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Bacterial communities structure in constructed wetlands for municipal and industrial wastewater treatment in Tanzania

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ABSTRACT

Bacteria are the largest and most essential microorganisms in wastewater treatment systems. Despite the vital role bacteria play in enhancing wastewater treatment, its community structure and diversity remain poorly known. This study elucidated bacterial diversity and community structure in constructed wetlands (CWs) for municipal and industrial wastewater treatment using a culture-independent approach. Analysis of physicochemical parameters and Illumina high-throughput sequencing of V3 and V4 hypervariable regions of 16S rRNA gene bacterial community was performed. The results showed that the *Proteobacteria* were dominant (48.66%) phyla across all CWs. The *Gammaproteobacteria* class (27.67%), family *Comamonadaceae* (35.79) and Genus *Flavobacterium* (4.35%) were dominant across studied CWs. Bacterial abundance increased from the inlet to the outlet, with CWs having a higher abundance around the outlet, showing a good performance. The Shannon–Wiener index showed the highest species diversity ($H = 1.45$) in Banana Investment Limited CWs treating industrial wastewater. This study found significant bacterial diversity across studied CWs, with higher abundance and diversity at the outlet indicating better performance than those with low abundance and diversity at the outlet. We recommend a longitudinal investigation at each CWs section to confirm and establish the role of bacterial diversity on the performance pattern of CWs.

Key words: bacterial diversity, constructed Wetlands, Illumina sequencing, Tanzania, wastewater treatment

HIGHLIGHTS

- Bacterial community structures across different points and types of constructed wetlands (CWs) were assessed.
- Proteobacteria were dominant phyla recovered across different types and points of CWs.
- Industrial wastewater CWs had wider bacterial community diversity than municipal wastewater CWs.
- Surface flow CWs had the highest bacterial reads and bacterial assemblage.

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GRAPHICAL ABSTRACT

Wastewater sampling in subsurface flow CWs



DNA extraction for bacterial analysis

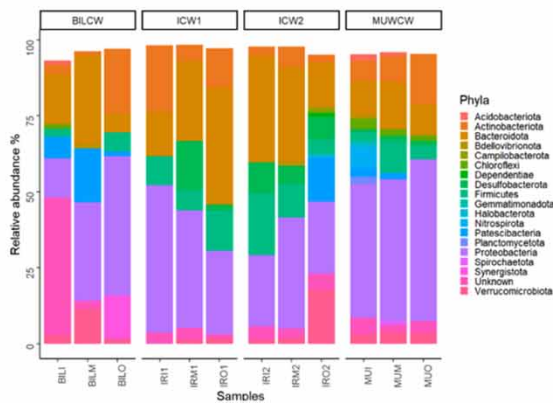


Wastewater sampling in Surface flow CWs



Sampling sites	Total_OTUs	Shannon	Simpson	Invsimpson
ICW1	150	0.89	0.26	1.36
ICW2	320	1.34	0.42	1.78
BILCW	391	1.45	0.5	2.16
MUWCW	562	1.37	0.37	1.59

Phyla Relative abundance



1. INTRODUCTION

Domestic and industrial wastewater has a complex and variable composition of organic and inorganic contaminants, making them a significant source of surface and underground water pollution (Bassin *et al.* 2017). Even though industries contribute significantly to the economy and earn substantial amounts of valuable foreign exchange, their effluents pose a significant threat to natural ecosystems (Holeton *et al.* 2011; Edokpayi *et al.* 2017; Selvarajan *et al.* 2018). Municipal wastewater treatment plants (WWTPs) receive wastewater from

domestic and industrial discharge with high pollutant levels for treatment before being discharged into the environment (EPA 2004).

Several wastewater treatment technologies have been used to improve pollutant removal from wastewater. For decades, wastewater stabilization ponds (WSPs) have been one of the essential wastewater treatment systems used in treating municipal wastewater (Quiroga 2013). The WSPs are large man-made impoundments in which wastewater, such as blackwater, greywater or fecal sludge, is treated. Wastewater treatment in WSPs occurs naturally under the influence of sunlight, wind, microorganisms and algae (Quiroga 2013). While WSPs are most commonly used for treating domestic wastewater, they are also successfully used for treating industrial wastewater, including water that contains agro-industrial wastes. Their capacity to remove some pollutants, such as microorganisms biological and physical parameters, is somehow limited (Kayombo *et al.* 2005). Constructed wetlands (CWs), biological sand filters and other decentralized solutions are becoming an increasingly relevant alternative to conventional systems when treating wastewater from industries, small communities dwellings and municipals due to their efficiency, low establishment costs and low operation and management requirements (Wilderer & Schreff 2000; Bakir 2001). Modern technologies have improved the WSPs design by adding CWs at the end of the system to polish the treated water from preliminary WSPs treatment systems before it can be released into the environment/reused (Vymazal 2005).

CWs are engineered wastewater treatment systems encompassing several treatment modules, including biological, chemical and physical processes akin to natural wetlands (Vymazal 2005). CWs constitute complex, integrated systems of water, plants, animals, microorganisms and the environment (USEPA 2000). The presence of a complex microbial community created by interactions with wastewater is mainly responsible for the degradation performance of the system (Sleytr *et al.* 2009).

The diversity of microorganisms in this environment may be critical for proper functioning and maintenance (Ibekwe *et al.* 2003). To improve the design and efficiency of these systems, detailed knowledge of the microbial community structure should be acquired to understand the areas where most of the biological processes occur (Truu *et al.* 2009; Dong & Reddy 2010). The extent, volume and bulk of wastewater treatment in CWs largely depend on the wetland's design, vegetation types and microbial composition (Rajan *et al.* 2019).

The CWs are home to various microorganisms, including bacteria, fungi and algae, which aid in removing inorganic and organic pollutants (Urrea-Valencia *et al.* 2021). Bacteria are the largest and most essential group in wastewater treatment systems due to their capacity for biodegradation and diversity in various environments (Seviour & Nielsen 2010; Adrados *et al.* 2014; Rajan *et al.* 2019). In addition, bacteria play an essential role in biogeochemical cycles, such as pollutant removal from wastewater (Rajan *et al.* 2019). According to Ibekwe *et al.* (2003), the bacterial composition and diversity differ from one type of treatment plant to the next, depending on the type of waste being treated, its location and the design of the wetland system. In his study on the characterization of the microbial composition of soil samples from two CWs treating dairy wash-water using PCR-DGGE, he found a higher percentage of *Nitrosospira*-like sequences and *Nitrosomonas*-like sequences, confirming that wetland systems are highly dependent on microbial activities and diversities for optimal wastewater treatment and the nature of wastewater to be treated. On the other hand, the nature of influents plays a role in determining the composition and structure of microbial communities found in WWTPs, with Proteobacteria, Bacteroidetes and Actinobacteria dominating the microbiota of domestic influents (Adrados *et al.* 2014; Desta *et al.* 2014; Gao *et al.* 2016; Selvarajan *et al.* 2018) while industrial wastewaters such as textile influents are dominated by *Planctomycetes*, *Chloroflexi*, *Acidobacteria* and *Chlorobi* (Meerbergen *et al.* 2017).

Recent studies (Collins *et al.* 2004; Desta *et al.* 2014), on the other hand, show that vegetation types promote the growth of specific microbial communities and plant diversity aids in the harboring of different microorganism species that respond differently to factors such as nutrient availability, contaminant loads, specific plant species and combinations chosen, thereby increasing microbial-based wastewater treatment capacity. Furthermore, Rajan *et al.* (2018) reported that CWs design and configuration heavily influence microbial diversity in treatment systems. The diversity of microorganisms is critical for effective pollutant removal (Urrea-Valencia *et al.* 2021). In general, biological waste treatment is currently gaining interest because of its significant contribution to the biodegradation of numerous waste materials. Microbial diversity is an important component that supports biological wastewater treatment, and its application has been shown to have positive effects and they are indispensable in modern cities as wastewater production and impacts on the ecosystem are escalating day to day (Urrea-Valencia *et al.* 2021).

