the samples, one litre of each of the final samples was filtered through sterile 0.22 µm filter membranes (Merck Millipore, Burlington, MA) to trap bacterial cells. The filter membranes were aseptically removed from the filtration apparatus and cut into four pieces using a sterile pair of forceps and scissors. The pieces were then placed along the bottom of a 50 ml sterile conical tube with the upper surface of the filter facing the centre of the tube. Thirty millilitres of extraction buffer were added to the tube. The trapped biomass was washed off the filters by vortexing the tubes vigorously, and the cell suspension was transferred to a clean microcentrifuge tube. The tube was incubated in a heating block at 65°C for 30 minutes, with gentle vortexing after every 10 minutes. After the incubation period, the tube was allowed to cool to room temperature, and an equal amount of chloroform: isoamyl alcohol (24:1 v/v) was added and mixed by gentle inversion. The mixture was then centrifuged at 13,200 rpm for 5 minutes at room temperature, and the supernatant was transferred to a new

50 ml tube. Total genomic DNA was then precipitated, cleaned, and resuspended in nuclease-free water. The concentration and purity of the extracted DNA were assessed using 1% agarose gel electrophoresis (28) and a NanoDrop spectrophotometer (29), then stored at -40°C.

Next Generation Sequencing

In this study, the 16S rRNA gene was targeted and amplified using the primers F27 "AGRGTTYGATYMTGGCTCAG" and R1492 "RGYTACCTTGTTACGACTT" (30). The amplified product was then sequenced on the PacBio Sequel platform using PacBio Barcoded M13 Primers for Multiplex SMRT Sequencing. A positive control sample containing 17 known bacterial isolates was used as a mock to test the sequencing and analysis pipelines, while negative controls were excluded from the analysis as they did not yield amplicons.

Analysis of PacBio SMRT sequences

PacBio sequences obtained were processed and visualised using the RS_ReadsOfInsert protocol in the SMRT Analysis software version 2.3 to obtain demultiplexed consensus sequences with a minimum of three full passes. The resulting sequence data were processed using the Divisive Amplicon Denoising Algorithm2 (DADA2) pipeline (31) in R version 4.2.1, R Core Team (2022) as follows. First, the F27 and R1492 primers were removed from the raw sequences and the quality of the reads was inspected. The sequences were then filtered using the parameters; minQ=2, minLen=500, maxLen=1600, maxN=0, rm. phix=FALSE, maxEE=2 and dereplicated to combine all identical sequencing reads into “unique sequences” with a corresponding “abundance” equal to the number of reads with that unique sequence. After denoising, the DADA2 algorithm was used to infer the true sequence variants from the unique sequences in all samples. Chimeric sequences were then identified and removed from the resulting amplicon sequence variants (ASVs) using the “removeBimeraDenovo()” algorithm. Taxonomy was then assigned at species level to the amplicon sequence variants trained on the SILVA database (32) in the DADA2 package which provides a native implementation of the naive Bayesian classifier method (silva_nr99_v138.1_train_set.fa.gz and silva_species_assignment_v138.1.fa.gz.).

To assess the reliability of the sampling depth, the ASV tables were rarefied and the precision of the rarefaction curves was estimated using the bootstrapping method (33). Alpha diversity measures were calculated using *vegan* package version 2.6-2 (34) in R from the number of ASVs. Chao1 estimate of species richness based on abundance, Shannon diversity estimate based on species richness and evenness emphasising more on species richness and Simpson diversity index based on species richness and evenness putting more weight on species evenness (35–37). To test whether there was any significant difference between parameters measured on the alpha diversity, non-parametric Kruskal-Wallis test was performed in R. Beta diversity of bacterial communities between different samples was computed using principal coordinate analysis (PCoA) implementing Bray-Curtis dissimilarity and Jaccard’s distance metrics and compared by Permutational Analysis of Variance (PERMANOVA) (38). Weighted and Unweighted UniFrac distance metrics coupled with the principal coordinates analysis (PCoA) were used to test if the phylogenetic lineages between samples were significantly different (39). An agglomerated phylogenetic tree was constructed using the Neighbor-Joining

algorithm to visualise the evolutionary relationships of different bacterial families between the positive and negative sites (40). Differential abundance analysis was performed using the Deseq algorithm in R to evaluate the bacterial genera that were significantly different between the positive and negative sites (41)

Data analysis

All statistical data analysis was performed in XLSTAT (42) and R statistical program version 4.2.1 (R Core Team, 2022). Physical characteristics of positive and negative sites were represented in percentages and compared using the Z-test. Physicochemical data was summarised using mean and standard deviation. Student's t-test and Permutational Multivariate Analysis of Variance (PERMANOVA) were used to compare physicochemical parameters between the positive and negative sites at 95 % confidence interval (38,43–45). The Principal components analysis (PCA) was conducted to identify the relationships between different parameters and the sites. To test whether there was any significant effect associated with the physicochemical parameters on the alpha diversity of bacteria, non-parametric Kruskal-Wallis test was performed (46).

RESULTS

Mosquito larvae distribution

We sampled a total of 65 sites during the study period and evaluated them for the presence (positive) or absence (negative) of mosquito larvae as described in Figure 2A. During the dry season, we sampled 35 sites, of which 19 (54.28%) were positive and 16 (45.72%) were negative (P = 0.321, 95% CI: 0.068, 0.228), indicating that the number of positive and negative sites was not significantly different. However, in the rainy season, we found that 26 out of 30 sampled sites (86.67%) were positive and only 4 (13.33%) were negative for mosquito larvae (P < 0.0001, 95% CI: 0.637, 0.843). The overall positive rate for the presence of mosquito larvae was 69.23% while the negative rate was 30.77% (P < 0.0001, 95% CI: 0.242, 0.518).

We collected 1,360 mosquito larvae and reared them into adult mosquitoes, which were then identified as *Anopheles gambiae* or *Culex* sp. with 68.34% (P < 0.0001, 95% CI: 0.221, 0.499) and 71.32% (P < 0.0001, 95% CI: 0.306, 0.574) abundance, respectively. The average percentage of *Anopheles gambiae* across both seasons was 69.83% (P < 0.0001, 95% CI: 0.263, 0.567). We found that 63.15% of the positive sites had *Anopheles gambiae* larvae only, 5.20% had *Culex* sp. larvae only, and 31.65% had both *Anopheles gambiae* and Culex sp. larvae (X2 = 5.991, df = 2, P < 0.0001) (Figure 2B). This suggests that during the study period, more habitats were suitable for the breeding of *Anopheles gambiae* compared to other species.

Physical characteristics of proliferation sites

Most of the sites were natural habitats (94.28%) while only 5.71% were artificial, such as man-made dams and road culverts (P < 0.0001, 95% CI: 0.8295, 0.9704). Natural habitats included marshy areas, shallow rivers, roadside pools, and animal hoof-prints. 65.71% of the sites had mud substrates while 34.28% had sand (P = 0.006, 95% CI: 0.179, 0.461). In terms of permanence, 65.7% of the sites were semi-permanent, while the rest were permanent (P < 0.0001, 95% CI: 0.158, 0.442). Most of the sites were fully exposed to sunlight (94.28%, P < 0.0001, 95% CI: 0.8295, 0.9704) and had a shallow depth of less than 1m (77.14%, P < 0.0001, 95% CI: 0.413, 0.667) with an average size of less than 10m2 (94.28%, P < 0.0001, 95% CI: 0.8295, 0.9704). In terms of vegetation, 82.86% of the habitats had some form of vegetation while 17.14% had no vegetation at all (P < 0.0001, 95% CI: 0.546, 0.774). The habitats were grouped into four categories based on the type of vegetation present. The majority of the habitats had only algae (54.28%, P < 0.0001, 95% CI: 0.144, 0.458). Some habitats had a combination of algae, submerged, and emergent vegetation (11.42%), others had algae and emergent vegetation (8.57%), and a few had algae with only emergent vegetation (8.57%).

