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Ecological, Molecular, and Antimicrobial Characterization of Cyanobacteria Isolated from different Springs in Khurmal-Halabja, Kurdistan Region, Iraq

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ABSTRACT

Cyanobacteria are known to produce biologically active compounds with potential antimicrobial properties. This study aimed to perform molecular identification and evaluate the antimicrobial activity of cyanobacteria from four springs in Khurmal-Halabja, in the Kurdistan Region of Iraq between September 2024 and February 2025. During this study period, physicochemical parameters such as temperature, pH, and nutrient levels were determined and monitored. Isolates of cyanobacteria were cultured on BG-11 medium, and through morphological studies combined with 16S rRNA gene sequencing, nine species were identified, including *Nostoc commune, Phormidium autumnale (Microcoleus autumnais), Aphanothece caldariorum, Microcoleus vaginatus, Microcoleus pseudoautumnalis, Gloeocapsa rupicola, Limnothrix planktonica, Thermocoleostomius sinensis,* and *Pseudanabaena yagii.* Ethanol extracts from four selected species were tested against *Staphylococcus aureus, Staphylococcus epidermidis*, and *Escherichia coli* by the agar well diffusion method. *Microcoleus pseudoautumnalis* exhibited the strongest activity, showing a 16-mm inhibition zone against *Staphylococcus aureus* while *Microcoleus vaginatus* had a broader spectrum of activity. There was greater susceptibility demonstrated by Gram-positive strains compared to Gram-negative strains. The research illustrates that cyst-forming cyanobacteria from Khurmal may be relevant for biotechnological and ecological applications.

Keywords: Cyanobacteria, Antimicrobial, Ethanol, Molecular Identification, Natural Springs.

1 Introduction

Cyanobacteria, often known as blue-green algae, are believed to be some of the planet's oldest living organisms, having been around for billions of years. Cyanobacteria, which are Gram-negative, photoautotrophic bacteria, can survive in soil, freshwater, and saltwater. Although cyanobacteria and bacteria have similar beginnings and basic anatomical characteristics, their ecological, biological, and physical properties are substantially different. The largest photosynthetic prokaryotes are cyanobacteria, which can fix CO2 and create O2 in addition to using chlorophyll-a to perform photosynthesis and capture solar energy [1].

Metabolites with distinct structures and physiological activities are prevalent in cyanobacteria. Recent studies have demonstrated the pharmacological qualities of several bioactive compounds present in freshwater blue-green algae, including anti-inflammatory, antifungal, anticancer, and antibacterial properties [2].

Cyanobacteria's bioactive compounds are usually extracted with particular objectives in mind. One of these is the discovery of new compounds for use in biology, agriculture, or medicine. It also aims to improve understanding of the interactions between individual organisms and their ecological environments. New species must be tested for each of these reasons [3]. Biologically active chemicals have been shown to be extracted from microalgae. Different strains of cyanobacteria are known to produce a variety of intracellular and extracellular compounds with antibacterial, antifungal, antiviral, and antialgal activities. Several factors influence the synthesis of antimicrobial agents, such as the temperature during incubation, the pH of the culture medium, the duration of the incubation period,

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the components of the medium, and the light intensity [4]. The aim of this study is to examine the molecular identity, diversity, and antimicrobial potential of cyanobacteria that were isolated from natural springs in Khurmal-Halabja, Kurdistan Region of Iraq, it specifically combines 16S rRNA gene sequencing and morphological characterization to precisely identify cyanobacterial species, analyzes the physicochemical properties of their spring habitats, and assesses the antibacterial activity of ethanol extracts against specific Gram-positive and Gram-negative bacteria.

2 METHODOLOGY

2.1 STUDY AREA

The territory of Iraqi Kurdistan is located approximately between the SN latitude of 34° 30′ and 37° 20′ and the WE longitude of 42° 20′ and 46° 20′ [5]. The Halabja Governorate is located approximately 240 kilometers northeast of Baghdad, 14 kilometers from the Iranian border, and 78 kilometers southeast of Sulaimani. In 2021, it had a population of about 118,924, according to data from the Sulaimani Statistical Directorate. Khurmal is a district in the Halabja Governorate, characterized by cold, rainy winters and hot, dry summers [6]. It is widely recognized for both its agricultural productivity and tourism potential. In the present study four sites (springs) were selected, three of these are located within the Khurmal district: Site 1: Khurmal Mineral Spring, Site 2: Ganjan Spring, Site 3: Khurmal Grand Mosque Spring; the remaining site, Site 4 (Shiramar Spring) is located in a village surrounding the Khurmal district. All four selected sites are freshwater springs. The Global Positioning System (GPS) model (GARMIN e-Map) was used to determine the precise location of the sites under study, as indicated in Table1 and Figure1.

