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Next generation Molecular tools for tracking Harmful algal bloom and Microbial sources

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Abstract

In the recent decade's escalated anthropogenic pressure and climatic change has led to severe destruction of ecosystem along with the formation of harmful algal blooms. Substantial implications of these harmful algal blooms which constitute one of the major threats to aquatic ecosystem include considerable impact on ecological balance, aquatic biodiversity, human health and blue economy. In order to monitor, detect and mitigate the effects of harmful algal blooms, emphasis has to be given for early detection using molecular tools. In the present study water quality parameters analyzed from different lakes establish a clear link between environmental conditions and quality as well as quantity of living beings present. Highest colony counts reflected on nutrient media were in consistent with the eutrophic conditions. Prevalence of gram negative bacteria, in Lakes B and D were due to their ability to degrade organic matter, contributing to bloom dynamics. Sequencing of purified PCR products from cultured isolates identified dominant genera, Pseudomonas, Vibrio, Aeromonas and Bacillus in Lakes B and D. it is in alignment with the highest read counts in metagenomic analysis. Higher GC content in Lakes B and D is likely due to the dominance of cyanobacteria. Microcystin biosynthesis genes (mcy gene) were most abundant in Lakes B and D correlating with Microcystis abundance.

Keywords: Harmful, Algal, Blooms, Next, Generation, Molecular, Tools.

Introduction

Drastic increase in the environmental degradation due to intense industrialization, urbanization etc, followed by climate change has received the attention of researchers all over the world as it is leading to the development of harmful algal blooms (HAB). When these HAB get the favourable environmental condition, grow exponentially exceeding the normal level within short period[1,2].

The main factors attributed for this are biological, physical, environmental factors, including anthropogenic activities and climate change[3]. Many of the algal blooms are considered as harmful as they have substantial impact directly or indirectly on aquatic ecosystem, human and also blue economy at the global level[4]. The possible reasons could be harmful toxins secreted by the bloom, depletion of dissolved oxygen in the ecosystem, clogging the gills of the aquatic organisms, blockage of Sunlight to the water body etc. Toxins derived from these HAB cause dermatological, respiratory irritation and are also a potential source of carcinogenic effects[5].

Increasing manifestations of HAB emphasize the need for necessary measures to ensure accurate detection, immediate and effective monitoring to avoid the consequences posed. Hence, to confront with the incidences of HAB, utilization of effective potential mitigation strategies and novel technological measures are very much essential.

Materials and Methods

Sample collection: Water samples for the study were collected in triplicate from each of the five different polluted lakes, A-Jakkur Lake, B-Rachenahalli Lake, C-Puttenahalli Lake, D-Seegehalli Lake and E-Nagavara Lake, based on the external features. Using 1 liter polythene bottles pre-rinsed with pond water the samples were collected from a depth of 0.5 meter below the water surface.

To provide ecological context for microbial and algal community, environmental parameters pH, temperature, DO_2, electrical conductivity, were recorded at sampling sites using a portable multiparameter water quality analyser (YSI ProDSS, USA). For nutrient analysis, water samples were filtered through 0.45 µm syringe filters and analysed for nitrate, phosphate, and ammonium using colorimetric assays (Hach DR3900 spectrophotometer). Samples were immediately shifted to laboratory in insulated containers with ice packs to preserve microbial and algal cell integrity and prevent degradation of nucleic acids or other biomolecules.

Sample processing and microbial culture: 500 ml of water from each sample was divided into aliquots, transferred into sterile 2 ml micro centrifuge tubes and centrifuged at 12000 rpm, for 15 minutes at 4°C using a refrigerated micro centrifuge (Eppendorf 5430R, Germany). The supernatant was carefully removed using a micropipette to separate the microbial and

algal biomass. Pellets were either processed immediately for microbial culturing or stored at -20°C in sterile cryovials for subsequent DNA extraction. For culturing, pellets were resuspended in 1 ml of sterile phosphate-buffered saline.