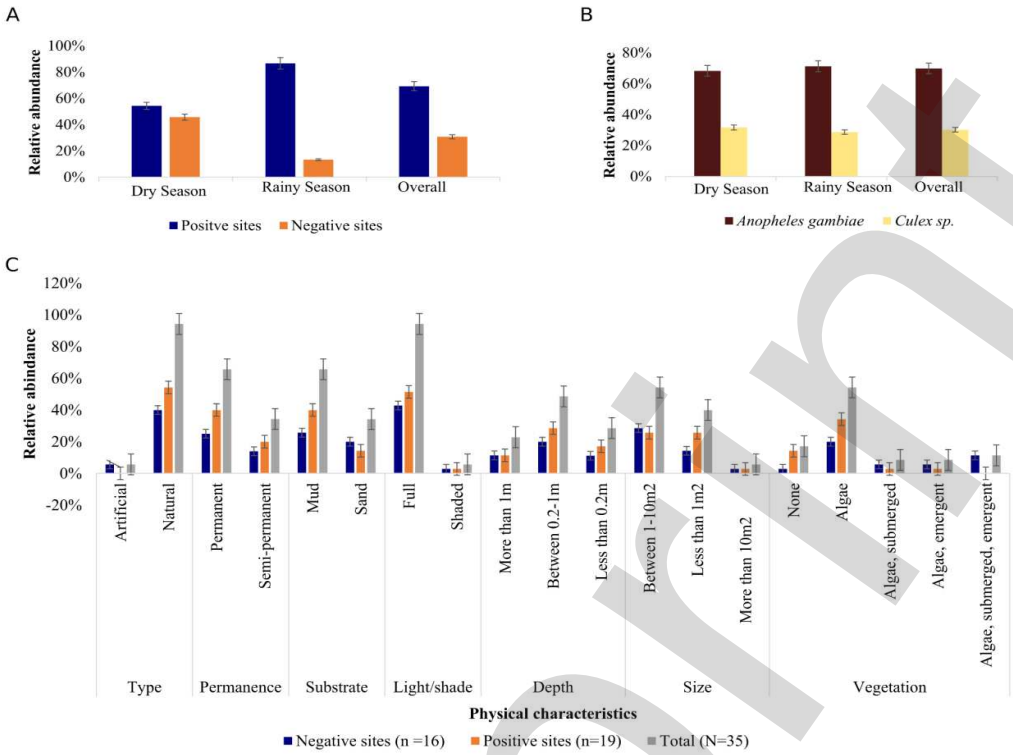


Figure 2: A) *Abundance of mosquito larval habitats (N=65)* ***B)*** *Abundance of mosquito species in larval habitats (N=1360)* ***C)*** *Physical characteristics of larval habitat (N=35)*

Physicochemical parameters of the sites

The mean temperature at positive sites was significantly lower than that at negative sites during the dry season (t = 1.729, df = 19, P = 0.0416), but there was no significant difference in temperature between positive and negative sites during the rainy season. A pairwise comparison of the individual physicochemical parameters using the Student's t-test between the dry and rainy seasons showed that salinity (t = 1.692, df = 33, P = 0.01104), electrical conductivity (t = 1.689, df = 33, P = 0.01617), total dissolved solids (t = 1.690, df = 33, P = 0.01204), and ammonia (t = 1.675, df = 33, P = 0.00029) were significantly lower during the rainy season compared to the dry season, while the other variables were not significantly different. The mean, standard deviations, and P-values for the Student's t-test of the physicochemical parameters between positive and negative sites evaluated during the dry and rainy seasons are summarised in *Table 1*.

338 ***Table 1:*** *Comparison of physicochemical parameters in the sites between dry and rainy seasons*

Parameters		**Temp**	**PH**	**DO**	**BOD_5**	**Salinity**	**E.C**	**TDS**	**TUR**	**NO_3^-**	**NO_2^-**	**NH_3**	**PO_4^{3-}**
(N = 30)		**(^{0} C)**	**(mg/l)**	**(mg/l)**	**(mg/l)**	**(ppt)**	**(us/m)**	**(mg/l)**	**(NTS)**	**(umol/l)**	**(umol/l)**	**(umol/l)**	**(umol/l)**
Dry season	Mean	31.74	8.431	6.57	3.94	2.653	4951.8	2861.9	350.7	23.21	5.4685	23.87	3.97
	SD	±2.27	±0.76	±4.2	±4.26	±3.989	±6831	±3938	±366	±12.4	±3.5	±12.8)	±3.79
Rainy	Mean	32.06	8.43	5.84	2.72	0.628	1650	875.86	469.6	24.14	4.5	12.81	3.58
season	SD	±1.44	±0.52	±2.0	±1.63	±1.04	±2162	±1161	±349	±7.1	±2.99	±8.91	±3.23
Overall	Mean	31.9	8.433	6.20	3.32	1.624	3274.1	1852.6	411.1	23.67	4.98	18.25	3.776
	SD	±1.89	±0.65	±3.29	±3.23	±3.04	±5259	±3228	±360	±10.0	±3.262	±12.28	±3.49
P- value		0.511	0.986	0.39	0.152	0.01104	0.0161	0.0120	0.200	0.722	0.254	0.0003	0.6638

339 Temp – Temperature, DO – Dissolved oxygen, BOD5 – Biological oxygen demand after 5-days incubation, E.C – Electrical conductivity, TDS –
340 Total dissolved solids, TUR – Turbidity, NH_3 - Ammonia

Metagenomic analysis of 16S rRNA gene sequences

Diversity of bacterial communities

From the rarefaction curves plot, a plateau phase was achieved for all samples except F6 as shown in *Figure 3.* This means that the sampling intensity was adequate to deduce the correct bacterial diversity from the samples examined. Several alpha diversity indices for the positive and negative sites were also computed and compared shown (*Figure 4*). All the alpha diversity indices were highest in sample F7 and lowest in sample F6 and they were highly variable between the sites, an indication that the bacterial community richness, evenness and abundance were not similar between sites ($F = 2.928$, $df = 8$, $P = 0.032$). However, the physicochemical parameters tested had no effect on the diversity indices of bacterial communities between sites (Kruskal-Wallis chi-squared = 6, df = 6, P = 0.4232). Samples taken from positive sites were noted to have higher alpha diversity indices in comparison to those from negative sites and therefore indicating that the presence of mosquito larvae was correlated with high bacterial richness and evenness ($R^2 = 9.822$, $df = 1$, $P = 0.00197$). For beta diversity analysis The Positive and negative samples clustered differently on the plot (*Figure 5 and 6*), although the differences were not statistically significant ($R^2 = 0.18157$, $df = 1$, $P = 0.353$). This suggests that the presence or absence of the mosquito larvae was not correlated with the bacterial community structure.