So far, in Tanzania, most studies conducted on CWs systems and wastewater treatment systems have concentrated on the fate and removal efficiency of pollutants and pathogenic indicator microorganisms as well as

chemical and biological quality parameters (Mashaury *et al.* 2000; Kimwaga *et al.* 2004; Njau *et al.* 2011a, 2011b; Mairi *et al.* 2012; Mtavangu *et al.* 2017; Mahenge & Malabeja 2018). However, the diversity of bacterial communities of CWs used in polishing wastewater has yet to be thoroughly investigated. Understanding the types of bacterial communities and areas where most bacterial groups are found will be useful to wastewater engineers in improving the design for increased efficiency of wastewater treatment systems. The present study objectives were to (1) determine the abundance of bacterial communities in different types of CWs that treat industrial and municipal wastewater at various points of the systems (i.e., inlet, middle and outlet zones) and (2) establish the bacterial communities diversity in the studied CWs. We hypothesized that CW's sections/points (inlet, middle and outlet zones) would have a different abundance of bacterial groups involved in organic and inorganic matter biodegradation. We further hypothesized that different types of CWs would have different abundance and bacterial groups involved in the biodegradation of organic and inorganic matter. Moreover, the study hypothesized that the nature of influents plays a crucial role in determining the composition and structure of microbial communities found in wastewater treatment systems.

Here, we present for the first time a detailed study of the bacterial communities present in CWs systems treating municipal wastewater, i.e., surface flow CW and subsurface CW (both receiving wastewater generated from domestic sources and some inputs from industrial sources) and subsurface CWs treating banana winery industrial wastewater in Tanzania. Our research determines the composition of bacterial communities at various points along the CW, which may aid in selecting microbe seeding sites in systems that need to improve performance.

2. MATERIALS AND METHODS

2.1. Study area

This study was conducted in three regions of Tanzania with well-established CWs for wastewater treatment (Figure 1). Arusha is located between 3.3869°S latitude and 36.6824°E longitude in northern Tanzania. The Kilimanjaro region is situated between 3.0674°S latitude and 37.3556°E longitude in the northeastern part of Tanzania. On the other hand, the Iringa Region is located between 7.77°S latitude and 35.69°E longitude in the southern part of Tanzania. The specific locations where samples were taken are shown in Figure 1.

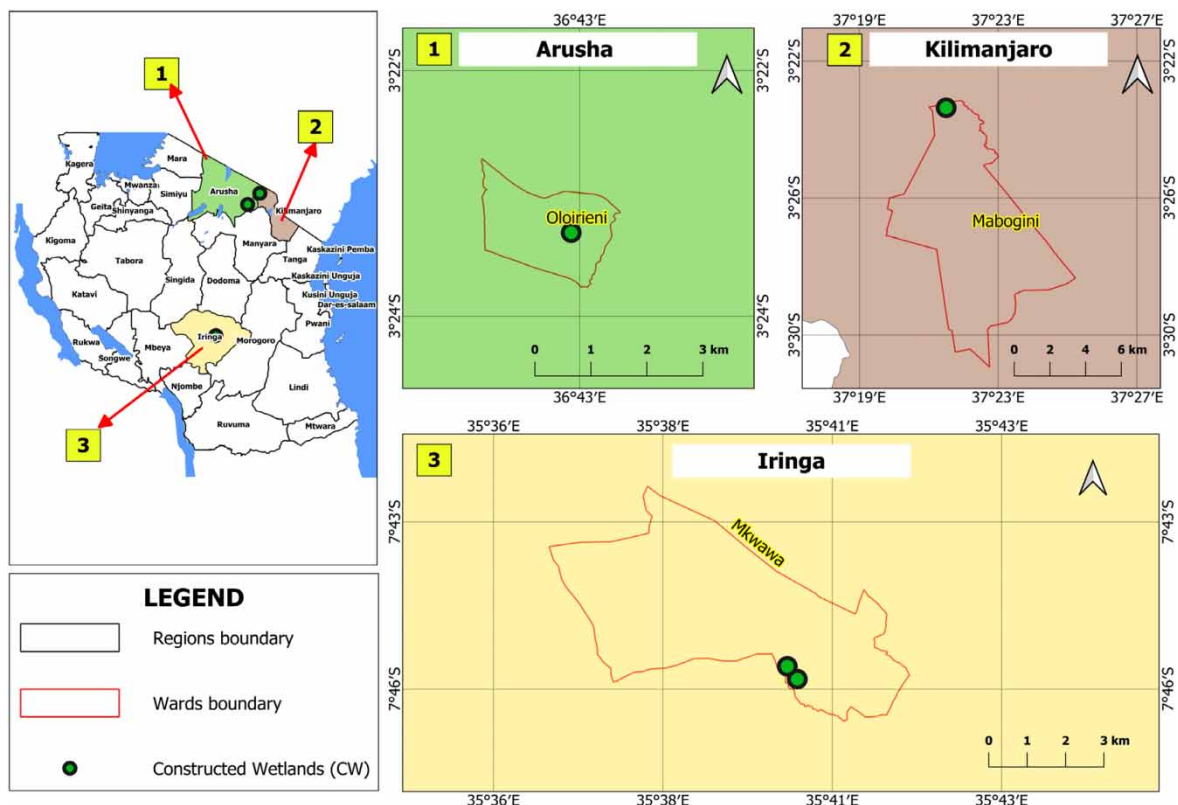


Figure 1 | Map of the study areas selected for a sample during data collection.

2.2. Sample collection and physicochemical parameters analysis

Wastewater samples for characterizing and establishing bacterial community diversity and composition in CWs were collected in 1 month (February 2021) in four different CWs, namely Banana Investment Limited CWs (BILCW), Moshi Urban Water Supply and Sanitation Authority CWs (MUWCW) and Iringa Urban Water Supply and Sanitation Authority CWs (two CWs, i.e., ICW1 and ICW2) (Figure 1). Of the four CWs studied, BILCW was treating industrial wastewater from a banana wine factory while the remaining were treating municipal wastewater. Triplicate samples were collected using a sterile plastic beaker in the four CWs at different points (i.e., at the inlet, medium and outlet zones), making a total of 36 wastewater samples. All samples were filled in 1 L plastic bottles, stored in a cool box and transported for laboratory analyses at the Nelson Mandela African Institution of Science and Technology (NM-AIST) Molecular Lab. Physicochemical parameters were measured on-site at each sampling point, and average measurements were taken. Temperature, total dissolved solids (TDS) and pH were measured in triplicates using a Multi-Parameter meter (Hach 40HQD). Biological oxygen demand (BOD) was analyzed by a closed manometer following standard methods (APHA 2012). Similarly, chemical oxygen demand (COD) was determined spectrophotometrically (HACH DR 2800) following standard methods (APHA 2012).

2.3. Extraction of genomic DNA

Before DNA extraction, 100 mL of wastewater samples were filtered using a 47 mm membrane filter with a pore size of 0.45 μm (HAWG047S1, Millipore Corporation, Billerica, MA 01821) placed within a sterile stainless steel vacuum filter holder (Sartorius, Germany) attached to a water aspirator (FBL, China) (Salem *et al.* 2011; Mtavangu *et al.* 2017). After filtration, the membrane filter containing the retained microbes was cut into small pieces, suspended in the PowerBeads tube and processed for DNA extraction. DNA was extracted and purified from 12 representative wastewater samples using a DNeasy PowerSoil kit according to the manufacturer's protocol (Qiagen, Germantown, MD, USA). The purities and concentrations (in $\text{ng } \mu\text{L}^{-1}$) of the extracted DNA were determined spectrophotometrically using the NanoDrop™ One Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, USA); the purity and quantity of DNA were within the recommended ranges for PCR (El-Ashram *et al.* 2016). The extracted DNAs were stored at a temperature of -80°C pending for further downstream analysis.

2.4. Metagenomic analysis of 16S gene sequencing

Purified DNAs were sent to Inqaba Biotechnical Industries (South Africa), a commercial NGS service provider, for metagenomic analysis of 16S gene sequencing through the Illumina system. Briefly, purified genomic DNA samples were PCR amplified using a universal primer pair 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) (Herlemann *et al.* 2011) targeting the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene. The resulting amplicons were purified, end-repaired, and Illumina-specific adapter sequences were ligated to each amplicon (NEBNext Ultra II DNA library prep kit). Following quantification, the samples were individually indexed using NEBNext multiplex oligos for Illumina (Dual Index Primers Set 1), and another AMPure XP bead-based purification step was performed. Amplicons were then sequenced on Illumina's MiSeq platform using a MiSeq v3 (600 cycles) kit. 20Mb of data (2×300 bp long paired-end reads) were produced for each sample. Briefly, the removal of primers was done using a cutadapt (<https://github.com/qiime2/q2-cutadapt>). Reads were processed through vsearch version 2.23.0 (<https://github.com/torognes/vsearch>), and taxonomic information was determined based on the Ribosomal Database Project's 16 s database v16 (<http://rdp.cme.msu.edu/index.jsp>). Operational Taxonomic Units (OTUs) contributing less than 1% of the total data have been excluded.

