RESEARCH PAPER

Assessment of Bacterial Community Structure and Diversity in Water Samples through Environmental DNA(eDNA) Study

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Abstract: Understanding microbial diversity in aquatic environments is essential for environmental monitoring and ecological assessments. This study utilized metagenomic sequencing to assess the bacterial community structure and diversity in three water samples (D1AW, D2AW, and D5AW). We employed 16S rRNA gene sequencing targeting the V3-V4 region, with successful PCR amplification confirmed by agarose gel electrophoresis. Quantification using a Qubit Fluorometer indicated sufficient DNA concentrations for sequencing, with D1AW having the highest DNA concentration. Taxonomic analysis at the phylum level revealed significant differences among samples, with D1AW exhibiting the highest bacterial diversity. Sample D1AW had 424 Operational Taxonomic Units (OTUs), while D2AW and D5AW each had 123 OTUs. Rarefaction curves showed that D1AW had captured most of its microbial diversity, while D2AW and D5AW exhibited increasing diversity with additional sequencing. Beta diversity analyses indicated that D1AW and D5AW shared similar microbial communities, while D2AW presented a distinct microbial profile. Alpha diversity metrics highlighted D1AW's superior richness and evenness compared to the other samples. These findings underscore the impact of environmental factors on microbial diversity and the necessity of adequate sequencing depth for comprehensive community characterization. This metagenomic approach provides valuable insights into bacterial community variations and their ecological implications in aquatic environments.

Keywords:16S rRNA; Gene Sequencing; Metagenomics; QIIME2; Microbial Diaversity; Next-generation sequencing

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Introduction

Microbial communities in aquatic environments play an integral role in shaping ecosystem dynamics, influencing biogeochemical cycles, and maintaining ecological balance. These microorganisms, particularly bacteria, are essential for nutrient recycling, organic matter decomposition, and overall environmental health [Hill et al., 2000]. The study of microbial diversity within aquatic ecosystems is crucial, not only for understanding their contribution to ecosystem function but also for assessing the health and stability of these environments. Recent technological advancements, especially in molecular biology, have significantly enhanced our ability to explore microbial communities in unprecedented detail [Prasad et al., 2021].

The 16S rRNA gene has emerged as a pivotal molecular marker for examining bacterial diversity. Its combination of conserved and variable regions allows for accurate taxonomic classification of bacteria, making it invaluable for studies focused on microbial community composition. Next-generation sequencing (NGS) technologies, in particular, have revolutionized microbial ecology by enabling comprehensive analysis of 16S rRNA sequences across large datasets, providing deeper insights into the taxonomic diversity and complexity of microbial communities [Cao et al., 2017]. The scalability and precision of NGS have opened up new avenues for examining microbial diversity in diverse environments, from terrestrial soils to marine ecosystems, with enhanced accuracy [Tringe and Rubin, 2005].

However, despite these advancements, challenges remain in characterizing microbial communities, especially in environmental samples [Raajaraam and Raman, 2024]. Environmental samples, such as water and sediment, often contain diverse microbial populations, including many species that are difficult or impossible to culture in the laboratory [Stewart, 2012]. Additionally, the presence of contaminants, inhibitors, or degradation products in environmental matrices can complicate DNA extraction and PCR amplification, leading to potential biases in microbial diversity assessments. Therefore, the development and optimization of robust molecular methodologies are essential for accurately analyzing microbial communities in these complex environments [Stinson, Keelan, and Payne, 2019].

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This study focuses on characterizing the bacterial diversity in water samples D1AW, D2AW, and D5AW, collected from the coastal waters near Beyt Dwarka, Gujarat, India (22°27'53.6"N 69°08'06.9"E). These samples were subjected to high-throughput 16S rRNA gene sequencing to investigate the microbial communities inhabiting these waters. The primary objective is to identify dominant bacterial taxa, assess microbial richness and diversity, and explore potential ecological functions within this coastal ecosystem. Given the significant role that microbial communities play in aquatic ecosystems—ranging from nutrient cycling to supporting aquatic life and regulating water quality—understanding their composition and structure is critical for ecosystem management and conservation [Glasl et al., 2017].