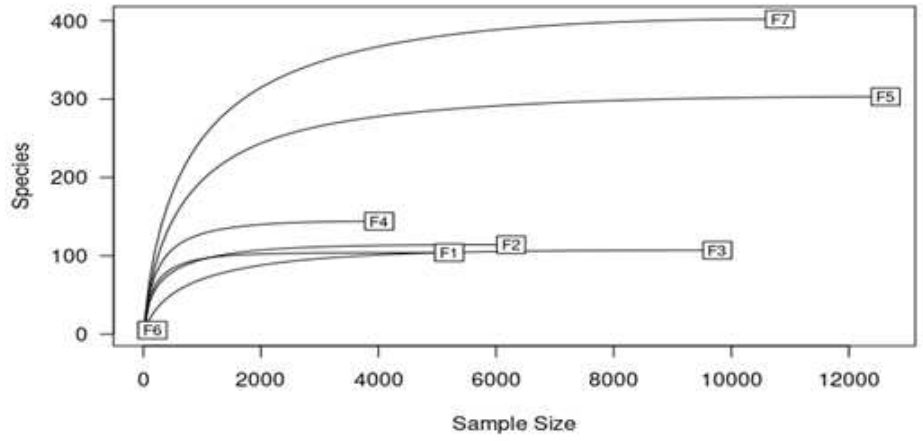


Figure 3: **Rarefaction curves for the samples analysed.** Curves reached a plateau except for sample F6 meaning that the sampling depth was adequate.

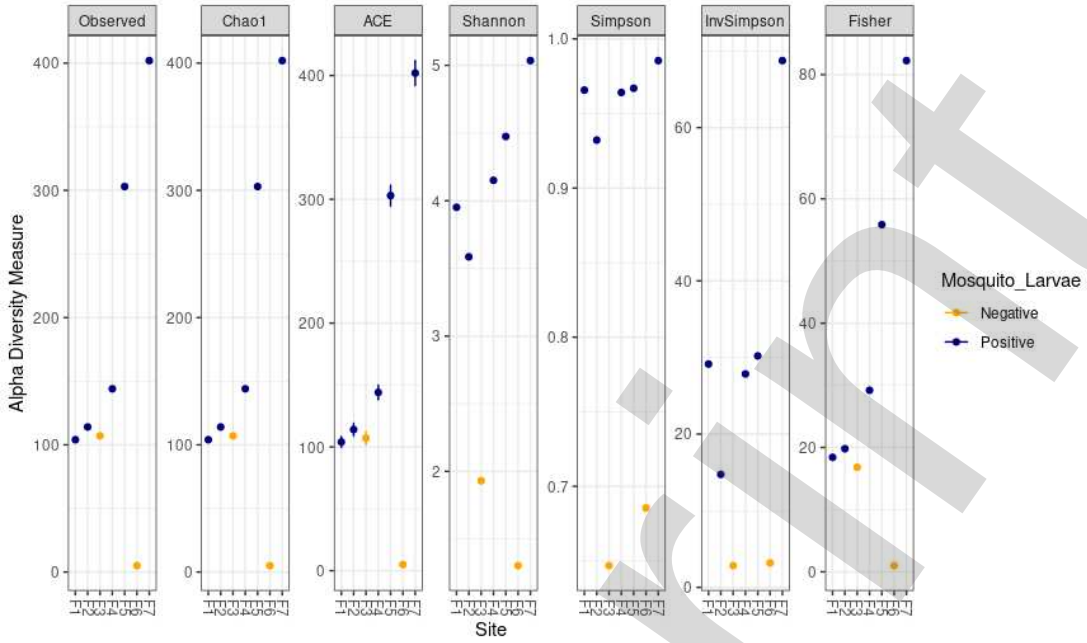


Figure 4: **Alpha diversity indices using different methods for each sample**. The negative (yellow) indicates sites where mosquito larvae were not detected while positive (blue) represents the sites where mosquito larvae were found. Positive sites were observed to have higher alpha diversity indices compared to negative ones that did not have mosquito larvae.

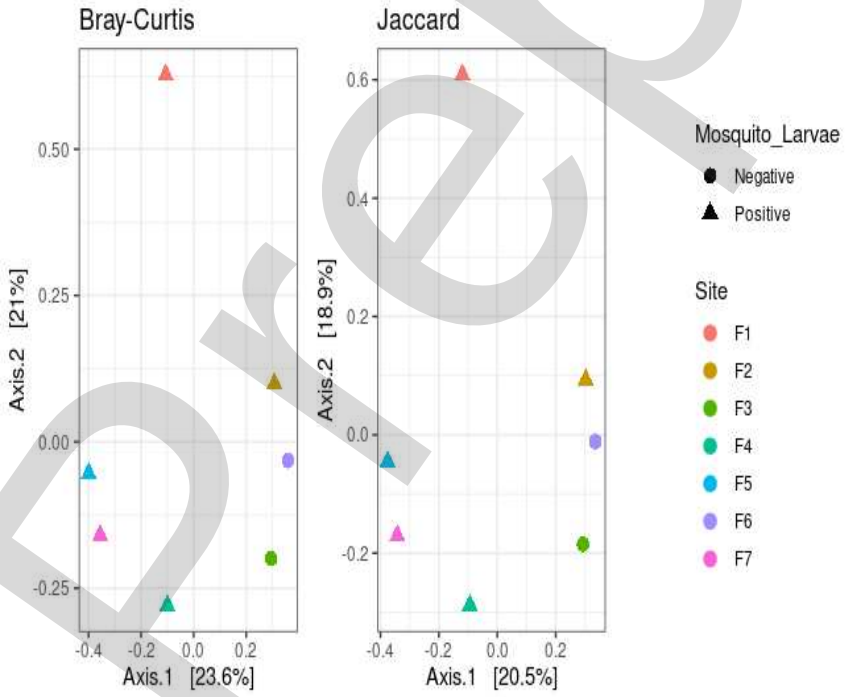


Figure 5: **Beta diversity analysis using Bray-Curtis and Jaccard's distance matrices.** The PCOA plot represents each sample as a dot, which is coloured according to their sampling site and shaped according to the presence (positive) or absence (negative) of mosquito larvae. First, this two-dimension PCOA plot

shows 44% of the total variance between the samples using Bray's dissimilarity matrix and 39% of total variance using Jaccard's dissimilarity matrix. The samples that did not have mosquito larvae clustered in the bottom right of the grid.