Table 1. Actual locations of the various springs' sites (GPS)

Site	Name of sites	Latitude	Longitude	Altitude
1	Khurmal mineral spring	35 O 18 ' 19.322 "N	46 O 02 ' 14.130 "E	687
2	Ganjan spring	35 O 18 ' 14.011 "N	46 O 02 ' 19.890 "E	562
3	Grand Mosque spring	35 O 18 '13.369 "N	46 O 02 ' 19.200 "E	586
4	Shiramar spring	35 O 19 ' 05.596 "N	46 O 00 ' 46.042 "E	567

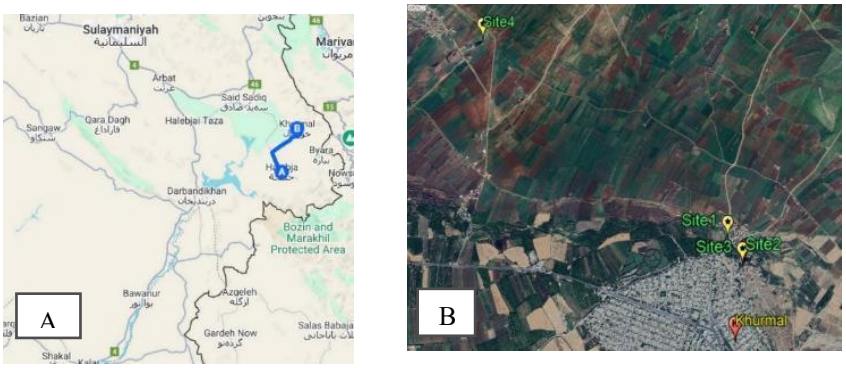


Figure 1. Maps show: a-Halabja governorate b- Location of study sites (Khurmal). (Google earth, 2024)

2.2 SAMPLE COLLECTION:

For six months, from September 2024 to February 2025, water and stone samples containing cyanobacteria were collected from four distinct springs in the Khurmal district. The samples were cultured directly in BG-11 medium [8], and the cyanobacteria were tansferred to the same medium [7]. Following colonization, the sub-culturing method was then employed to prepare unialgal growth [7].

2.3 PHYSICAL AND CHEMICAL ANALYSIS:

Several parameters were measured in the field at the sampling locations. A mercury thermometer with a range of 0 to 50 degrees ºC that graduated to 0.1 degrees ºC was used to monitor water temperatures. A portable multimeter (EUTECH INSTRUMENT) (CYberScan PC 300) was used to measure the concentration of hydrogen ions (pH). A portable dissolved oxygen meter, DO2 meter

HI9142 (HANA instrument), was used to measure dissolved oxygen directly in the field. The results were given in milligrams per liter(mg/L). Following that, water samples were gathered in sterile plastic bottles, stored as needed, and brought to the lab for additional examination. A flame emission photometer was used to test the concentrations of sodium in the lab. The results were calibrated using the proper standard solution, and they were reported in milligrams per liter. Utilizing a UV-visible spectrophotometer, the concentrations of nitrate and sulfate were measured; the results were reported in milligrams per liter(mg/L). According to the protocol outlined in APHA (2017) [9] the concentrations of alkalinity, calcium were determined using the conventional EDTA titrimetric method, and the results were expressed in mg/L.

2.4 MORPHOLOGICAL IDENTIFICATION OF CYANOBACTERIA (LIGHT MICROSCOPE ANALYSIS)

A light microscope was used to observe different strains of cyanobacteria. In order to identify these individuals, traditional botanical methods have been used, with a particular emphasis on the species' morphological characteristics. In traditional morphological taxonomy, observable characteristics such as cell arrangement, pattern, shape, size, and division process are examined by light microscopy [10], [11]. Using a microscope device, images of the morphological traits of isolated cyanobacteria were captured, with magnification ranging from40X [12].

2.5 MOLECULAR EXAMINATION OF SOME CYANOBACTERIA SPECIES:

2.5.1 DNA EXTRACTION OF CYANOBACTERIA

According to the manufacturer's instructions, genomic DNA was extracted from cyanobacterial cultures using the Beta Bayern Tissue DNA and Cyanobacteria Preparation Kit (Beta Bayern GmbH, 90453 Bayern, Germany). In summary, 500 µL of culture was centrifuged at 12,000 × g for 60 s, and the pellet was lysed using 400 µl of lysis buffer supplemented with 12 µl of proteinase K (20 mg/mL). The samples were incubated at 80°C for 5 to 10 minutes before being cooled to room temperature. DNA was eluted in 50 µl of elution buffer that had been heated to 70 °C after being bound to a silica spin column, and wash with BDW1 and BDW2 buffers. Purified DNA was stored at -20°C until it was needed.