2.5. Data analysis

Statistical analyses were performed using R-studio software. The Shannon–Wiener diversity index and Simpson index, as described by Nolan & Callahan (2006), were calculated to show the diversity and richness of bacterial communities across different studied CWs in the study area. The Mann–Whitney *U* test on the physicochemical parameters at the influent and influent of studied CWs were assessed using Jamovi software version 2.3.18. The GO and KEGG enrichment analyses were conducted using the EDGE bioinformatics software (<https://edge-bioinformatics.org/>), and the ShinyGO enrichment tool (<http://bioinformatics.sdstate.edu/go/>) was also employed for the analysis and graphical representation of the data.

3. RESULTS AND DISCUSSION

3.1. Physicochemical parameters of sampled wastewater

The measured physicochemical parameters results are shown in Table 1. The temperature values of wastewater ranged from 24.85 ± 0.15 °C to 27.13 ± 0.21 °C which are within ranges reported in various studies such as Bidu *et al.* (2021), Mtavangu *et al.* (2017) and Njau *et al.* (2011a, 2011b) who conducted their studies in Moshi, Arusha and Iringa regions and reported wastewater temperature to range from 24 to 35 °C. Metrological data show that air temperature is maximum in December and minimum in June. Moderate temperatures can support a wide range of bacterial organisms. A study by Hassan *et al.* (2021) revealed that temperature influences microbial activities in CWs and can affect the treatment process. Lower temperatures slow down microbial activities, whereas higher temperatures may have detrimental effects on microorganisms. Our finding matches well with those of Christofilopoulos *et al.* (2019) and Nguyen *et al.* (2019), who reported that temperatures of 20–30 °C are suitable for the growth of plants and bacteria. pH measured shows slight to moderate values ranging from 7.3 ± 0.10 to 8.3 ± 0.20 . This finding indicates that the recorded pH and temperature condition in all the wastewater treatment systems was moderate and, therefore, can support a wide variety of bacterial communities that engage in the biodegradation of organic and inorganic pollutants. Studies by Kumar & Chandra (2020) reported that wastewater's pH strongly affects microbial-enhanced activities in CWs. The TDS concentration was high in BILCW ($1,649.42 \pm 1.23$ mg/L), while it ranged between 494.9 ± 0.10 and 772 ± 15.00 mg/L in other visited sites. Higher TDS could have an implication that provides surface area for bacterial attachment. From the obtained data, BOD and COD were lower in Banana Investment Limited CWs, which had higher bacterial abundance and diversity at the effluent and were higher in Iringa Water Supply and Sanitation Authority, which had lower bacterial abundance and diversity along the system. These findings concur well with those reported by Olutiola *et al.* (2010), who reported a significant decline of BOD and COD in wastewater treatment ponds with increased bacterial composition.

Table 1 | Average physicochemical parameters of 36 wastewater samples collected and measured from different sections/points of studied CWs

Sampling sites	Location	Temp. (°C)	pH	TDS (mg/L)	BOD ₅ (mg/L)	COD (mg/L)
ICW1	In	25.67 ± 0.83	8.15 ± 0.16	772 ± 15.00	86 ± 47.50	88 ± 21.80
	Medium	24.93 ± 0.37	8.2 ± 0.21	716 ± 9.00	nd	nd
	Out	24.83 ± 0.15	7.9 ± 0.11	667 ± 3.00	63.52 ± 13.58	73 ± 20.11
ICW2	In	24.87 ± 0.41	8.3 ± 0.20	638 ± 12.00	86 ± 47.50	97.1 ± 13.57
	Medium	25.01 ± 0.32	7.5 ± 0.15	520 ± 11	nd	nd
	Out	24.83 ± 0.35	8.0 ± 0.15	556.33 ± 4.04	68.08 ± 1.86	90.21 ± 12.65
BILCW	In	24.95 ± 0.31	7.5 ± 0.12	$1,649.42 \pm 1.23$	36.90 ± 9.0	44.41 ± 5.78
	Medium	24.89 ± 0.25	7.4 ± 0.11	$1,640 \pm 1.40$	nd	nd
	Out	25.00 ± 0.50	7.3 ± 0.10	$1,638.15 \pm 1.10$	19.72 ± 4.00	34.41 ± 4.61
MUWCW	In	26.53 ± 3.2	8.3 ± 0.14	494.9 ± 0.10	56.34 ± 13.50	74.22 ± 11.36
	Medium	26.89 ± 31	7.9 ± 0.12	497 ± 0.32	nd	nd
	Out	27.13 ± 0.21	7.6 ± 0.15	431.90 ± 0.20	28.29 ± 16.55	54.23 ± 12.31

Key: ICW1, Iringa Water Supply and Sanitation Authority (ICW1); ICW2, Iringa Water Supply and Sanitation Authority (ICW1), BILCW, Banana Investment Limited CW; MUWCW, Moshi Urban Water Supply and Sanitation Authority; nd, not determined.

Statistical analysis revealed no significant difference in measured physicochemical parameters between the different sections of the studied CWs (Table 2). This could be due to the slight difference between the parameters measured of inlet and outlet, the sample collected in a single sampling event and the smaller sample size, which limits the power to detect whether there is a significant difference between the influent and effluent. These findings concur with those reported in the studies by Hernandez-Rivera *et al.* (2023) and Berego *et al.* (2022), who also reported no significant difference between measured physicochemical parameters in the study on assessing coliphages as indicators of primary wastewater treatment efficiency by CWs. However, some effluent values measured, i.e., BIL and MUWCW, were within the permissible limit by the Tanzania Bureau of Standards (TBS) and World Health Organization.

Table 2 | Mann–Whitney *U* test on physicochemical parameters of 36 wastewater samples collected and measured from different sections/points of studied CWs results measured at a different station

S/no	Parameter	Site	Sampling point	Mean	Standard deviation	Standard error	P-value
	Temperature	ICW1	Inlet	25.67	0.074	0.043	$P = 0.100$
			Outlet	24.83	0.055	0.032	
		ICW2	Inlet	24.87	0.010	0.006	$P = 0.077$
			Outlet	24.83	0.006	0.003	
		BILCW	Inlet	24.95	0.010	0.006	$P = 0.077$
			Outlet	25.00	0.058	0.033	
	MUWCW	Inlet	26.53	0.010	0.007	$P = 0.100$	
		Outlet	27.13	0.049	0.008		
	pH	ICW1	Inlet	8.14	0.006	0.003	$P = 0.072$
			Outlet	7.87	0.058	0.030	
		ICW2	Inlet	8.30	0.100	0.058	$P = 0.077$
			Outlet	8.03	0.058	0.033	
BILCW		Inlet	7.47	0.058	0.033	$P = 0.059$	
		Outlet	7.30	0.000	0.000		
MUWCW		Inlet	8.33	0.058	0.033	$P = 0.072$	
		Outlet	7.57	0.058	0.033		
TDS		ICW1	Inlet	772.00	1.530	0.882	$P = 0.100$
			Outlet	667.00	1.000	0.577	
		ICW2	Inlet	638.00	0.577	0.333	$P = 0.072$
			Outlet	556.00	0.577	0.333	
	BILCW	Inlet	1,649.00	0.006	0.003	$P = 0.072$	
		Outlet	1,638.00	0.075	0.043		
MUWCW	Inlet	495.00	0.252	0.145	$P = 0.077$		
	Outlet	432.00	0.058	0.033			
BOD ₅	ICW1	Inlet	86.70	0.577	0.333	$P = 0.072$	
		Outlet	63.52	0.012	0.007		
	ICW2	Inlet	86.00	0.577	0.333	$P = 0.072$	
		Outlet	68.08	0.006	0.003		
	BILCW	Inlet	36.90	0.100	0.058	$P = 0.077$	
		Outlet	19.72	0.006	0.003		
	MUWCW	Inlet	56.34	0.006	0.003	$P = 0.072$	
		Outlet	28.29	0.012	0.007		
	COD	ICW1	Inlet	88.00	0.058	0.033	$P = 0.072$
			Outlet	73.00	0.006	0.003	
		ICW2	Inlet	97.15	0.058	0.033	$P = 0.072$
			Outlet	90.21	0.012	0.007	
BILCW		Inlet	44.41	0.000	0.000	$P = 0.059$	
		Outlet	34.41	0.006	0.003		
MUWCW	Inlet	74.22	0.006	0.003	$P = 0.077$		
	Outlet	54.23	0.049	0.028			