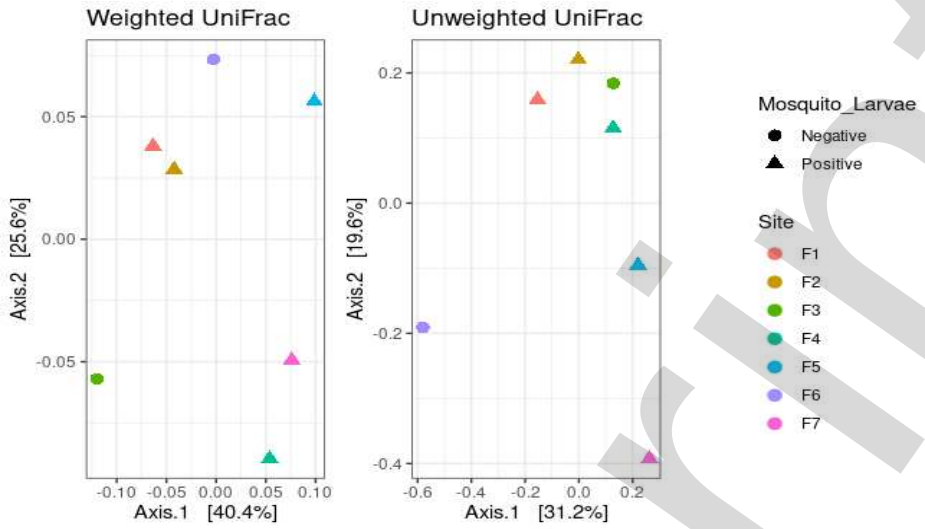


Figure 6: **Beta diversity using weighted and unweighted UniFrac distance matrices.** PCOA plot shows 51 % of the total variance between the samples using unweighted UniFrac distance matrix and 65% of total variance using weighted UniFrac distance matrix. The results indicate that there was no significant difference between the positive and negative sites.

Assignment of taxonomic units

Sequencing of the full 16S rRNA generated 203,934 reads from 7 environmental samples with an average of 29133 reads per sample. The average length of the reads was approximately 1450 base pairs, which aligns with the expected full length of the 16S rRNA gene. After applying various quality control measures such as trimming, filtering, and denoising, we were left with 50,804 reads that represented 935 amplicon sequence variants (ASVs). These ASVs were taxonomically assigned to 17 phyla, 37 classes, 72 orders, 83 families, and 138 genera. The majority of the ASVs (71.76%) were classified up to the genus level, while a smaller proportion was assigned up to the family (16.36%), order (6.73%), and class (3.3%) levels. The most commonly recovered phylum was Proteobacteria, which accounted for 48.16% of the total ASVs, followed by Bacteroidota (32.91%) and Actinobacteriota (10.94%). These three phyla were present in all of the samples. Additionally, Actinobacteriota, Verrucomicrobiota, and Patescibacteria were present in all samples except sample F6. The results of the taxonomic assignment are visualised in *Figure 7, Figure 8, Figure 9, Figure 10, Figure 11*. Phylogenetic analysis demonstrated that there were more families associated with the positive sites than the negative sites (*Figure 12*). Further, differential abundance analysis revealed that 19 genera of bacteria were significantly more abundant in the positive sites than in the negative sites (*Figure 13*).

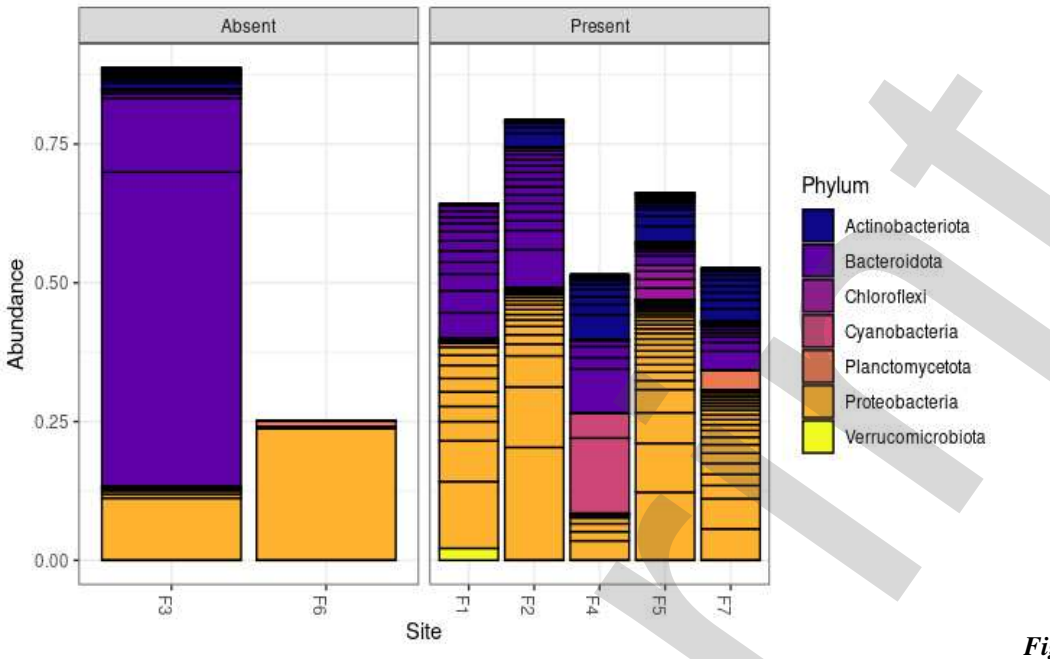


Figure 7: Phylum bar plot. Absent = Negative, Present = Positive, the bar plot represents bacterial phyla as observed in the positive and negative sampling sites. The figure illustrates that microbial phyla in the positive sites were more diverse compared to those in positive sites.