By utilizing cutting-edge molecular techniques and bioinformatics analyses, this research aims to contribute to the broader understanding of microbial diversity in coastal waters. Specifically, the study provides insights into the microbial dynamics within samples D1AW, D2AW, and D5AW, with implications for ecological functioning and the health of the marine ecosystem surrounding Beyt Dwarka [Fuhrman, Cram, and Needham, 2015]. The findings will help illuminate the complex interactions within microbial communities and their potential roles in maintaining ecosystem balance and resilience in aquatic environments [Cadotte, Carscadden, and Mirotchnick, 2011].

Sample Collection and Preparation

Water samples D1AW, D2AW, and D5AW were collected from the coastal waters near Beyt Dwarka, Gujarat, India (22°27'53.6"N 69°08'06.9"E), under strict sterile protocols to minimize contamination risks. The collected samples were kept on ice during transportation and were immediately stored at -20°C until further processing. Before extracting DNA, each sample was thoroughly homogenized using a sterile mortar and pestle to ensure uniform distribution of microbial content across the sample [Thomsen et al., 2012].

DNA Extraction and Quantification

Genomic DNA was isolated from 0.25 grams of each sample using the Alexgen Soil DNA Kit (Cat no. AG-SD50), strictly adhering to the manufacturer's guidelines. The lysis of cells was achieved through both mechanical disruption and chemical treatment to enhance the DNA yield. Purification was carried out using a silica membrane, followed by sequential washing and the final elution of the DNA in 100 µL of nuclease-free water. To assess the quality and concentration of the extracted DNA, we utilized the Qubit® 4.0 Fluorometer (Thermo Fisher Scientific, USA), and the samples were also checked via gel electrophoresis to confirm DNA integrity [Lee et al., 2003].

16S rRNA Gene Amplification and Library Preparation

For each sample, the bacterial 16S rRNA gene's V3-V4 hypervariable regions were targeted for amplification. The primary PCR amplification employed the primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'), generating an amplicon of approximately 459 base pairs. Following the initial amplification, a second PCR was performed with overhang adapter primers: V3-F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and V4-R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') [Fadeev et al., 2021]. The PCR products were purified using AMPure XP beads (Beckman Coulter, USA) to remove contaminants, and their concentrations were measured using a Qubit® 4.0 Fluorometer. The amplicons were indexed with the Nextera XT Index Kit, followed by an additional round of purification using AMPure XP beads. The final library quality was confirmed on an Agilent TapeStation 4150 system with High Sensitivity D1000 ScreenTape®, ensuring the libraries were of the appropriate size and concentration for sequencing [Pal and Pal, 2022].

Sequencing

The indexed PCR libraries for samples D1AW, D2AW, and D5AW were pooled in equimolar ratios for sequencing on the Illumina MiSeq platform (Illumina, San Diego, CA, USA). Paired-end sequencing was carried out with a read length of 2 × 300 bp. Cluster generation was achieved through hybridization onto the oligonucleotide-covered flowcell surface, followed by bridge amplification to generate dense clonal DNA clusters [Daniel and Harbison, 2021].

Bioinformatics and Data Analysis

The raw sequence data generated from the Illumina platform were first processed using Cutadapt to trim adapters and filter low-quality bases. Cleaned reads were imported into QIIME2 (version 2022.2) for further analysis. Quality control, denoising, and merging of paired-end reads were performed using the DADA2 plugin. Chimeric sequences were filtered out, and high-quality sequences were clustered into Operational Taxonomic Units (OTUs) at a 97% similarity threshold, using the SILVA 138 database for reference. OTUs present at very low abundances (<0.01% of total reads) and singletons were excluded to minimize noise and improve data accuracy [Zakaria et al., 2024; Song et al., 2024].

Taxonomic assignment was performed using the SILVA 16S rRNA gene database, with a confidence threshold set at 0.8. Relative abundances were calculated for each sample to identify the dominant taxa. Alpha diversity metrics, such as Shannon's index, observed OTUs, and the Chao1 richness estimator, were computed to quantify microbial richness and evenness. For beta diversity, Bray-Curtis dissimilarity

and UniFrac distances were utilized, and Principal Coordinates Analysis (PCoA) was conducted to visualize compositional differences between the samples. A phylogenetic tree was constructed using the FastTree algorithm to explore evolutionary relationships among the bacterial taxa identified [Xia and Sun, 2023; Wang et al., 2015].

Data Availability

The raw sequencing data from this study have been deposited in the NCBI Sequence Read Archive under accession number- PRJNA1372649.