3.2. Bacterial diversity in the studied areas

The current study clarifies bacterial diversity using next-generation sequencing (NGS) technology. The Shannon–Wiener index showed the highest species diversity in Banana Investment Limited CWs (BILCW) and the lowest in Iringa Water Supply and Sanitation Authority CWs (ICW1) as shown in Table 3. Richness was also estimated

Table 3 | Summary of diversity indices in studied CWs

Sampling sites	Total OTUs	Shannon	Simpson	Invsimpson
ICW1	150	0.89	0.26	1.36
ICW2	320	1.34	0.42	1.78
BILCW	391	1.45	0.5	2.16
MUWCW	562	1.37	0.37	1.59

Key: ICW1, Iringa Water Supply and Sanitation Authority (ICW1); ICW2, Iringa Water Supply and Sanitation Authority (ICW1); BILCW, Banana Investment Limited CW; MUWCW, Moshi Urban Water Supply and Sanitation Authority.

using the Simpson index, which showed that ICW2 was richer than other studied sites. From the comparison between the different sections of the wetlands, it has been observed that BILCW and MUWCW had higher bacterial diversity around the outlet zone. The other wetlands had higher diversity at the inlet and middle part. A comparison between bacterial diversity and the physicochemical parameters shows that BIL and MUWCW, which had higher bacterial diversity at the effluent zone, had good BOD and COD removal efficiency compared to ICW1 and ICW2.

Further analysis of the boxplot (Figure 2) indicates that the diversity means for the BILCW is significantly higher than the rest of the studied areas. These findings correlate to some physicochemical parameters obtained from BIL CWs monitoring units, which showed significantly lower BOD, thereby supporting a higher diversity of bacterial communities. Similarly, the richness index box plot shows that ICW2 is richer than the other studied.

Beta diversity (Figure 3) shows a distant variation of different species at the studied cluster. The diversity within site-specific was also varied due to the relatively higher distance of the plotted beta diversity graph.

3.2.1. Microbial diversity at the phylum level

A comparison across the wetlands revealed higher bacterial reads in samples from two surface flow CWs, with total reads of 111,268 and 94,609 in the two subsurface CWs of BILCW and MUWCW. Further analyses of the results revealed that Proteobacteria had the highest relative abundance compared to all other phyla found in nearly all wetland sites, followed by phylum *Bacteroidota*, *Actinobacteriota* and *Firmicutes* as seen in Figure 4. Moreover, the significant presence of *Desulfobacterota* and *Verrucomicrobiota* was also found. The dominance of phylum *Proteobacteria* followed by *Bacteroidota*, *Actinobacteriota* and *Firmicutes* as subdominant phyla concur with other studies done in CWs especially the one executed by Hua *et al.* (2018). Their study explored the bacterial community compositions of vertical flow CWs using Illumina high-throughput sequencing; they found that the dominant phyla in the whole process were *Proteobacteria* and *Bacteroidota*. In addition, the study by Lang *et al.* (2018) on the variation of bacterial and archaeal community structure in full-scale CWs reported that *Proteobacteria* is the dominant phyla. In most municipal WWTPs, including CWs, phylum *Proteobacteria* has been reported to be dominant, and they tend to be responsible for organic and nutrient removal (Cyzdik-Kwiatkowska & Zielińska 2016). It's possible to infer the member of proteobacteria, which has been observed to be dominant in this study, may play an essential role in organic and nutrient recycling at the effluent, accounting for wetland efficiency. Moreover, the findings from this study highlight the potential of the phylum *Proteobacteria* in wastewater treatment systems, and thereby, they can be used as seeds in the areas of the wetlands where most of the microbial processes occur, which in this study was identified to be around the outlet zone of the studied wetlands as we noted wetlands with higher bacterial abundance along these sites had good removal efficiency.

The phylum *Bacteroidota* (formerly known as *Bacteroidetes*) is one of the diverse lineages and most abundant in WWTPs. In different WWTPs, their main role is the degradation of complex organic substances, such as proteins, starch, cellulose and fibers (Yang *et al.* 2017; Al Ali *et al.* 2020). Members of this phylum can also degrade dead cells and exopolysaccharides into simpler organic compounds, such as ethanol and lactate, which can then be utilized by other members of that community (Al Ali *et al.* 2020). All other bacteria in this phylum *Bacteroidota* exhibit various metabolisms (Kondrotaitė *et al.* 2022). This phylum might be implicated in the degradation of complex organic substances; therefore, its importance in CWs for enhancing the removal of complex organic compounds generated from industrial and pharmaceutical products. The bacterial species of phylum *Actinobacteriota* play a significant role in organic matter decomposition in many organic matter-rich environments (Lu *et al.* 2022). *Firmicutes* as subdominant phylum in WWTPs, as many previous studies have already reported

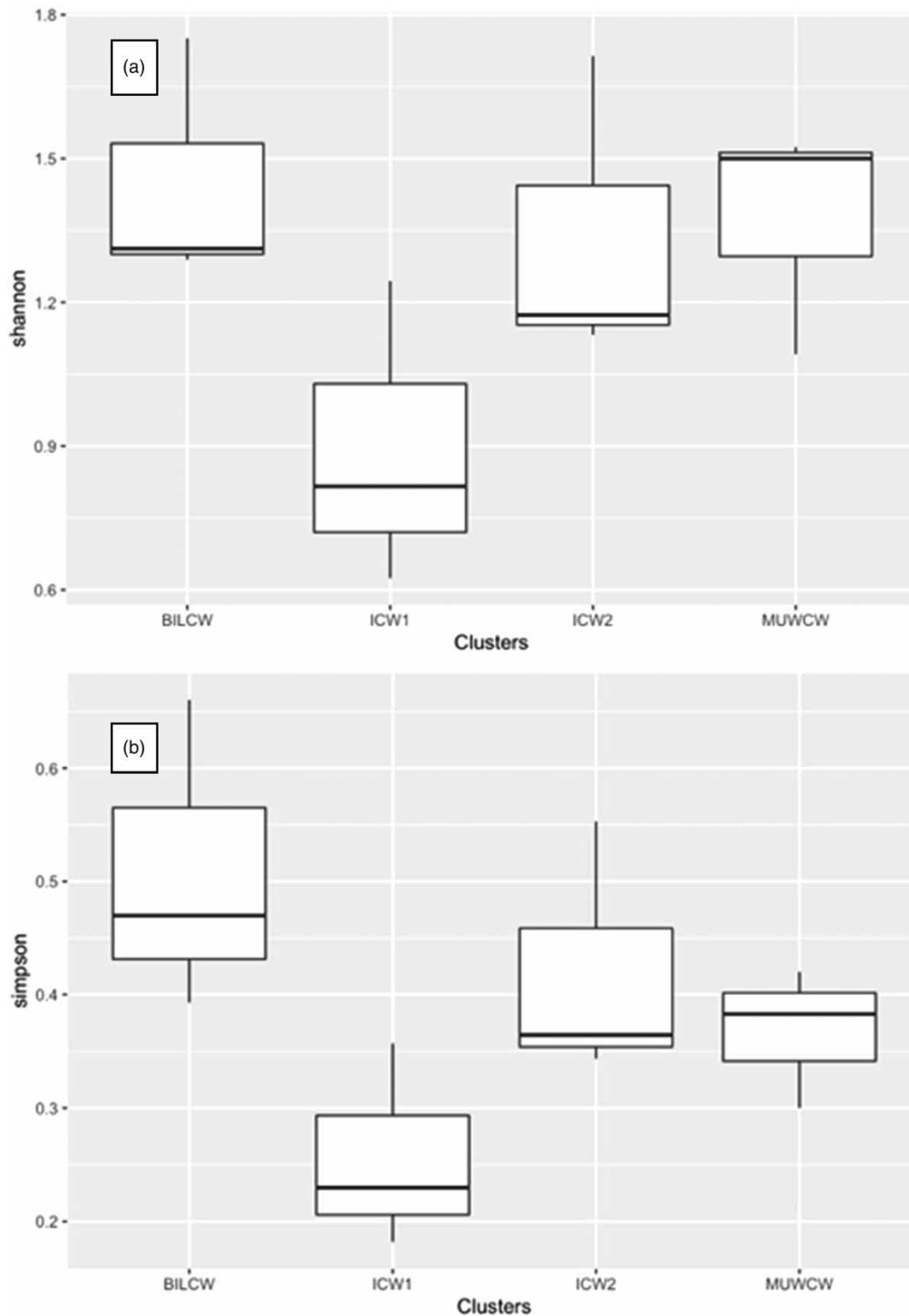


Figure 2 | Box plot presentation for bacterial diversity indices, Shannon (a) and Simpson (b) indices between different types of CWs studied.

their presence in such areas, they play an important role in the anaerobic degradation of methyl *tert*-butyl ether (MTBE) (Weimin *et al.* 2012).