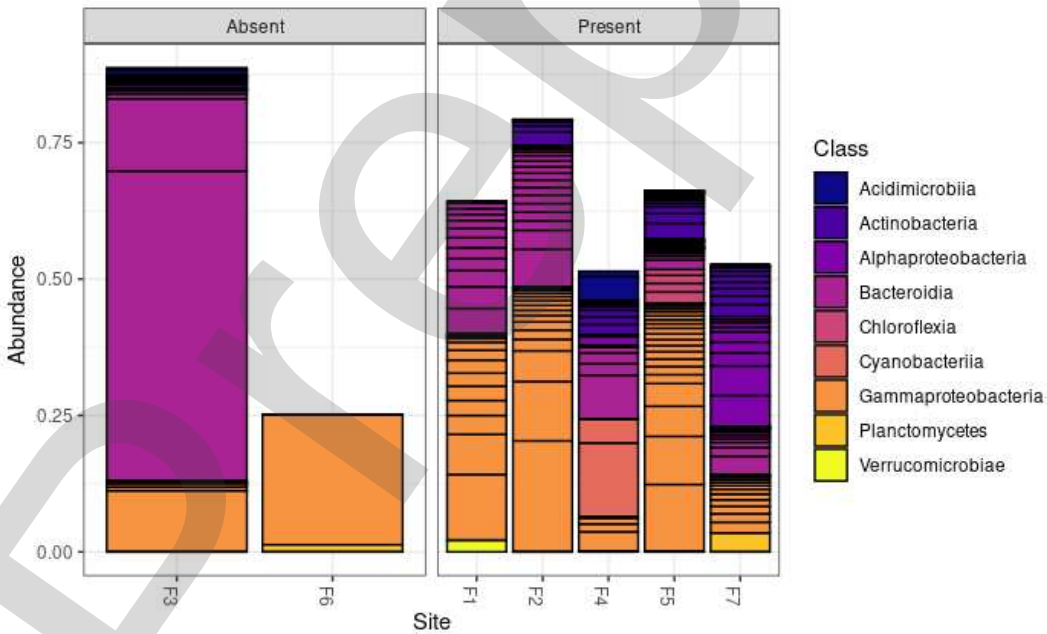
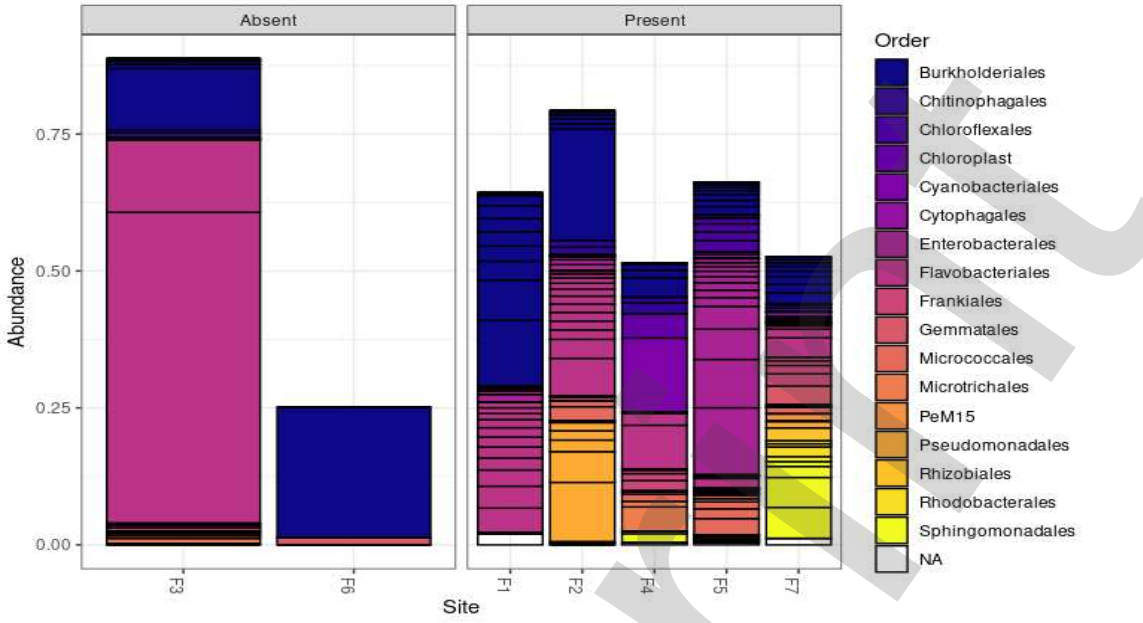
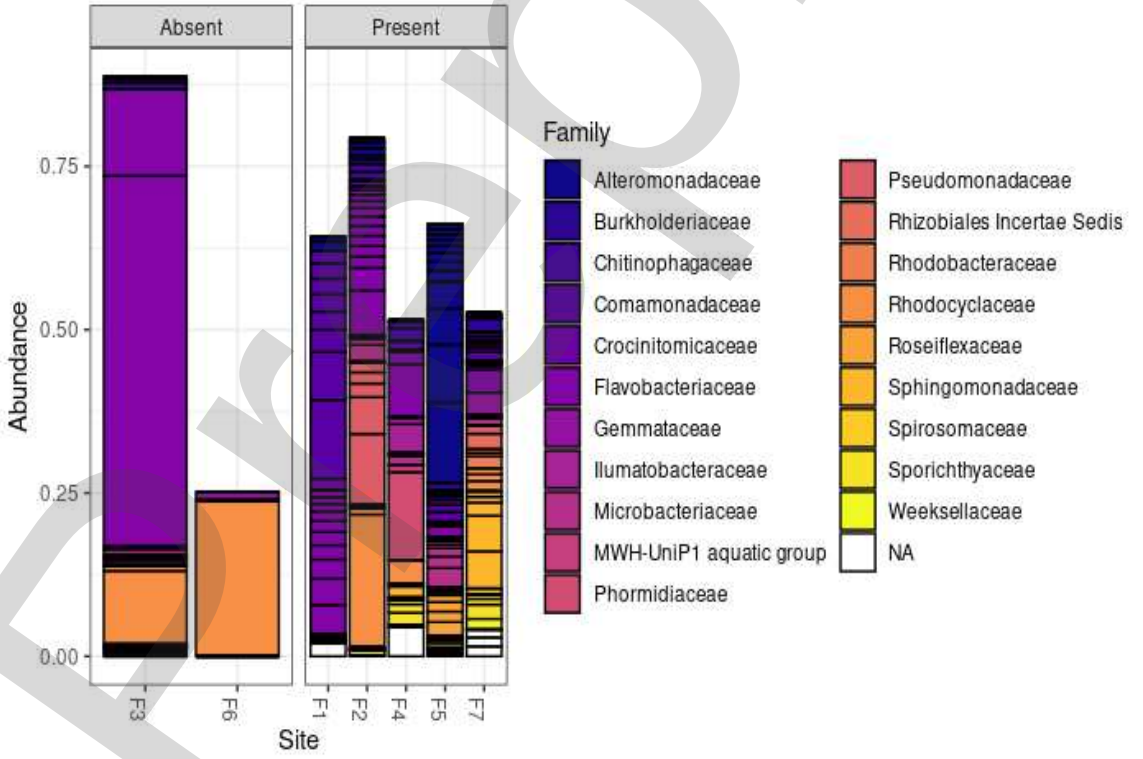


Figure 8: **Phylum plot showing the most abundant classes of bacteria in the positive and negative sampling sites.** Absent = Negative, Present = Positive



404
405 ***Figure 9:*** **Order bar plot showing the most abundant orders of bacteria observed in positive and**
406 **negative sites.** Absent = Negative, Present = Positive



407
408 ***Figure 10:*** **Family bar plot showing the most abundant families of bacteria detected in both**
409 **positive and negative sites.** Absent = Negative, Present = Positive

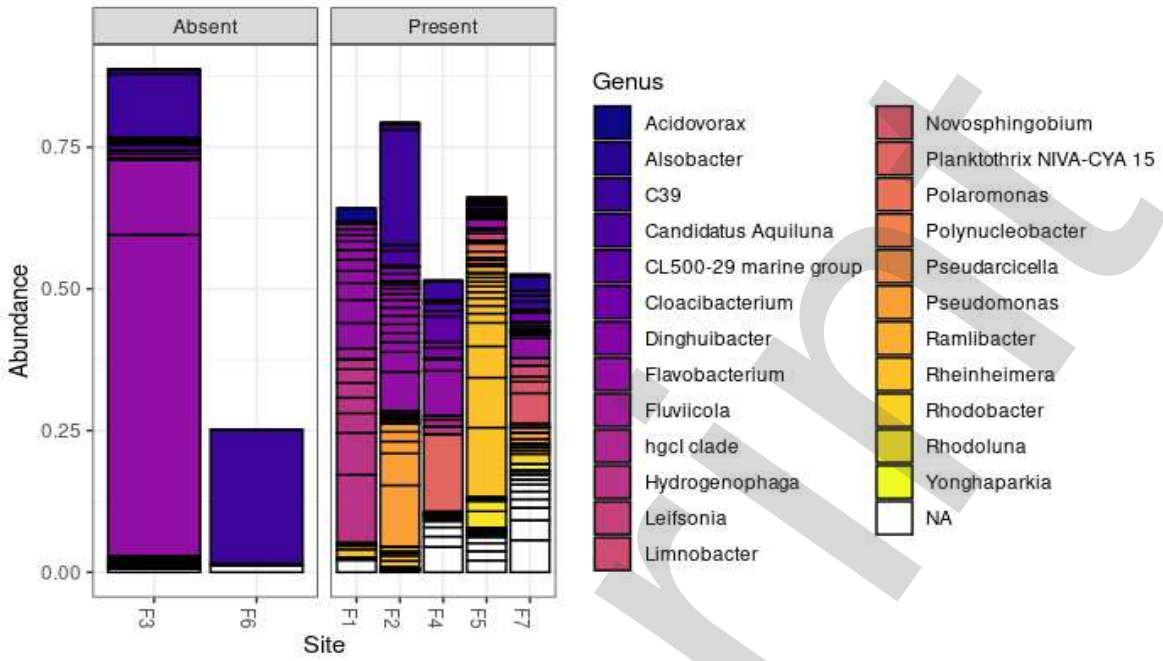


Figure 11: Genus bar plot. Absent = Negative, Present = Positive, the bar plot represents bacterial genera as observed in the positive and negative sampling sites. The figure illustrates that bacterial genera in the positive sites had more features compared to those in positive sites.