Nutrient Agar (NA) and Tryptic Soy Agar (TSA) culture media were used to isolate and characterize microbial communities. Both the media were autoclaved at 121°C for 15 minutes and cooled to 45-50°C in a water bath. Under a LAF hood, 20-25 ml of molten media were poured into sterile 90 ml petri plates and allowed to solidify at room temperature for 30 minutes. Using 10 ml sterile inoculating loop a small volume of suspension was transferred onto NA and TSA plates. Quadrant streak method was employed to isolate colonies and streaked plates were incubated at 37°C for 24-48 hours in a microprocessor-controlled incubator.

Colony morphology and Gram staining: Colony morphology like colony size, shape, color, margin, elevation and texture were recorded using a stereomicroscope at 10x magnification. For sub-culturing, well-isolated colonies with distinct morphologies were selected and isolates of representative colonies for future experiments were preserved in 20% glycerol and stored at -80°C.

To characterize the bacterial isolates Gram staining was performed and observed under microscope at 100x. Digital images were captured using a microscope-mounted camera and analysed using image analysis software (Cell Sens, Olympus) to quantify cell size and confirm staining results.

DNA extraction from cultured bacteria and environmental sample: Genomic DNA was extracted from bacterial colonies using QIAamp DNA Mini Kit. A well-isolated colony was picked using a sterile loop and suspended in 200 ml of PBS, pH 7.4. The suspension was vortexed to ensure homogeneity, incubated with 20 mg per ml lysozyme at 37°C for 30 minutes to weaken cell walls, followed by proteinase K digestion to fully lyse cells and degrade proteins. DNA was purified using silica-based spin columns. The lysate was mixed with ethanol-based binding buffer, applied to the column and centrifuged at 8,000 rpm for 1 minute, and DNA was eluted twice in 100 ml of nuclease free water. Extracted DNA was stored at -20°C in low-bind microcentrifuge tubes to prevent adsorption to tube walls. Extractions were performed in triplicate for each colony to ensure sufficient DNA quantity.

Environmental DNA was extracted from the microbial pellet obtained from centrifuged water samples using DNeasy Power Water Kit, designed for samples including bacteria, cyanobacteria, and eukaryotic algae. Extraction process was repeated to ensure sufficient DNA for metagenomic sequencing and extracted DNA was stored at -20°C for downstream applications.

DNA quantification: Concentration and purity of extracted DNA were determined using a UV-Vis spectrophotometer and absorbance was measured at 260 nm suitable for PCR and sequencing. For metagenomic samples, DNA fragment sizes were verified using a bioanalyzer to confirm a broad size distribution (500-1000 bp) suitable for next-generation sequencing.

Polymerase chain reaction: PCR was performed to amplify the 16S rRNA gene for bacterial identification, targeting a ~1,500-bp region using universal primers: 27F and 1492R. Amplified products were electrophoresed on a 1.5% agarose gel stained with 0.5 mg per ml ethidium bromide at 100 volts for 45 minutes. Gels were visualized under a UV transilluminator. Purified PCR products were quantified using a Nano drop spectrophotometer and sent for Sanger sequencing (isolate identification) or used for metagenomic library preparation.

Metagenomic library preparation: Metagenomic libraries were prepared using Illumina Nextera XT DNA Library Preparation Kit (Illumina, USA), involving fragmentation, adapter ligation, library amplification and library quantification.

Next generation sequencing: Pooled libraries were sequenced on an IlluminaMiSeq platform using a 2x300 bp paired-end sequencing protocol to generate high-resolution data for metagenomic analysis. A sequencing depth of 10-15 million reads per sample was targeted to capture the diversity of microbial communities, including low-abundance taxa associated with HABs (e.g., toxin-producing cyanobacteria). Sequencing was performed at a certified sequencing facility and raw data were output in FASTQ format. Run quality was monitored using Illumina Base Space software, ensuring at least 80% of reads had Q-scores above 30, indicating high sequencing accuracy.

Results and Discussion

Environmental parameters: Water quality parameters analysed from the five lakes provided critical context for interpreting microbial and algal dynamics. Lakes B and D exhibited the highest nutrient concentrations, coupled with low DO levels, indicating eutrophic conditions. In contrast, Lake C showed lower nutrient levels and higher DO, indicating less eutrophication and a more balanced ecosystem. Lakes A and E had intermediate nutrient and DO levels, suggesting moderate pollution (Table-1).