A comparison of bacterial phyla abundance across the different parts of the wetlands showed that the bacterial phyla population increased from the inlet towards the outlet zone, with the phyla *Proteobacteria* dominating almost all parts of the wetland, followed by the *Bacteroidota*, *Firmicutes* and *Actinobacteriota* (Figure 4).

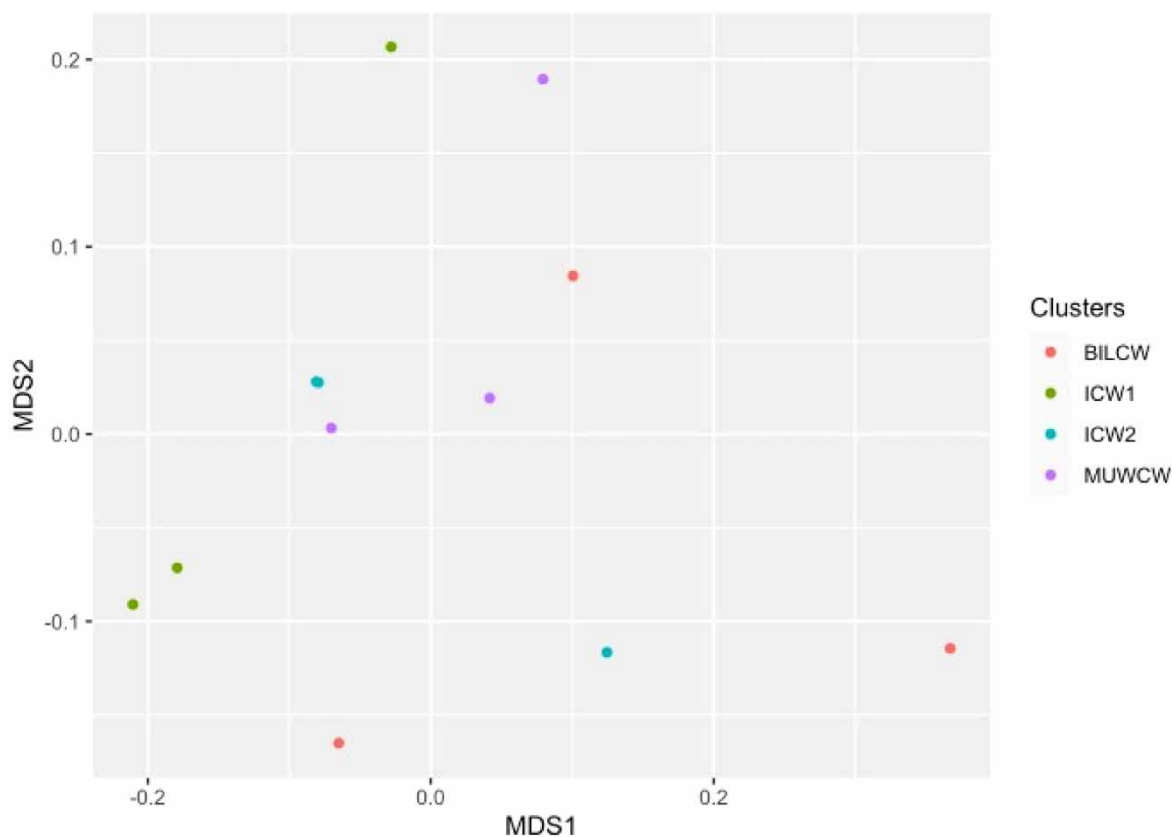


Figure 3 | Beta plot diversity for bacterial communities for samples collected in the study area.

Unlike wetlands that treat domestic wastewater, BILCW, which treat industrial effluent, had a higher abundance of *Bacteroidota* phyla at the inlet zone. The dominance of this family in industrial CWs may be implicated in degrading complex organic compounds used in the brewing of banana wineries. In general, *Proteobacteria* and *Bacteroidota* were found in higher abundance at the inlet and near the outlet zone, indicating that most microbial activities would have been occurring in these areas. Our results also showed that CWs with higher bacterial abundance at the effluent zone had a good performance. These results could snapshot which sections in CWs are appropriate for applying bacterial seeding technology to enhance wastewater treatment efficiency. This finding agrees with other studies (Desta *et al.* 2014; Selvarajan *et al.* 2018); their study found that CW treating domestic and industrial wastewater harbors a large abundance of *Proteobacteria* followed by *Bacteroidota*, *Actinobacteriota* and *Firmicutes*. Furthermore, these findings are well matched with those reported by Gao *et al.* (2016), who found that the relative abundance of *Bacteroidota* was larger than that of other phyla in a wetland treating industrial wastewater. Moreover, the highest abundance of *Proteobacteria*, *Bacteroidota*, *Firmicutes* and *Actinobacteria* indicates the prevalence of significant denitrification processes in the studied CWs. These findings are supported by the study conducted by Wang *et al.* (2022a, 2022b) who reported that *Proteobacteria*, *Bacteroidetes*, *Firmicutes* and *Actinobacteria* are popular denitrifying bacteria in CWs.

Moreover, we found abundance in surface flow CWs (ICW1 and ICW2) with bacterial abundance ranges between 32 and 48%. Subsurface CWs treating municipal wastewater (MUWCW) were also found to have a good representative of bacterial abundance with an abundance range between 21 and 53%, whereas the one treating industrial wastewater represented a relatively moderate abundance of bacteria. The reason could be because of the larger size of the CW and the volume of wastewater treated by the municipal treatment systems. In general, understanding and harnessing the functions of *Proteobacteria* and other dominant phyla in CWs for wastewater treatment are essential for optimizing the efficiency and performance of these systems for enhanced environmental protection by releasing treated wastewater within the permissible limits for effluent discharge.

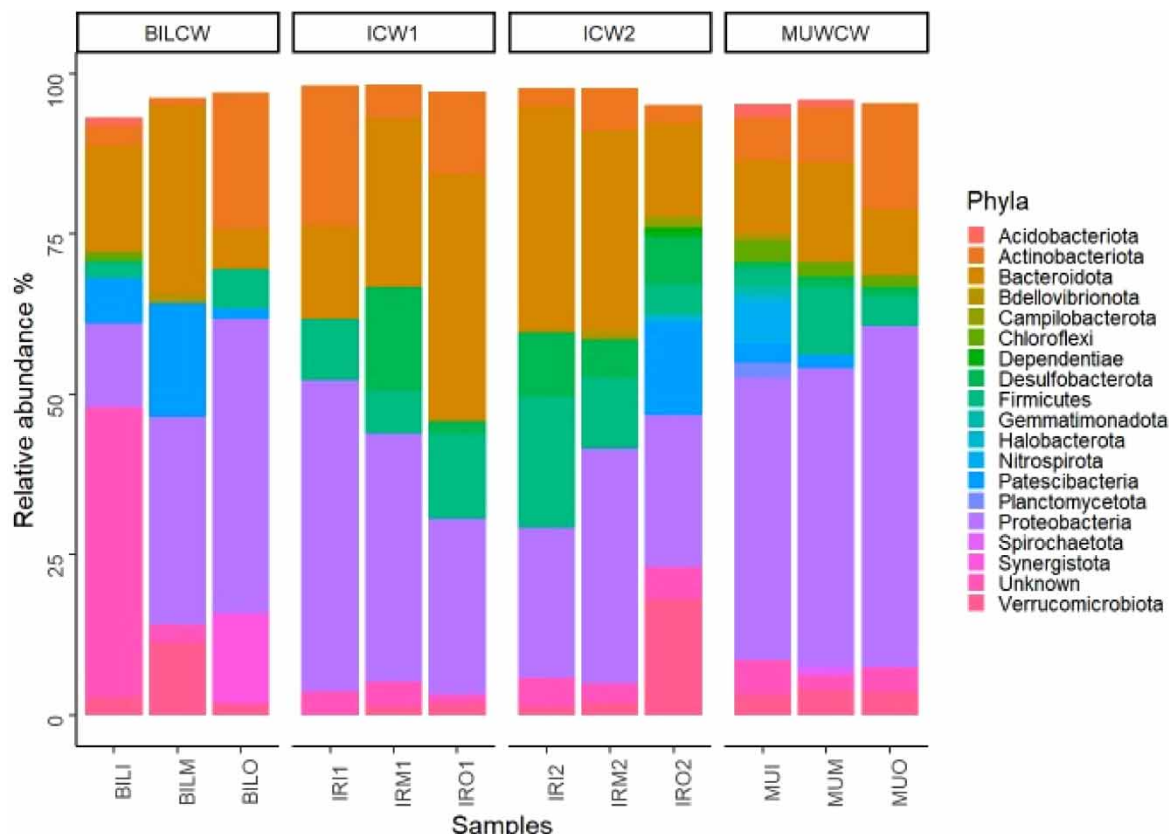


Figure 4 | Phyla relative abundance and taxonomic classification of different identified bacterial phyla for samples collected in studied CWs from Arusha, Iringa and Kilimanjaro regions CWs.