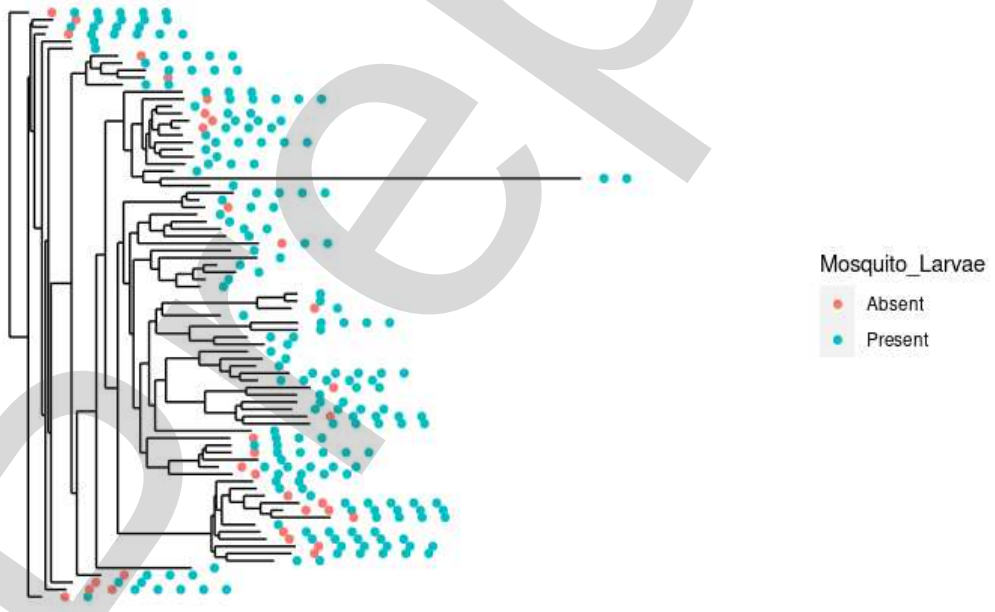


Figure 12: **Family agglomerative phylogenetic tree.** Absent = Negative sites, Present = Positive sites. The tree is generated using the Neighbor-Joining algorithm and shows bacterial families as observed in the positive and negative sites. The figure illustrates that in the positive sites there were more bacterial families compared to negative sites.

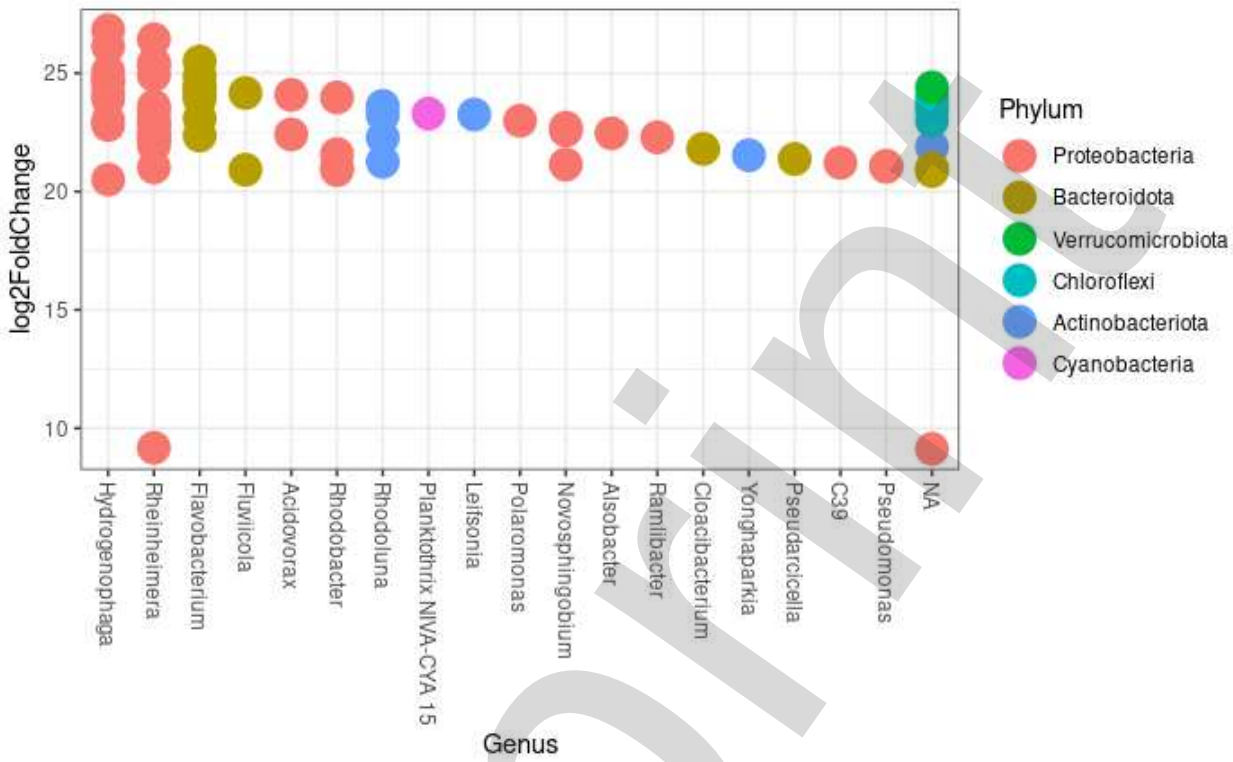


Figure 13: **Differential abundance analysis of bacterial genera.** The figure shows bacterial genera whose abundance was significantly different between the positive and negative sites.

DISCUSSION

Mosquitoes Proliferation sites

During the dry season, the number of positive sites was comparable to that of negative sites. However, during the rainy season, there were more positive sites than negative ones. This result indicates that the rainy season's rainfall patterns can impact the reproduction and distribution of mosquitoes in the environment. The increased humidity and availability of water during the rainy season create favourable conditions for mosquito proliferation. The presence of positive sites even during the dry season is a concern as it contributes to malaria transmission year-round.