Results and Discussion

Amplicon Quality Control PCR amplification targeting the V3-V4 region of the 16S rRNA gene was successfully achieved for all three samples (D1AW, D2AW, D5AW). Agarose gel electrophoresis confirmed the amplification with distinct bands at the expected size of approximately 459 bp [Figure 1]. The clarity and intensity of these bands indicate efficient amplification without significant primer-dimer formation or non-specific amplification, ensuring high-quality templates for sequencing [Rintala et al., 2001].

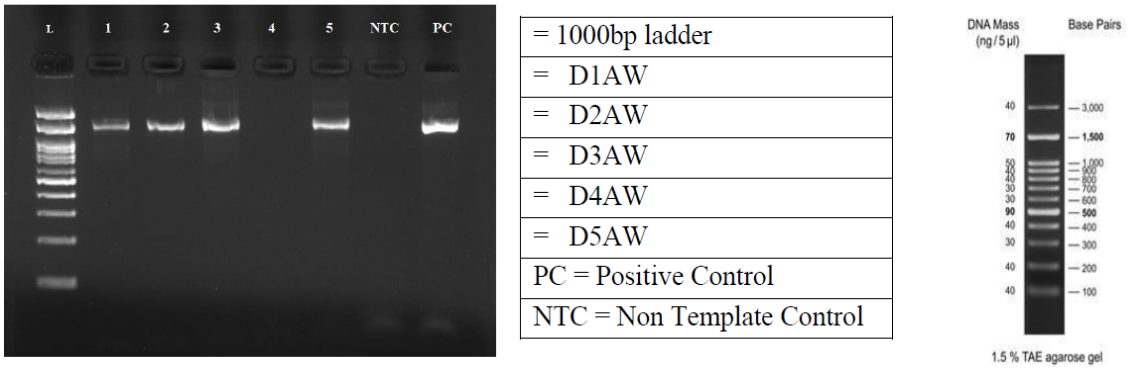


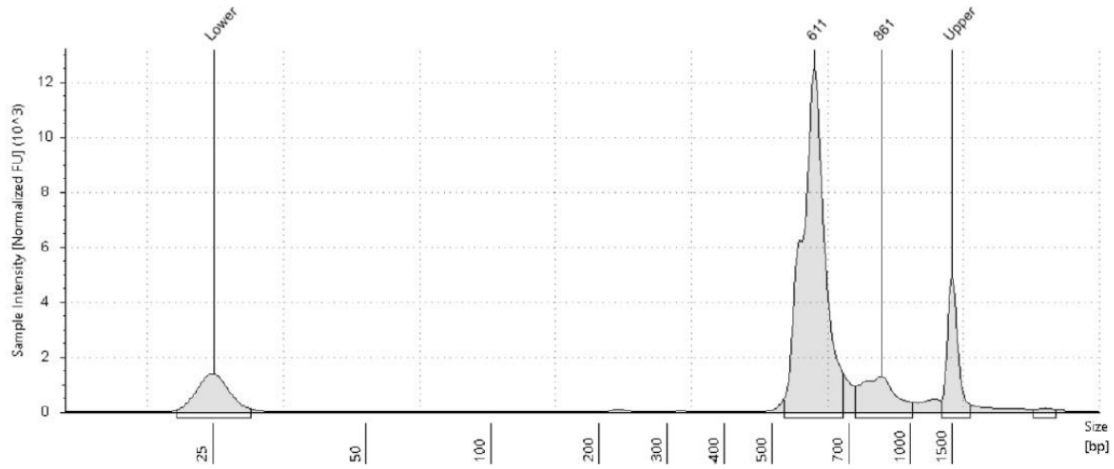
Figure 1: QC of amplicon on 1.8% Agarose Gel (A- series)

DNA Quantification Using Qubit Fluorometer DNA quantification using the Qubit Fluorometer provided precise measurements of DNA concentrations. Sample D1AW had the highest concentration at 2.40 ng/µL, yielding 120 ng from 50 µL. Samples D2AW and D5AW had lower concentrations of 0.244 ng/µL and 0.560 ng/µL, respectively, resulting in 12.2 ng and 28 ng of total DNA (Table 1). All samples passed quality control, as indicated by the "QC Pass" status, confirming their suitability for downstream applications [Li et al., 2021].