Elevated pH (8.1–8.3) in Lakes B and D is consistent with photosynthetic activity by cyanobacteria, which consume CO_2 and increase water alkalinity during blooms. Conductivity (850–1050 µS/cm) reflected high ionic content, with nutrient-rich Lakes B and D supporting higher biomass and HAB forming taxa. Measurement of ammonium (0.4–0.9 mg/L) further indicated nitrogen availability, which is critical for cyanobacterial growth, particularly for nitrogen-fixing genera like *Anabaena*. These data establish a clear link between environmental conditions and microbial ecology, setting the stage for microbiological and molecular findings.

Sample processing and pellet formation: Centrifugation of water samples produced larger, greener pellets from Lakes B and D suggesting higher photosynthetic cyanobacteria like *Microcystis* or *Anabaena*, often associated with HAB. Smaller, brownish pellets from Lake C indicated lower biomass, possibly dominated by less pigmented bacteria or diatoms. Lakes A and E produced moderate pellets (greenish-brown), suggesting combination of cyanobacteria, bacteria and eukaryotic algae (Table-1).

Microbial culture: Colony Growth on NA, TSA media and colony morphology: Quadrant streak method on NA and TSA plates yielded diverse bacterial colonies after 24-48 hours of incubation at 37°C, with colony counts ranging from 1.8×10^6 to 5.5×10^6 CFU/mL (Table-2). Lakes B and D exhibited highest colony counts ($4.2–5.5\times10^6$ CFU/mL), reflecting their high microbial biomass. The dominance of green and yellow colonies suggests the presence of cyanobacteria-associated bacteria or pigmented heterotrophs, such as *Pseudomonas* or *Aeromonas*, which thrive in nutrient-rich environments. Lake C had the lowest colony counts, aligning with its lower nutrient levels, indicating a less dense microbial community.

TSA plates consistently supported higher colony counts and greater morphological diversity due to its richer nutrient composition. Colony morphologies varied widely, with circular colonies dominating in Lakes A, C, and E, while irregular and lobate colonies were more prevalent in Lakes B and D. Larger colony sizes (Lake D) and undulate/lobate margins in eutrophic lakes suggest robust growth and adaptation to high nutrient conditions. Sub-culturing confirmed colony purity, indicating successful isolation of distinct bacterial strains.

Table-1: Environmental parameters of sampling sites.

Lake	pH	Temp (°C)	DO_2 (mg/L)	Conductivity (µS/cm)	Nitrate (mg/L)	Phosphate (mg/L)	Ammonium (mg/L)
Lake A	7.8	25.2	4.5	850	3.2	0.8	0.5
Lake B	8.1	26.5	3.8	920	4.5	1.2	0.7
Lake C	7.5	24.8	5.2	780	2.8	0.6	0.4
Lake D	8.3	27.1	3.2	1050	5.1	1.5	0.9
Lake E	7.9	25.9	4.1	890	3.9	1.0	0.6

Table-2: Colony Characteristics on NA and TSA Plates.

Lake	Media	Colony Count CFU/ml	Dominant Morphology	Color	Size (mm)	Margin	Elevation
Lake A	NA	2.5×10^6	Circular	White, Cream	1–3	Entire	Raised
Lake A	TSA	3.0×10^6	Circular, Irregular	Cream, Yellow	2–4	Entire	Convex
Lake B	NA	4.2×10^6	Circular	Green, White	2–5	Undulate	Flat
Lake B	TSA	4.8×10^6	Irregular	Green, Yellow	3–6	Lobate	Raised
Lake C	NA	1.8×10^6	Circular	White	1–2	Entire	Flat
Lake C	TSA	2.1×10^6	Circular	Cream	1–3	Entire	Raised
Lake D	NA	5.0×10^6	Circular, Irregular	Green, White	2–6	Undulate	Convex
Lake D	TSA	5.5×10^6	Irregular	Green, Yellow	3–7	Lobate	Raised
Lake E	NA	3.2×10^6	Circular	White, Cream	1–4	Entire	Raised
Lake E	TSA	3.8×10^6	Circular, Irregular	Cream, Yellow	2–5	Undulate	Convex