The majority of the sampled sites were found to contain *Anopheles gambiae* larvae, with only a few containing *Culex* mosquitoes. This indicates that the conditions in these sites were more conducive to the proliferation of *Anopheles gambiae* than other species. The co-occurrence of both mosquito genera in the same habitat was also observed in this study. This result is in agreement with previous research that showed that the two genera can coexist in the same habitat despite having different breeding requirements (47,48). These findings are consistent with those found in southern Ghana during the rainy season which showed a significant presence of *Anopheles* mosquitoes in urban areas (49). However, low abundance of *Anopheles* mosquitoes was reported during the rainy season in the Korhogo area of northern Cote d'Ivoire (50), which is in contrast to these findings. It is important to note that heavy rains and floods

441 can wash away the proliferation sites of *Anopheles* mosquitoes, eliminating the mosquito eggs
442 and larvae (51). In this study, no floods were witnessed in the study areas during the rainy
443 season, which may account for the high proportion of *Anopheles* mosquito larvae observed.

444 **Physical characteristics of proliferation sites**

445 Most proliferation sites identified were natural in the form of marshes, swamp margins, edges
446 of shallow rivers, roadside pools and animal hoof-prints. This is in agreement with past studies
447 which found that *Anopheles* mosquitoes prefer to breed near human settlements along the
448 edges of shallow rivers, transient roadside puddles, marsh margins, and tree holes (52–54).
449 Additionally, similar mosquito proliferation sites were discovered in Western Kenya and in
450 Ethiopia (55,56). In contrast to these results, Hinne and others (57) categorised the majority of
451 anopheline larval habitats found in Ghana's three main ecological zones as man-made. The low
452 abundance of artificial mosquito proliferation sites in this region could be explained by the low
453 levels of infrastructural development and less human activities on the environment since the
454 local community is composed of small-scale farmers cum pastoralists and traders in a rural set-
455 up.

456 More sites sampled had mud substrates and were semi-permanent. Faehler and others (58)
457 suggested that the type of soil in a larval habitat and its quality can determine the chances of
458 survival and influence the development of *Anopheles* mosquito larvae. *Anopheles gambiae* s.l.
459 proliferate in habitats with hydromorphic and holomorphic soil substrates due to their ability to
460 retain water for a longer time and also to provide a conducive saline environment for growth of
461 the mosquito larvae (59). Semi-permanent and temporal mosquito larval habitats were also
462 observed in Western Kenya (60). This might be because there are fewer predators for the
463 larvae in smaller temporary habitats than in larger permanent habitats (61).

464 A majority of the sites observed were exposed to full sunlight and had a shallow depth of less
465 than 1m with an average size of less than $10m^2$. The growth of algae, a vital source of
466 nourishment for developing mosquito larvae, depends on the presence of sunlight in a larval
467 habitat (61). Sunlight also warms the water to a suitable temperature that is conducive for
468 growth and development of the mosquito larvae (57,62,63). *Anopheles* mosquitoes prefer
469 breeding in small and shallow water bodies as those observed in this study (51,52,55,56,64).
470 Small and shallow water bodies are more suitable for mosquito breeding since they are less
471 vulnerable to water currents and tides which can wash away the mosquito eggs and larvae as
472 compared to large and deep water bodies (65). These sites are also unsuitable for habitation by
473 other organisms which may be competitors or predators of the mosquito larvae (66). On the
474 other hand, small and shallow water bodies are more likely to dry faster especially if they are
475 not associated with a larger water body.

476 The most prevalent type of flora found in the sites was algae. Since algae provides the larvae
477 with nourishment, it was positively correlated with the presence of *Anopheles* mosquito larvae
478 at potential proliferation sites (57,62). The proportion of sites with high vegetation cover
479 consisting of algae, emergent and submerged vegetation was very low and none was positive
480 for the mosquito larvae, an indication that the presence of mosquito larvae was inversely

correlated to the amount of vegetation in the water body. High levels of vegetation growth generally interfere with light penetration in the water and hence affect the growth of algae and the temperature of the water body (57,67,68).

Physicochemical parameters of the sites

Generally, the physicochemical parameters evaluated in this study were observed to be high. High levels of physicochemical parameters in environmental water samples can be attributed to pollution either from human settlements or from agrochemicals used in farms (48,69). Except for the temperature, which was lower in the positive sites than in the negative sites during the dry season, there was no other noticeable difference between the positive and negative sites during either the dry or rainy seasons. According to this study, there was no apparent difference in temperatures between the dry and rainy seasons. Notably, temperatures reported in both seasons encouraged the presence of *Anopheles* mosquito larvae, and this was consistent with the findings obtained in different places (70,71). This indicates that mosquitoes have become less selective to environmental parameters, allowing them to breed in locations with varying levels of physicochemical parameters.

Although electrical conductivity, total dissolved solids, salinity, and ammonia were significantly lower in the rainy season than in the dry season, there was no evidence that these variables could influence the mosquito larval presence or absence at the sites throughout the two seasons. However, it is notable that the levels recorded for conductivity, total dissolved solids, salinity, and ammonia in both seasons were favourable for mosquito breeding, which was comparable to the findings of a study conducted on a Nigerian university campus (72). The low levels of these parameters during the rainy season can be attributed to the dilution of environmental surface water by rainwater (64,71). However, Emidi and others (47) reported a positive correlation between *Anopheles* mosquito larval abundance, salinity, and conductivity. Mosquitoes prefer breeding in sites with alkaline pH (10,73,74), which concurs with the findings of this study since most of the sites had alkaline pH levels except one negative site, which was slightly acidic. High pH levels in the sites were positively correlated with high levels of dissolved oxygen, biological oxygen demand, and nutrients.

Dissolved oxygen, biological oxygen demand, pH, and nutrients evaluated in this study were positively correlated and negatively correlated with turbidity. The presence of high levels of nitrates, nitrites, ammonia, and phosphates can be attributed to the use of fertilisers containing ammonium and phosphorus in the farms since most of the sites were adjacent to the farms, while turbidity is associated with silt, mud, algae, and plant pieces (75). The high level of nutrients has been reported to promote excessive growth of water plants and microorganisms in the water bodies which reduces turbidity of the water making it more suitable for the proliferation of mosquitoes (76,77). Similar research in Western Kenya revealed that the amount of nutrients in the proliferation sites had no effect on whether *Anopheles* mosquito larvae were present or absent (78). Excessive growth and multiplication of microorganisms in water bodies affects their biological oxygen demand and is an indicator of water pollution (79). Finding *Anopheles gambiae* mosquitoes larvae in polluted water is uncommon since the species is believed to prefer proliferating in clean, unpolluted water in the environment.

However, the presence of *Anopheles* mosquito larvae in unclean polluted water has been reported (47), which shows that the mosquitoes could have become more adapted to survive in polluted water to enhance their chances of survival. This might have an impact on how mosquitoes are distributed and abundant in the environment, which would then have an impact on how quickly and frequently malaria spreads.

Metagenomic analysis of bacterial communities

According to beta diversity analysis, the bacterial composition was not correlated to the presence or absence of mosquito larvae but rather may have been influenced by the sites' locations. Previous studies also found no association between bacterial composition and the occurrence or absence of mosquito larvae in potential proliferation sites (78,80,81). However in other studies, the structure of bacterial communities in mosquito larval habitats was correlated with the presence of mosquito larvae (82,83). While the two different situations could not be explained with certainty, it is hypothesised that the geographical location of these sites may have an impact on the bacterial compositions in mosquito larval habitats. There was a difference in bacterial communities between sylvatic and domestic proliferation sites of *Aedes aegypti* in Gabon and these bacterial communities are also correlated with those present in the midgut of the adult mosquitoes (84). This similarity in the bacterial community profiles could suggest that the origin of bacteria in the sites was the same for both positive and negative sites.