Table: 1

Sample ID	Concentration (ng/µL)	Volume (µL)	Yield (ng)	Remarks
D1AW	2.40	50	120	QC Pass
D2AW	0.244	50	12.2	QC Pass
D5AW	0.560	50	28	QC Pass

Library Pooling and Quality Control For sequencing, D1AW, D2AW, and D5AW were included in Pool-1. The pooling was performed based on their concentrations to ensure optimal sequencing depth. The quality of the pooled libraries was assessed using the Agilent TapeStation 4150 system with D1000 ScreenTape® [Figure 2]. The TapeStation profiles confirmed that the libraries were of the expected size and free from contamination, making them suitable for sequencing [Daniels et al., 2023].



Sample Table

Well	Conc. [ng/µl]	Sample Description	Alert	Observations
C1	41.4			

Figure 2: TapeStation 4150 profiles of Pool-1.

Data Generation Sequencing results yielded high-quality paired-end (PE) reads for all samples. Sample D1AW produced 450,952 reads, D2AW 416,656 reads, and D5AW 413,516 reads. The average read length was consistent at approximately 300 bp, with total data generated ranging from 124.38 Mb for D5AW to 135.63 Mb for D1AW (Table 2). This extensive sequencing coverage provided a thorough representation of the microbial communities in each sample.

Table: 2

Sample ID	# PE Seq	Total Reads (R1+R2)	Avg. Read Len (bp)	Data (bp)	Data (Mb)
D1AW	225,476	450,952	300.8	135,636,114	135.63
D2AW	208,328	416,656	300.9	125,365,340	125.36
D5AW	206,758	413,516	300.8	124,388,938	124.38

Taxonomy Analysis

Feature Summary The feature summary of each sample after processing through QIIME2 reveals the number of reads joined, filtered, and denoised into Operational Taxonomic Units (OTUs). Sample D1AW exhibited the highest filtered feature count of 424 OTUs, while D2AW and D5AW both displayed 123 OTUs (Table 3). This suggests that D1AW had greater bacterial diversity, with D2AW and D5AW showing lower diversity. The number of denoised sequences was highest in D1AW with 50,240 sequences, compared to 53,685 in D2AW and 50,677 in D5AW.

Table: 3

Sample ID	PE Reads	Joined Filtered Reads	Denoised Sequences	Filtered Sequences (OTUs)	Filtered Feature Count/OTUs
D1AW	225,476	146,675	50,240	3,040	424
D2AW	208,328	138,583	53,685	5,474	123
D5AW	206,758	138,653	50,677	7,330	123

Comparative Taxonomy Analysis

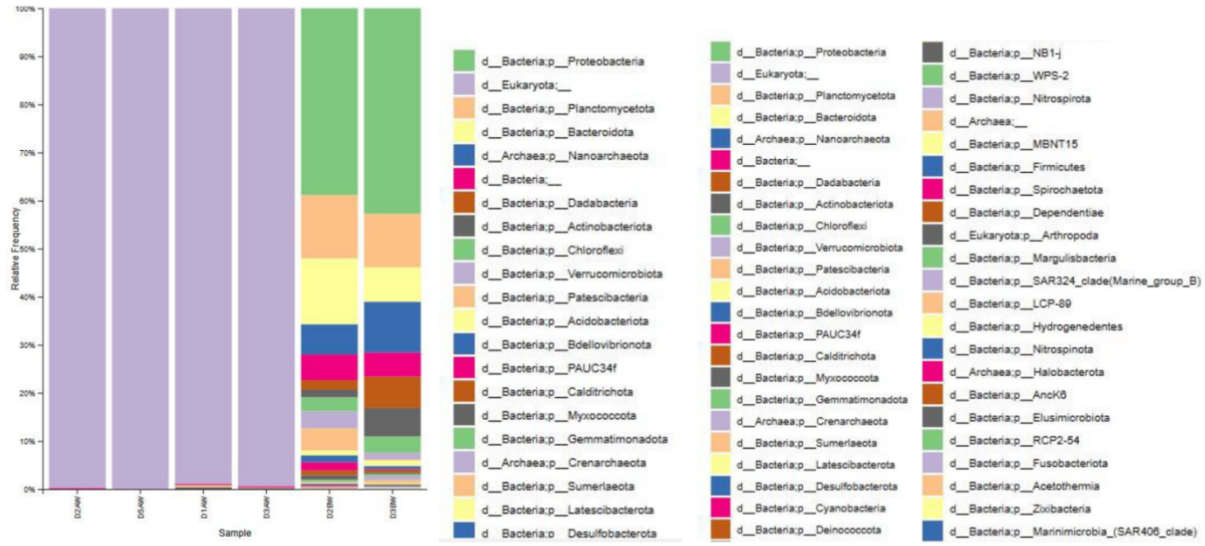


Figure 3: Comparative analysis of bacterial communities across samples D1AW, D2AW, and D5AW at the phylum