3.2.2. Microbial diversity at class level

The dominant class of the studied bacterial community was *Gammaproteobacteria*, with an average abundance of 27.67% (Figure 5). Class *Bacteroidia* was the second most represented across all the wetlands, with an average abundance of 19.98%. Class *Actinobacteria*, *Alphaproteobacteria* and *Clostridia* were also highly represented across all the wetlands, with an average abundance of 7.82, 8.24 and 6.05%, respectively. A comparison of abundances across the different types of wetlands revealed that surface flow CWs represented the highest-class abundances, with the maximum abundance representing 40.48%. Moreover, comparing the wetlands' different sites, the data show maximum class abundances that were found at the inlet zone of the surface flow CWs, followed by the outlet zone of subsurface CWs whereby the dominant class at the inlet zone was *Bacteroidia*, followed by class *Gammaproteobacteria*. Class *Gammaproteobacteria* was found to have a higher abundance at the outlet zone of most of the CWs. The role played by these bacteria includes acting as the main sulphur oxidizer, organic carbon turnover and cycling of nitrogen from wastewater (Brueser *et al.* 2000; Mathew *et al.* 2022). Members of this class may be implicated in the oxidation of sulfides generated from industrial applications, thereby improving sulfide removal efficiency in CWs and carbon turnover and nitrogen cycling.

The hierarchy of classes found in the present study is similar to those observed in the study done by Desta *et al.* (2014), who reported significant abundances of class *Bacteroidia*, *Gammaproteobacteria* and *Clostridia*. A comparison of surface flow and subsurface CWs shows that OTUs of surface flow CWs have higher relative abundances than subsurface CWs. In addition, the relative abundance of OTUs for *Bacteroidia* in the medium zone was significantly lower than that of *Gammaproteobacteria*. The reported classes contain most bacterial species that can engage in phosphorus removal in WWTPs. Moreover, the high presence of class *Alphaproteobacteria* and *Gammaproteobacteria* indicates that these functional groups enhance nitrogen and phosphorus removal in the studied CWs. A study by Aguilar *et al.* (2019) and Ajibade *et al.* (2021) reported that *Alphaproteobacteria* and *Gammaproteobacteria* are the dominant classes related to nitrogen removal in CWs. They contain large numbers of nitrifying bacteria, ammonia oxidizing bacteria (AOB) and nitrate-oxidizing bacteria (NOB), which play important ecological functions in CWs and are largely involved in nitrogen removal. High

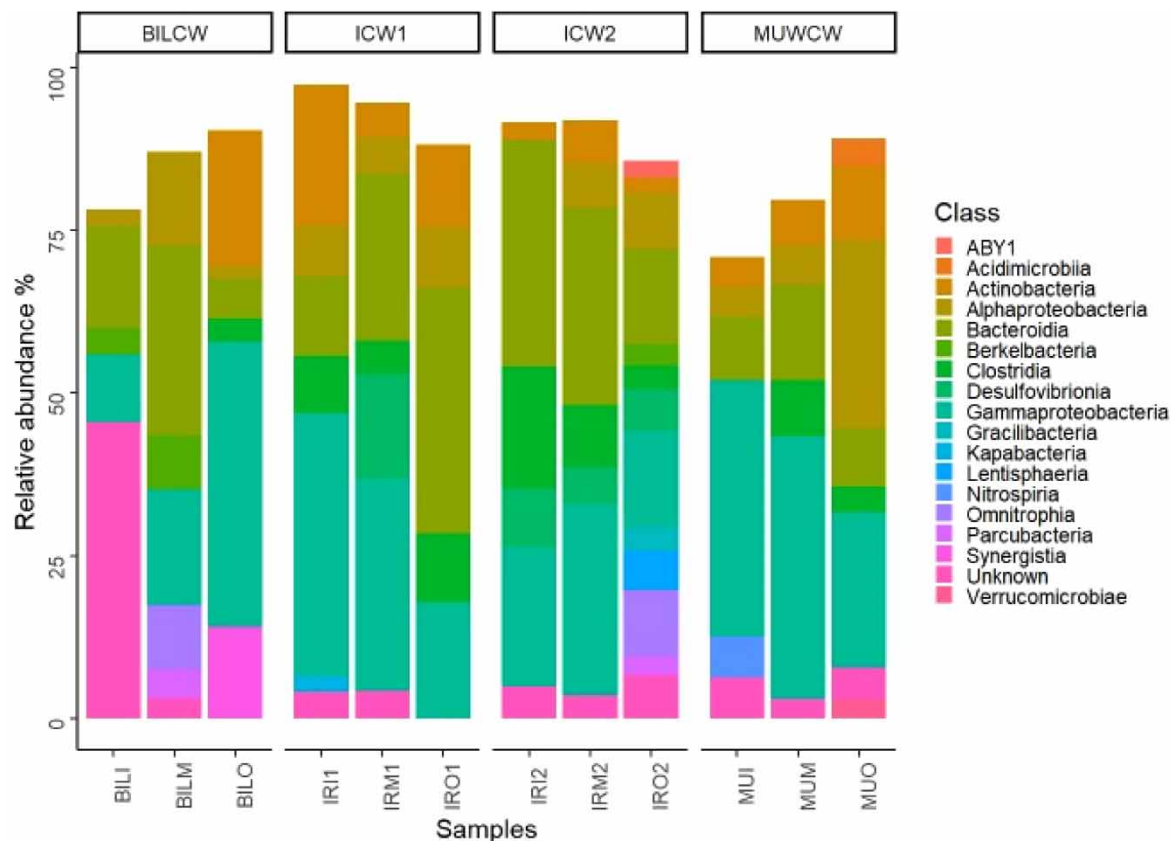


Figure 5 | Class relative abundance and taxonomic classification of different identified bacterial classes for samples collected in studied CWs from Arusha, Iringa and Kilimanjaro regions CWs.

concentrations of total phosphorus in sewage may be from detergents and cleaning products (Comber *et al.* 2013; Gomes de Quevedo & da Silva Paganini 2016). The presence of these bacteria classes helps to reduce the amount of phosphorus that can be discharged into the environment. The knowledge of the distribution of these bacterial classes may be useful in improving the design of CWs in phosphorus-rich production industries to help treat wastewater to the permissible limits for discharge into the environment. Generally, *Gammaproteobacteria*, *Bacteroidia*, *Actinobacteria* and *Alphaproteobacteria* in CWs can contribute to various important processes, including nitrogen and sulfur cycling, organic matter degradation and biofilm formation. Their adaptability, functional diversity and potential for pathogen removal make them valuable components of the microbial communities that drive wastewater treatment in CWs.

3.2.3. Microbial diversity at the family level

Family *Comamonadaceae* was dominant across all wetland effluents, occurring at a relative abundance of 2.33 and 21.12% and having an average relative abundance of 7.73% (Figure 6). This was followed by family *Rhodocyclaceae* 5.94% and *Flavobacteriaceae* 4.35%. Most other families were represented by a relative abundance of less than 1%. Family *Comamonadaceae* constitutes of large group of heterotrophic denitrifying bacteria. Also, this family constitutes phosphorus-accumulating bacteria such as *Hydrogenophaga*, which could be the reason for its dominance. These findings are supported by other studies, such as Zhang *et al.* (2018) and Ge *et al.* (2015), who also noted the dominance of the family *Comamonadaceae* in the studied CWs. A comparison across the wetlands revealed that surface flow CWs represented significantly higher family abundances than other wetlands. Different from other wetlands family, *Rhodocyclaceae* was reported in the wetland treating industrial effluent; this indicates that they present high denitrification in wastewater as different studies show that *Rhodocyclaceae* perform denitrification function. The results herein match well with those reported by Shi *et al.* (2021), in their study, they reported that *Rhodocyclaceae* is a key functional microbial group in a wastewater treatment system.