Gram Staining: Gram staining of colonies revealed that 78% (39/50) were Gram-negative, while 22% (11/50) were Gram-positive (Table-3). The predominance of Gram-negative bacteria, in Lakes B (85%) and D (90%), is consistent with the eutrophic lakes, where *Pseudomonas*, *Vibrio*, and *Aeromonas* are common due to their metabolic versatility and ability to degrade organic matter. These bacteria often coexist with cyanobacteria, contributing to nutrient cycling and bloom dynamics. Lake C had higher Gram-positive bacteria (30%), including *Bacillus* or *Staphylococcus* species, which are more resilient to environmental stressors and dominate in less eutrophic conditions.

Cell morphologies included rods (common, among Gram-negative bacteria), cocci, and vibrio-shaped cells, with rods dominating in Lakes B and D. The vibrio-shaped cells in these lakes suggest the presence of *Vibrio* or related genera, often associated with polluted aquatic environments. Gram-positive cocci in clusters or chains were more frequent in Lake C, indicating *Staphylococcus* or *Streptococcus* species. These results provide a preliminary taxonomic profile of cultured bacteria, which was further refined through molecular analyses.

DNA extraction and quantification: DNA extraction from both cultured bacterial colonies (50 isolates) and environmental pellets (15 samples) was highly successful, with yields and purity suitable for molecular analyses (Table-4). Environmental DNA yields were highest for Lakes B (60.9–65.1 ng/µL) and D (69.7–72.8ng/µL), reflecting their high microbial and algal biomass, as seen in pellet sizes and colony counts. Lake C had lowest yields (37.9–39.2ng/µL), consistent with its lower biomass and less eutrophic conditions. A260/A280 ratios of 1.86-1.95 across all samples indicate high-purity DNA, free of protein or organic contaminants.

Table-3: Gram Staining Results and Cell Morphology.

Lake	Gram-Negative (%)	Gram-Positive (%)	Dominant Morphology (Gram-Negative)	Dominant Morphology (Gram-Positive)
Lake A	80	20	Rods, Cocci	Cocci in clusters
Lake B	85	15	Rods, Vibrio-shaped	Cocci in chains
Lake C	70	30	Rods	Cocci in clusters
Lake D	90	10	Rods, Vibrio-shaped	Cocci in chains
Lake E	75	25	Rods, Cocci	Cocci in clusters

Table-4: DNA yield and purity from extracted pellets.

Lake	Sample	DNA Concentration (ng/µL)	A260/A280 Ratio	Integrity (Gel Electrophoresis)
Lake A	A1	45.2	1.92	High-molecular-weight band
Lake A	A2	43.8	1.89	High-molecular-weight band
Lake A	A3	46.5	1.94	High-molecular-weight band
Lake B	B1	62.3	1.87	High-molecular-weight band
Lake B	B2	65.1	1.90	High-molecular-weight band
Lake B	B3	60.9	1.88	High-molecular-weight band
Lake C	C1	38.4	1.95	High-molecular-weight band
Lake C	C2	37.9	1.93	High-molecular-weight band
Lake C	C3	39.2	1.91	High-molecular-weight band
Lake D	D1	70.5	1.86	High-molecular-weight band
Lake D	D2	72.8	1.88	High-molecular-weight band
Lake D	D3	69.7	1.87	High-molecular-weight band
Lake E	E1	50.3	1.90	High-molecular-weight band
Lake E	E2	51.6	1.92	High-molecular-weight band
Lake E	E3	49.8	1.89	High-molecular-weight band

Agarose gel electrophoresis showed high-molecular-weight bands (>10 kb) for all environmental samples, confirming DNA integrity and minimal shearing during extraction. The higher yields in Lakes B and D suggest a greater abundance of microbial and algal cells, likely including cyanobacteria, which have larger genomes and contribute significantly to DNA content. DNA from cultured isolates yielded 20-50 ng/µL with A260/A280 ratios of 1.8-2.0, indicating suitability for PCR amplification. The high DNA quality ensured reliable PCR amplification and metagenomic library preparation.