level. The bar plot illustrates the relative abundance of major bacterial phyla identified in each sample. Each bar represents a different phylum, with the length corresponding to its relative abundance within the respective sample. Notable variations among the samples highlight differences in microbial community composition. For additional taxonomic levels and detailed visualizations, please refer to the interactive HTML file located in the "05_Taxonomy_barplot/index.html".

Intra-Sample Analysis: α-Diversity Alpha diversity, reflecting the microbial diversity within each sample, was assessed using Chao1 richness estimator, observed OTUs, and Shannon entropy. D1AW showed the highest alpha diversity, with a Chao1 value of 424.1373 and Shannon entropy of 7.115507, indicating a more complex and diverse bacterial community (Table 4). In contrast, D2AW and D5AW exhibited lower diversity, with Chao1 values of 123.1887 and 123.0588, and Shannon entropy values of 3.555844 and 3.591353, respectively.

Table: 4

Sample ID	Chao1	Observed Features	Shannon Entropy
D1AW	424.1373	424	7.115507
D2AW	123.1887	123	3.555844
D5AW	123.0588	123	3.591353

D1AW's higher diversity could be linked to greater environmental heterogeneity or nutrient availability, while D2AW and D5AW showed similar, lower diversity, suggesting more homogenous conditions or environmental stress.

Rarefaction Analysis-

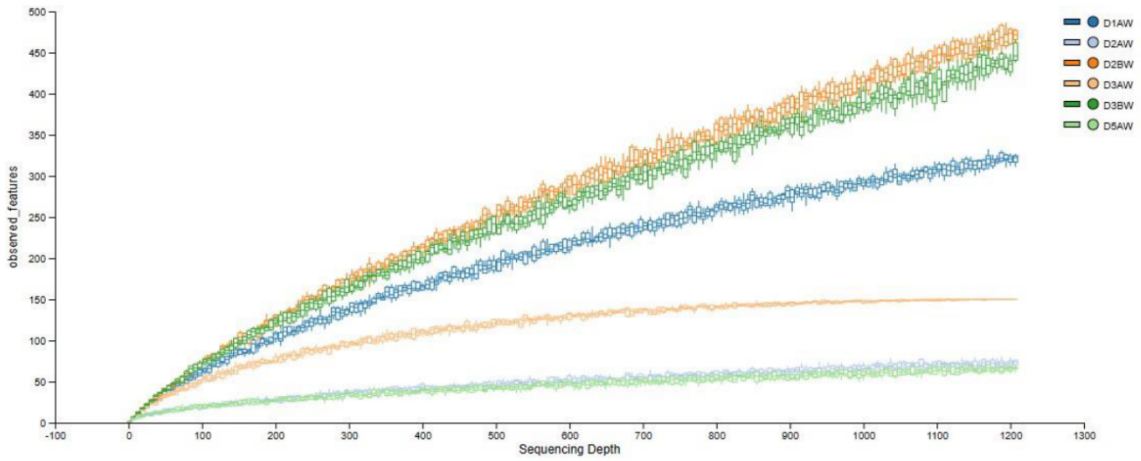


Figure 4: Rarefaction Curves of Observed OTUs for Microbial Diversity in Samples D1AW, D2AW, and D5AW

Rarefaction curves estimate species richness within a sample based on sequencing depth. The plot (Figure 4) shows that D1AW's curve reaches a plateau, indicating that the microbial diversity in this sample has been largely captured. Conversely, D2AW and D5AW show steeper slopes, suggesting that additional sequencing could reveal more diversity. This indicates that these samples have more complex microbial communities that require deeper sequencing for full coverage. The rarefaction analysis files are included in the deliverables under "09_Rarefaction_curve."

Inter-Sample Analysis: Beta Diversity Beta diversity assesses compositional differences between microbial communities across samples. Both weighted and unweighted beta diversity metrics were used to evaluate these differences.

Weighted Distance Matrix The weighted distance matrix (Table 5) incorporates both taxa presence and abundance. D1AW and D5AW have the smallest distance (0.65), indicating similar microbial communities. D2AW shows greater dissimilarity from both D1AW (1.03) and D5AW (1.07), suggesting distinct microbial communities.