Family *Nitrospiraceae* was represented in the CW treating industrial effluent at the inlet zone, indicating more nitrogen fixation is being done at this zone. Moreover, the abundance of different families was higher at the inlet

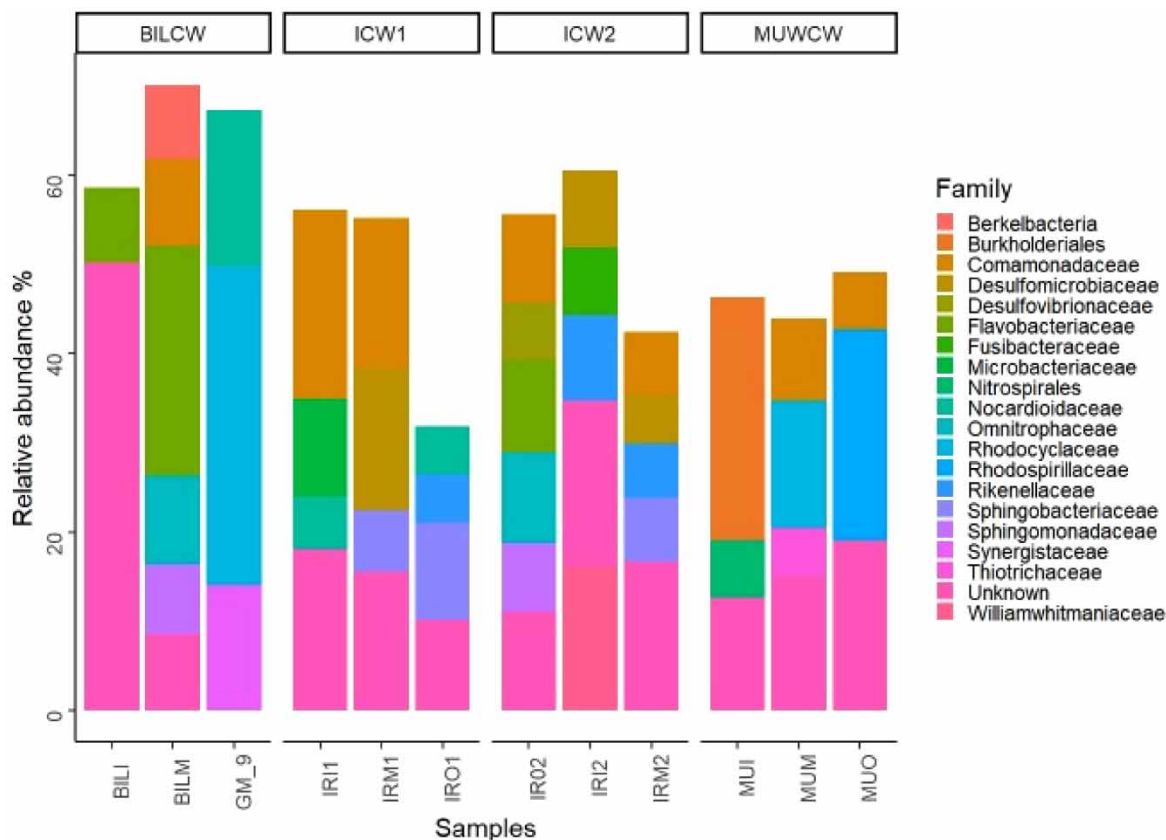


Figure 6 | Family relative abundance and taxonomic classification of different identified bacterial families for samples collected in studied CWs from Arusha, Iringa and Kilimanjaro regions CWs.

and medium zones of the studied wetlands. Across all wetlands families, *Comamonadaceae* was the most abundant since this constituted largely of the *Betaproteobacteria* group engaged in denitrification, and therefore, this is a clear indication that these systems had higher nitrate removal. These results are consistent with those reported by [Verduzco Garibay et al. \(2021\)](#), who also noted that the family *Comamonadaceae*, *Flavobacteriaceae* and *Rhodocyclaceae* represent higher abundances in wastewater treatment systems. Moreover, the high abundance of these families indicates the high removal of phosphate and nitrogen in the wastewater treatment systems. These findings are supported by studies by [Zheng et al. \(2021\)](#) and [Zhao et al. \(2021\)](#), who reported that the high abundance of family *Rhodoclyceae*, *Nitospirae* and *Comamonadaceae* enhances phosphate and nitrogen removal in wastewater treatment systems. The family *Comamonadaceae*, *Rhodocyclaceae* and *Flavobacteriaceae*, which were found to have higher abundance in studied CWs can contribute significantly to the functioning of CWs by participating in nutrient cycling, organic matter degradation, biodegradation of contaminants and establishment of symbiotic relationships with plants. Their diverse metabolic capabilities and ability to form biofilms make them valuable components of the microbial communities in constructed wetland ecosystems. Proper understanding and management of these bacteria can enhance the performance and effectiveness of CWs in treating wastewater and improving water quality.

3.2.4. Microbial diversity at the genus level

The genus *Flavobacterium* was the most dominant bacteria recovered in most of the studied CWs, with an average relative abundance of 4.35% ([Figure 7](#)). Genus *Desulfomicrobium* was the second most highly represented genus across most studied CWs, 3.48%. Genus *Nitrospira*, the most dominant nitrifying bacteria, was also recovered in CWs 2.53%. Besides, a significant abundance of the genera *Aeromicrobium* 2.38%, *Haematospirillum* 1.97, *Candidatus*, *Omnitrophus* 1.81% and *Fusibacter* 1.15% were recovered in the studied CWs. Additionally, in this study, a substantial diversity of heterotrophic nitrification-aerobic denitrification (HN-AD) bacteria was recovered, dominated by the *Hydregonophage*, whose presence depicts that heterotrophic bacteria can do

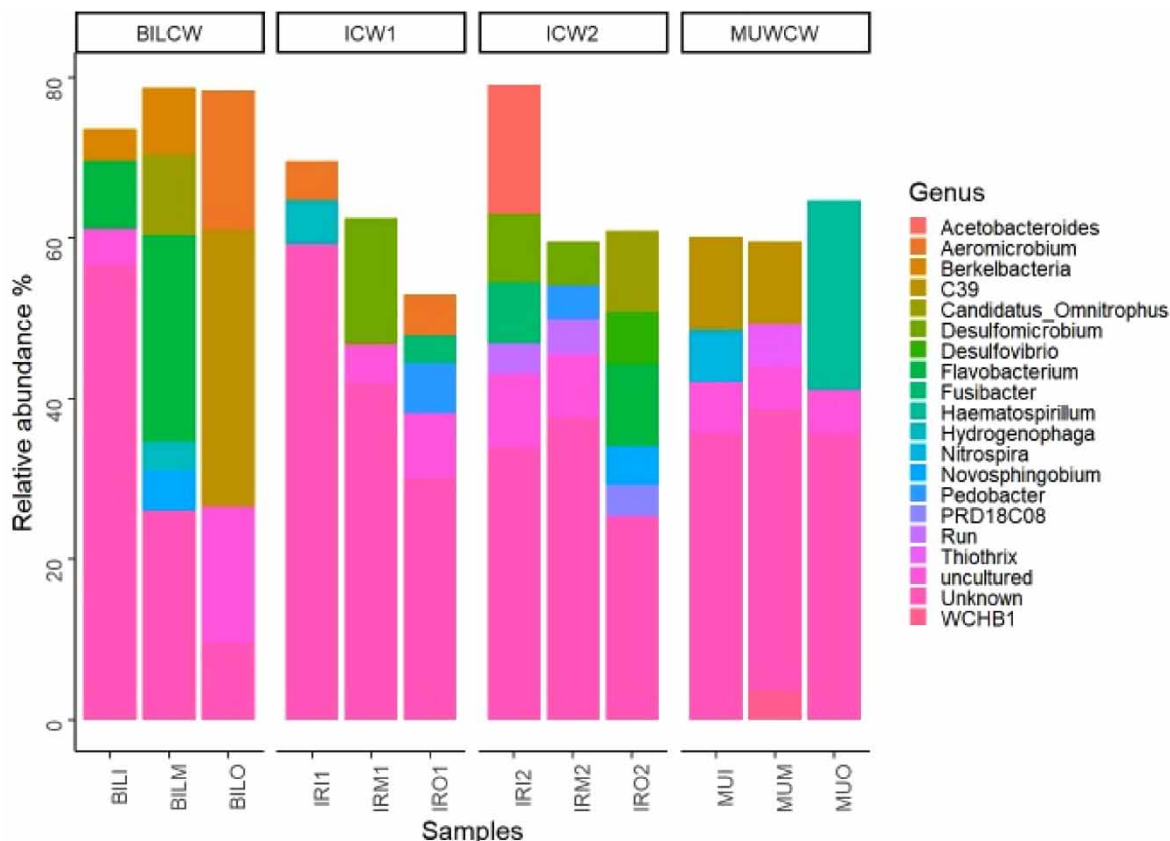


Figure 7 | Genus relative abundance and taxonomic classification of different identified genera for samples collected from Arusha, Iringa and Kilimanjaro regions CWs.