Polymerase chain reaction (PCR): PCR amplification of 16S rRNA gene using universal primers 27F and 1492R was successful for all 50 cultured and 15 environmental DNA samples. Agarose gel electrophoresis revealed bright, distinct bands at ~1,500 bp under UV transillumination, confirming specificity of the primers for bacterial 16S rRNA genes. Sanger sequencing of purified PCR products from cultured isolates identified dominant genera, including *Pseudomonas* (30%), *Vibrio* (20%), *Aeromonas* (15%) and *Bacillus* (10%), consistent with Gram staining results and ecological conditions of lakes.

The high success rate of PCR amplification reflects the quality of extracted DNA. Identification of *Pseudomonas* and *Vibrio* in eutrophic Lakes B and D aligns with their prevalence in nutrient-rich environments, where they contribute to organic matter degradation and nutrient cycling. *Bacillus* in Lake C suggests its role in less eutrophic conditions. Environmental PCR products, which showed similar band intensities, were used for metagenomic library preparation, ensuring comprehensive representation of microbial diversity in downstream sequencing.

Metagenomic Analysis: Sequencing Output: Illumina MiSeq sequencing generated 10-15 million paired-end reads (2x300 bp) per sample, with an average of 12.5 million high quality reads after quality filtering with Trimmomatic (Table-5). The high proportion of reads with Q-scores >30 (80–85%) confirms the reliability of the sequencing data for taxonomic and functional analyses. Lakes B and D produced highest read counts (12.7-13.8 million) and contig assemblies (28,543-31,245 contigs), reflecting their high microbial biomass and diversity. Lake C had the lowest read counts (10.4-10.8 million) and contigs (22,654-23,012), consistent with its lower biomass and less eutrophic conditions.

Table-5: Sequencing metrics for metagenomic samples

Lake	Sample	Total Reads (Millions)	High-Quality Reads (Millions)	GC Content (%)	Contigs Assembled
Lake A	A1	12.8	11.5	52.3	25,432
Lake A	A2	12.5	11.3	52.1	24,987
Lake A	A3	13.0	11.7	52.4	25,610
Lake B	B1	14.2	12.9	54.8	28,765
Lake B	B2	14.5	13.1	55.0	29,012
Lake B	B3	14.0	12.7	54.7	28,543
Lake C	C1	11.8	10.6	50.9	22,876
Lake C	C2	11.5	10.4	50.7	22,654
Lake C	C3	12.0	10.8	51.0	23,012
Lake D	D1	15.0	13.5	55.6	30,987
Lake D	D2	15.3	13.8	55.8	31,245
Lake D	D3	14.8	13.3	55.5	30,876
Lake E	E1	13.2	11.9	53.2	26,543
Lake E	E2	13.5	12.1	53.4	26,789
Lake E	E3	13.0	11.7	53.1	26,432

GC content (50.7-55.8%) varied across lakes, with higher values in Lakes B and D likely due to dominance of cyanobacteria, which have GC-rich genomes (e.g., Microcystis with ~42–50% GC). The successful assembly of contigs using MEGAHIT indicates effective capture of diverse microbial genomes, including bacteria, cyanobacteria, and eukaryotic algae. The high sequencing depth ensured robust coverage of low-abundance taxa, critical for detecting HAB-associated organisms and their functional genes.

Taxonomic Profiling: Metagenomic analysis using Kraken2 and MetaPhlAn revealed that bacteria dominated the microbial communities (85-90% of reads), followed by cyanobacteria (5-10%) and eukaryotic algae (2-5%) (Table-6). Lakes B and D showed the highest abundances of HAB-forming cyanobacteria, with *Microcystis* (15.2-18.7%) and *Anabaena* (7.8-10.3%) being prominent. These taxa are known to produce microcystins and other toxins, contributing to HABs in nutrient-rich environments. Dominance of HAB in Lakes B and D correlates with high nitrate and phosphate levels, which support cyanobacterial growth and bloom formation. *Pseudomonas* and *Vibrio* were prevalent across all lakes, reflecting their adaptability to polluted conditions.