The findings of this study indicate that the alpha diversities were not similar between the sites, an indication that the bacterial community richness, evenness and abundance was distinct and independent in each of the sites. Additionally, it was observed that the alpha diversities of bacteria were generally higher in the positive sites than in the negative sites and that these differences were not associated with any of the physicochemical parameters that were evaluated. Although the differences observed in alpha diversity between the sites could not be explained with certainty, it is suggested that they may be influenced by factors like age, the presence or absence of mosquito larvae, and the physical location of the sites.

This concurs with other studies that have suggested a positive correlation between bacterial abundance and the age of the larval habitats (4,85). Furthermore, it is suggested that mosquito larvae can modify the bacterial communities in their habitats either through feeding or the egestion of bacteria, which could also explain the higher alpha diversity in the positive sites (86). Mosquito larval activities such as feeding and excretion in the habitats may promote the development of optimal conditions for the growth of bacteria which could not find an ideal environment for growth in uncolonized sites and hence such bacteria may often go undetected (87).

The ASVs were assigned to 17 phyla, 37 classes, 72 orders, 83 families, and 138 genera. These phyla; Proteobacteria, Bacteroidota and Actinobacteriota dominated in all the sites accounting for 92.04 % of the total ASVs and have also been reported in other studies of mosquito habitats. A similar study conducted to identify the dominant bacterial communities associated with the larval habitats of *Anopheles darlingii* in the Amazon basin reported the same bacterial

phyla with the most dominant phyla being Proteobacteria, Firmicutes, Bacteroidota, and Actinobacteriota (88). These phyla were also observed in the larval habitats of *Anopheles coluzii* and *Anopheles gambiae* in Cameroon, as well as on three Kenyan Islands in Lake Victoria (78,89). A separate study carried out in Kenya, pointed out that these same phyla were prevalent in semi-natural habitats of mosquito proliferation, although the study also revealed that Cyanobacteria was the second most abundant phylum (90). Furthermore, the same phyla recorded were the most abundant in household water-storage containers in India (83).

At the class level, the most common groups of bacteria found were Gammaproteobacteria, Bacteroidia, Alphaproteobacteria, and Actinobacteria, accounting for a total of 76.67% of all bacteria detected. Gammaproteobacteria was also found to be the most prevalent class of bacteria in studies conducted in different places (88,91). Although Bacilli were not among the commonly detected groups in the current study, it was reported as one of the most abundant classes in a previous research (88). Similarly, Alphaproteobacteria and Cyanobacteria were found to be the most common classes associated with semi-natural mosquito habitats in Kenya (90). Meanwhile, Betaproteobacteria and Alphaproteobacteria were identified as the most abundant bacterial classes in household water-storage containers in India (83), while a study on Kenyan Islands of Lake Victoria found Betaproteobacteria to be the most common class of bacteria in the mosquito larval habitats (78). Other frequently found classes include Verrucomicrobiae, Planctomycetes, Microgenomatia, Gemmatimonadetes, Acidimicrobiia, Cyanobacteriia, Chloroflexia, and Saccharimonadia.

In this study, the most frequently observed orders of bacteria were Burkholderiales, Flavobacteriales, Chitinophagales, Sphigomonadales, Micrococcales, Rhizobiales, Sphigobacteriales, Enterobacterales, Frankiales, and Cytophagales. It was noted that Burkholderiales and Cytophagales were considered indicator species in water samples collected from the breeding sites of *Anopheles darlingii* (88). Many of the families detected in the study have been previously associated with *Anopheles* mosquitoes, with the most abundant being Commamonadaceae, Flavobacteriaceae, and Chitinophagaceae (78,88,92–96). In a different study, it was evident that the most abundant families in the larval habitats of *Aedes albopictus* in Italy were Sphingobacteriaceae, Spirosomaceae, Chitinophagaceae, Cellvibrionaceae, Burkholderiaceae, Caulobacteraceae, Planococcaceae, Cytophagaceae, and Blastocatellaceae (87).

There were 16 most commonly identified genera that were significantly abundant in the positive sites namely; *Flavobacterium*, *Acidovorax, Rhodoluna, Leitsonia, Polaromonas, Alsobacter, Cloacibacterium, Yonghaparkia, Ramlibacter*, *Dinghuibacter*, *Rheinheimera*, *Hydrogenophaga*, *Novosphingobium*, *Pseudomonas*, *Rhodobacter* and *Fluviicola*. Athough it was not clear about the role of these bacteria in mosquito breeding from this study, understanding the role of specific bacteria in mosquito breeding sites can help in developing effective mosquito control strategies, such as the use of bacteria as biological control agents or the manipulation of environmental conditions to limit the growth of certain bacterial species. It was found that *Bacillus*, *Pseudomonas*, *Micrococcus*, and *Serratia* were the dominant genera in bacterial

communities associated with mosquito proliferation sites in Ethiopia (97). A similar study reported the presence of *Rubrivivax*, *Hydrogenophaga*, *Rhodobacter*, *Pseudomonas*, and *Flavobacterium* in mosquito larval habitats in Western Kenya (78). These bacteria were discovered in the larval habitats of *Aedes aegypti* associated with domestic water storage containers in Thailand and Laos (68). Bacterial communities present in mosquito larval habitats may serve as indicator species for high potential proliferation sites and hence affect the larval survival and growth, adult fitness, vector abundance and distribution, and therefore impacting on malaria transmission (98).

Conclusion

Proteobacteria, Bacteroidota, and Actinobacteriota predominated in all the sites, making up 92.04% of the total ASVs classified. There were 16 most commonly identified genera that were significantly abundant in the positive sites. The presence of *Anopheles* mosquito larvae was found to be positively correlated with the rainy season and bacterial abundance, and negatively correlated with Electrical conductivity, total dissolved solids, salinity and ammonia. This shows that mosquito oviposition and subsequent population growth are possible in environmental water samples with a variety of physicochemical properties and bacterial community compositions. The findings of this study, along with comparisons to previous studies, suggest that both the time period and geographical location have a significant impact on the structure of the bacterial communities in mosquito larval habitats. This study provides further understanding of the bacterial communities in mosquito habitats and may have implications for predicting the potential of environmental water samples becoming proliferation sites. More studies to investigate the bacterial communities in the potential proliferation sites before and after oviposition may provide useful information on the role of mosquito larvae in shaping the water bacterial communities which can be used to predict the probability of environmental water collections becoming proliferation sites.

Author contributions

J. Mutinda, J.K. Nonoh, R.M. Ntabo and M.V. Omollo contributed to conceptualization and design of the study. Sample collection was done by K.O. Oduor, J. Mwangangi and J. Mutinda. Laboratory analysis was conducted by J. Mutinda, J.M. Gathiru and K.O. Oduor while S.M. Mwamburi, J. Mutinda, and K.O. Oduor carried out statistical analysis, interpreted the result of the study and wrote the first draft of the manuscript. All authors assisted in review, reading and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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Ethical approval

Before sampling, the owners of private land with mosquito breeding grounds provided informed consent. Research permit was obtained from the National Council for Science, Technology, and Innovation (Licence No: NACOSTI/P/21/10048). Because the study did not include human samples, national parks or endangered species, no special permits were required.

Consent for publication

Not applicable.

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