nitrogen removal through heterotrophic nitrification and anaerobic denitrification in the wastewater. Similar results were reported in the study by Zhang *et al.* (2020), who also recovered a good abundance of bacteria belonging to the genus *Hydrogenophaga* and can engage in HN-AD processes. A comparison between the different types of CWs shows that the genus *Desulfomicrobium* was highly represented in CWs treating industrial wastewater, indicating potential in sulfur convention compared to the other wetland that treats a mix of industrial and domestic wastewater. These findings concur with the study by Lu *et al.* (2023) on electrolysis-integrated CWs with pyrite filler for simultaneous enhanced phosphorus and nitrogen removal, which noted that the main functional bacteria were from the genus *Desulfomicrobium*.

The majority of other genera were represented by less than 0.01%. The *Flavobacterium* genus represents a group of bacteria that extensively engage in denitrification in wastewater treatment systems and floc-forming capabilities (Gonzalez-Martinez *et al.* 2018). The presence of a higher abundance of *Flavobacterium* may imply that they could play a role in CWs in reducing excess nitrogen in wastewater, thereby less harm to the environment from the effluent from these systems. Genus *Desulfomicrobium* was recovered at the inlet zone of the surface flow CWs. Among the harmful bacteria groups, *Enterobacteriaceae* was one of the bacteria groups found in the inlet of the CWs in IRI1. In all other wetlands, we have not recovered any harmful bacterial group. Similar findings were reported by Wang *et al.* (2022a, 2022b), who also noted a significant presence of *Enterobacteriaceae* in the studied wastewater from CWs. Although in this study we assessed the environmental samples using modern technologies, we did not find a significant abundance of the harmful bacterial group; this could be because of the method used to concentrate bacterial or could indicate a good removal efficiency of the wastewater treatment systems in the study area thereby making the treated wastewater good for reuse into economic activities such as urban farming.

The presence of a large set of uncultured or unidentified groups could be attributed by some bacteria that may not have been identified by this method either because their DNA is not captured during the extraction process or because their DNA sequences are not well represented in the references databases used for analysis these bacteria are referred as uncultured or unknown.

3.3. Functional prediction of the bacterial community in the studied CWs

The Kyoto Encyclopedia Genes and Genomes (KEGG) functional analysis was used to predict the functions of bacteria in the four studied CWs: Cellular process, metabolic process and nitrogen compound metabolic process. As shown in Figure 8, the abundance of cellular processes-related genes was highest in all samples, indicating that cellular processes play a great role in the microbial community and extensive growth of the microorganisms related to the abundance of the cellular processes. Genes related to metabolic process, organic substance metabolic process and nitrogen compound metabolic processes exist and were highly rich in the studied CWs, highlighting the biological significance of such bacterial genes in organic pollutants breakdown and removal of nitrogen compounds in the wastewater. A study by Wang *et al.* (2022a, 2022b) also indicated a higher abundance of functional genes relating to nitrogen conversion in CWs. The findings are also consistent with those reported by Cao *et al.* (2022) in their study on the microbial community involved in nitrogen and phosphorus removal, which found that the dominating genes in the examined CWs were for nitrogen and phosphorus removal.

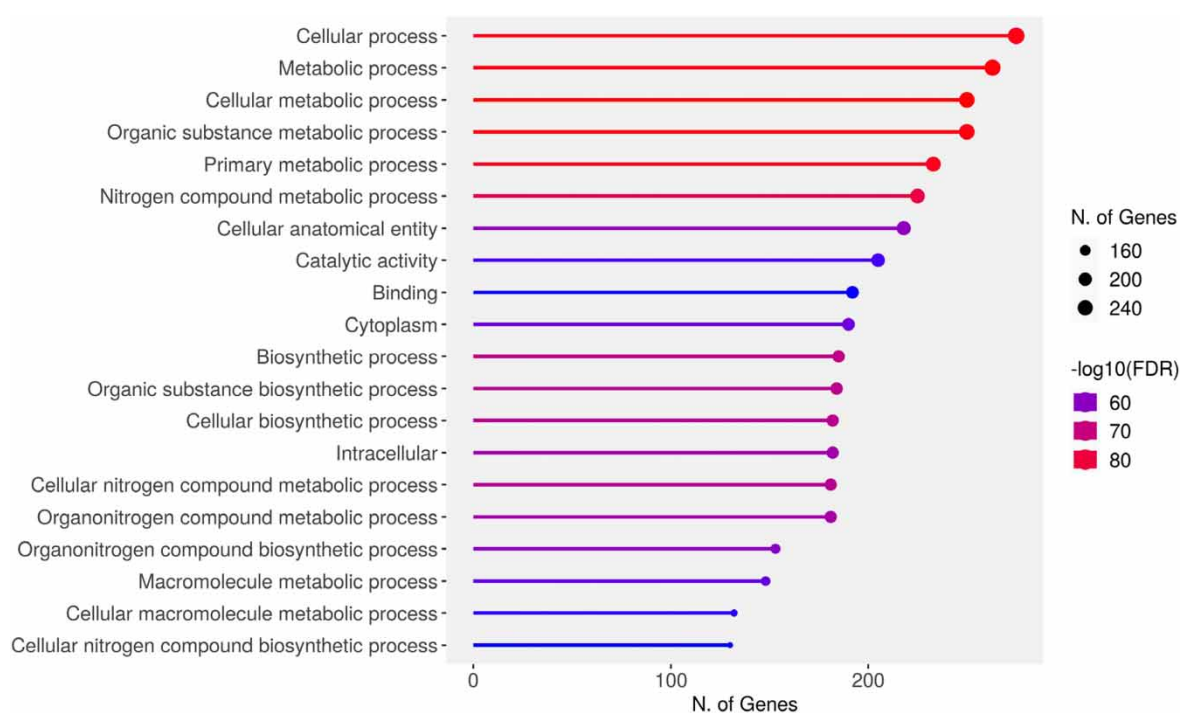


Figure 8 | Functional analysis of bacterial community in the studied CWs.

4. CONCLUSIONS

In conclusion, our study elucidates bacterial diversity in four different types of CWs for wastewater treatment: A subsurface CWs treating Banana brewing wastewater (BILCW), two surface flow CWs treating Municipal wastewater (ICW1 and ICW2) and a subsurface CW treating Municipal wastewater (MUWCW) using NGS. To the best of our knowledge and literature review, this is one of the first research papers in Tanzania that reports on bacterial communities in CWs for wastewater treatment. In summary, the phylum *Proteobacteria*, class *Gammaproteobacteria*, family *Comamonadaceae* and genus *Flavobacterium* were found to occur in a relatively higher percentage in all four studied CWs. Our study suggests that bacterial abundance and diversity differed along the different points of studied CWs, with BIL CWs and MUWCW having more bacterial abundance and diversity at the outlet zone and the other wetlands having higher abundance and diversity at the inlet and middle zones. These results also showed that the CWs with higher bacterial abundance and diversity at the outlet zone had good pollutant removal efficiency. A comparison between industrial wastewater treatment CWs and the one treating domestic wastewater revealed higher bacterial abundance and diversity in industrial CWs. The physicochemical parameters were found to play an important role in determining bacterial diversity

in the treatment systems. This study found that most bacterial activities happen at the outlet zone, thereby improving efficiency; this could be used to enhance CW design of CWs. This study observed that CWs, which host a significant abundance of bacterial community and higher bacterial composition identified to help remove pollutants from wastewater before it can be discharged into the environment. To have conclusive information on bacterial diversity at different sections of CWs and if they influence the performance of CWs systems, it is very important to perform a longitudinal investigation of bacterial community at each section and compare with the effluent removal efficiency for improving wastewater treatment technologies.

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DATA AVAILABILITY STATEMENT

Data cannot be made publicly available; readers should contact the corresponding author for details.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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