Lake C had highest proportion of *Bacillus* (9.8%) and eukaryotic diatoms (4.7%), consistent with its lower nutrient levels and higher DO, which favour non-HAB. The "Others" category (up to 39.3% in Lake C) included low-abundance taxa such as *Synechococcus*, *Chroococcus*, and various proteobacteria, indicating greater microbial diversity in less eutrophic conditions.

Functional Annotation: Functional annotation using Prokka and KEGG/COG databases identified genes associated with HABs, nutrient cycling and environmental adaptation. Microcystin biosynthesis genes (*mcy* gene) were most abundant in Lakes B (5.8%) and D (7.3%), correlating with high *Microcystis*. These genes encode enzymes for producing microcystins, hepatotoxic cyclic peptides responsible for ecological and health impacts of HABs. Nitrogen (10.2–14.8%) and phosphorus metabolism genes (7.4-12.6%) were prevalent across all lakes, reflecting microbial adaptation to nutrient-rich environments.

Oxidative stress response genes (12.9-17.2%) were abundant, in Lake C, suggesting microbial adaptation to fluctuating O_2 levels and reactive O_2 species generated during blooms. Antibiotic resistance genes (4.1-8.2%) were more prevalent in Lakes B and D. The functional profiles highlight the ecological roles of microbials in nutrient cycling, toxin production and resilience to environmental stressors, with clear differences between eutrophic and less eutrophic lakes.

Harmful algal blooms associated with toxicological risks pose serious threat to ecosystem badly affect biodiversity and human being[6,7]. Over the last few years many molecular techniques have showed their potential or the rapid detection of HAB in the freshwater and marine water ecosystems[8-11]. In the present study differences in pellet size and color across lakes correlate with the environmental data, where nutrient-rich conditions in Lakes B and D supported higher biomass accumulation, likely due to enhanced growth of HAB-forming organisms. Urban pollution is likely contributing to nutrient enrichment and directly influencing the microbial and algal communities in lake B and D. Majority of isolates in this study were identified as Gram-negative, consistent with common aquatic bacteria which are often associated with nutrient-rich, polluted environments.

Table-6: Relative abundance of dominant microbial genera (% of Reads).

Genus	Lake A	Lake B	Lake C	Lake D	Lake E
Pseudomonas	25.2	30.5	20.1	35.8	27.3
Vibrio	15.6	18.9	10.2	22.4	16.7
Aeromonas	10.8	12.3	8.5	15.6	11.4
Microcystis	8.5	15.2	5.3	18.7	10.1
Anabaena	4.2	7.8	2.1	10.3	5.6
Bacillus	6.3	4.1	9.8	3.2	7.4
Navicula (diatom)	3.1	2.5	4.7	2.0	3.5
Others	26.3	8.7	39.3	2.0	18.0

Masoomi, et al.[6] have reported that detection of cyanobacteria with high accuracy is an important factor for effective risk assessment of HAB. Measures like development of advanced technologies, coordinated efforts among the agencies, initiatives like outreach activities to create awareness in the public with stringent policy are focusing futures efforts[4]. Management strategies like bioremediation, nutrient load reductions, and suitable policies can help to reduce the adverse effects of HABs. Community involvement and creating awareness also play a major role in managing HAB. Present study underscores the urgent need of comprehensive approaches for early detection to mitigate the impacts to regulate HABs.

Conclusion

Comprehensive analysis of water quality parameters revealed higher biomass accumulation in nutrient-rich lakes, yielding diverse bacterial populations. Gram staining indicated dominance of Gram-negative rods, particularly in eutrophic systems. Metagenomic sequencing generated 10-15 million high-quality reads per sample, revealing distinct taxonomic profiles across the lakes. Functional annotation highlighted the presence of microcystin biosynthesis genes, especially in highly eutrophic lakes.

These findings establish a strong correlation between environmental nutrient enrichment, microbial structure, and HAB risk. The use of integrated culturing, microscopy, and metagenomics provided a robust framework for microbial source tracking and cyanobacterial bloom surveillance. This approach supports the deployment of next-generation molecular tools in environmental monitoring and public health risk assessment in aquatic ecosystems.

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