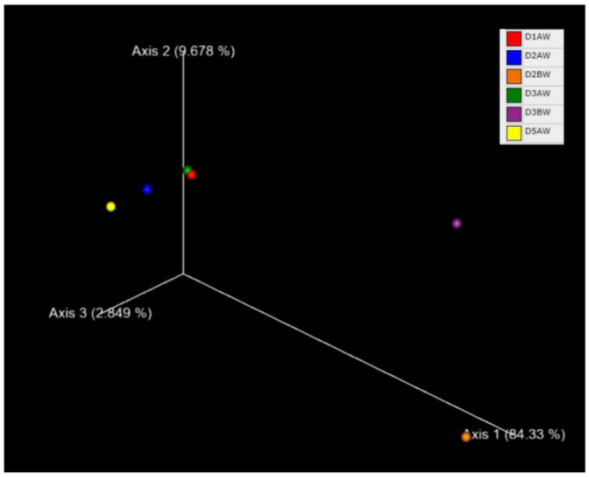


Figure 5: Principal Coordinates Plot Based on Weighted Distance Matrix

PCoA based on the weighted distance matrix (Figure 4) reveals a distinct separation of D2AW from D1AW and D5AW, which cluster closer together, highlighting compositional differences.

Table: 5

Sample ID	D1AW	D2AW	D5AW
D1AW	0	1.03	0.65
D2AW	1.03	0	1.07
D5AW	0.65	1.07	0

Unweighted Distance Matrix The unweighted distance matrix (Table 6) evaluates taxa presence without considering abundance. D1AW and D5AW are still closely related (0.68), while D2AW remains the most distinct (0.80 and 0.73 from D1AW and D5AW, respectively), suggesting a unique microbial profile.

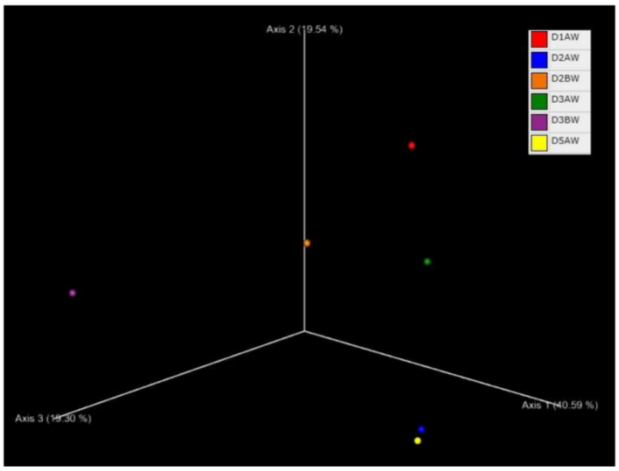


Figure 6: Principal Coordinates Plot Based on Unweighted Distance Matrix

The unweighted PCoA plot (Figure 5) highlights D2AW's distinct microbial community, while D1AW and D5AW are more similar.

Table: 6

Sample ID	D1AW	D2AW	D5AW
D1AW	0	0.80	0.68
D2AW	0.80	0	0.73
D5AW	0.68	0.73	0

Intra-Sample Analysis: Alpha Diversity Alpha diversity metrics indicate that D1AW has the highest richness and evenness, suggesting a more complex microbial community compared to D2AW and D5AW. Both D2AW and D5AW show lower richness and diversity, with D2AW having slightly lower evenness.

Discussion

The analysis of bacterial communities in water samples D1AW, D2AW, and D5AW provides significant insights into the microbial diversity and community structure present in these aquatic environments. This study utilized 16S rRNA gene sequencing to profile bacterial communities, revealing notable differences in diversity and composition across the samples.

Quality Control and Sequencing Depth

The initial quality control measures confirmed successful PCR amplification of the 16S rRNA gene V3-V4 region, as indicated by clear bands on the agarose gel (Figure 1). The Qubit Fluorometer results confirmed sufficient DNA concentrations for sequencing, with D1AW exhibiting the highest concentration among the samples. This suggests that D1AW potentially harbors a more complex microbial community, which is supported by subsequent diversity analyses.

Taxonomic Composition

Taxonomic analysis (Figure 1) at the phylum level revealed significant differences in bacterial community composition among the samples. D1AW demonstrated a greater diversity in bacterial phyla, which could be attributed to more varied environmental conditions or nutrient availability in its habitat. The relative abundance of different phyla varied across samples, with D2AW and D5AW showing less diversity. This variation could be indicative of differing environmental conditions, such as water chemistry or anthropogenic impacts, which may influence microbial community structure [Bissett et al. 2013].

Diversity Metrics

Rarefaction analysis (Figure 3) provided valuable insights into the sampling depth and coverage of microbial diversity [Sogin et al. 2006]. The rarefaction curves for D1AW reached a plateau, suggesting that the sequencing depth was adequate to capture the majority of microbial diversity in this sample. In contrast, D2AW and D5AW showed steeper slopes, indicating that additional sequencing could uncover more diversity. This observation highlights the importance of sequencing depth in accurately characterizing microbial communities and underscores the potential need for more extensive sampling in environments with higher microbial diversity. Alpha diversity metrics further emphasize the differences in microbial richness and evenness across the samples [Hagerty et al. 2020]. D1AW exhibited the highest values for Chao1 and Shannon entropy, reflecting a more complex and diverse microbial community. This increased diversity could be due to environmental factors such as habitat heterogeneity or nutrient gradients that support a wider range of microbial species. Conversely, D2AW and D5AW displayed lower diversity metrics, which could be related to more homogeneous environmental conditions or environmental stressors affecting microbial populations [Ryall, Eydallin, and Ferenci, 2012].

Beta Diversity

Beta diversity analyses, including weighted and unweighted distance matrices (Tables 5 and 6) and Principal Coordinates Analysis (Figures 3 and 4), revealed distinct patterns in microbial community composition [Lozupone et al. 2007]. The weighted distance matrix indicated that D1AW and D5AW shared similar microbial communities in terms of taxa composition and abundance. However, D2AW exhibited significant dissimilarity from both D1AW and D5AW, suggesting unique microbial profiles. The unweighted distance matrix reinforced this finding by highlighting the distinct set of taxa present in D2AW compared to the other samples [Liu et al. 2022]. These results underscore the impact of environmental factors on microbial community composition. The distinct microbial community in D2AW could be influenced by specific local conditions or pollution sources that differ from those affecting D1AW and D5AW [Liu et al. 2023].

The metagenomic analysis of water samples D1AW, D2AW, and D5AW reveals distinct variations in bacterial community structure and diversity, reflecting the influence of environmental conditions on microbial composition. The significantly higher alpha diversity observed in sample D1AW, as evidenced by Chao1 and Shannon entropy metrics, suggests that this sample harbors a more complex and diverse microbial community. The rarefaction curves further confirm that D1AW's microbial diversity is well-captured with the current sequencing depth, indicating a comprehensive coverage of its community [Liao et al. 2017]. In

contrast, D2AW and D5AW exhibited lower alpha diversity and steeper rarefaction curves, implying that additional sequencing may be required to fully characterize their microbial richness. Beta diversity analyses reveal notable differences in microbial composition between samples, with D2AW displaying greater dissimilarity from D1AW and D5AW. This divergence suggests unique environmental or ecological factors affecting D2AW, which may include variations in nutrient availability or pollution levels. The consistency in the composition between D1AW and D5AW, both in weighted and unweighted analyses, highlights their shared environmental characteristics but also underscores the need for further investigation into specific local factors influencing microbial communities. Overall, the study underscores the importance of sequencing depth and environmental context in shaping and understanding microbial diversity [Kassen and Rainey, 2004].

Conclusion

This study comprehensively characterizes bacterial communities in water samples D1AW, D2AW, and D5AW using 16S rRNA gene sequencing. The results demonstrate significant variations in microbial diversity and composition across the samples, with D1AW showing the highest richness and diversity, indicative of a complex and varied microbial ecosystem. In contrast, D2AW and D5AW present lower diversity and distinct microbial profiles, suggesting that environmental factors or ecological conditions substantially influence microbial community structure. The findings emphasize the critical role of adequate sequencing depth for thorough microbial characterization and the impact of environmental variables on microbial diversity. This research contributes valuable insights into microbial ecology and highlights the utility of metagenomic approaches in environmental monitoring, providing a foundation for future studies aimed at understanding and managing microbial communities in aquatic systems.

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Not applicabele

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Conflict of Interest

The authors declare no conflicts of interest related to the design, execution, interpretation, or reporting of this study.

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