2.5.2 PCR AMPLIFICATION OF 16S RRNA GENE

Primers specific to cyanobacteria were designed using Primer3(**https:// primer3.ut.ee**) and based on the NCBI sequences MT4488144 in order to amplify partial sequences of the 16S rRNA gene. The Micro-Gene Company (South Korea) synthesized the forward primer CYANO16S-F(5′-AGACTGCCGGTGACAAACC-3′) and the reverse primer CYANO16SR(5′-GGTGCGCTCTAACCAACTGA-3′).

Each PCR reaction was carried out in a 50 µL total volume, which included 25 µL of 2× Taq DNA Polymerase Master Mix (AMPLIQON A/S, Stenhuggervej 22), 3 µL of forward primer (20 pmol), 3 µL of reverse primer (20 pmol),15 µL of DNase-free water, and 4 µL of template DNA (50 ng/µL). Amplification was carried out in a Bioresearch PTC-200 Gradient Thermocycler with the following cycling parameters: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 40 seconds, annealing at 57°C for 40 seconds, and extension at 72°C for 1 minute; a final extension at 72°C for 10 minutes, and a final hold at 4°C. The anticipated amplicon size for this reaction was 372 bp [13].

2.5.3 DNA SEQUENCING AND ANALYSIS

The PCR product of the partial 16S rRNA gene was sequenced using ABI Prism Terminator Sequencing Kit (Applied Biosystems) at Macrogen Molecular Company of Korea. Chromatogram files was edited and base calls were checked using the Finch TV software. The genes sequences were applied to Basic Local Alignment Search Tool (BLAST) is a searching tool that applies the sequence alignment method (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and is available at the NCBI (National Center for Biotechnology Information) website to compare the laboratory (query) sequence with other biological sequence to find out more similarity with other biological sequences of bacteria in GenBank of NCBI.

2.6 CULTIVATION OF THE PURIFIED CYANOBACTERIA

A small portion of the uni-algal culture, which had been microscopically verified as such, was inoculated in to medium nutrient solutions in a 250 ml sterile conical flask to achieve the proper growth, and it was then cultured for 2-3 weeks using Jawad′s method. [14] the cultivated cyanobacteria were then centrifuged for 15 minutes at 10,000 rpm. The cyanobacteria pellet was then collected and dried in an oven set at 40 ºC. The dried cyanobacteria were then stored in a plastic bottle in the refrigerator. Through sub culturing into a different media nutrient solutions, these cultures were refreshed every two weeks to maintain the viability of the uni-algal growth [15].

2.7 PREPARATION OF CRUDE EXTRACT OF CYANOBACTERIA

After the cultivation period the culture was harvested by centrifugation at 4,000 rpm for 10 minutes and washed with distilled water (two times), dried under shade [16]. One gram of the isolates' dried biomass was extracted with ethanol in a mortar and pestle and left

at 4 ºC overnight. The supernatant was then collected after centrifugation at 10,000 ×g for 10 minutes to ensure complete extraction. At 40 degrees Celsius and under reduced pressure, the extracted solvent was concentrated. The dry residue was re-dissolved in DMSO (dimethyl sulfoxide) and stored at 4 °C until it was used in the bioassay [17].

2.7 ANTIMICROBIAL ACTIVITY

2.7.1 PREPARATION OF MICROBIAL INOCULUM

All bacterial strains employed for antibacterial activity were obtained from the Media Diagnostic Center in Erbil, Kurdistan Region, and crude extract was used for antimicrobial testing [18]. Two Gram-positive bacterial strains *Staphylococcus aureus* ATCC: "25923", *Staphylococcus epidermidis* ATCC: "12228", and one Gram-negative strain *Escherichia coli* ATCC: "25922", were tested against the crude solvent extract, an entire loopful of each bacterial strain was inoculated in to a tube with 5mL of tryptic soy broth from its 24-hour-incubated nutrient agar slant. The broth culture was incubated for two to six hours at 35 ºC until it reached the turbidity of 0.5 McFarland BaSO4 standard [19].

2.7.2 ANTIBACTERIAL ASSAY

Using the agar well diffusion method, the antibacterial activity of the cell-free extract that had been previously dissolved in DMSO (50 mg/ml) was assessed [20]. Briefly, 100 µL of each bacterial suspension were spread over the entire surface of Mueller–Hinton agar plates to inoculate them. Then, using a sterile cork borer, wells measuring 6 mm in diameter were punched, 50 µl of each extract was added to the wells, and the plates were incubated for 24 hours at a temperature that was suitable for the test microorganism. The diameters of all complete inhibition zones, including the well diameters, were measured after every plate was inspected for the existence of growth inhibition zones. Positive control (Cefuroxime, 30µg) and negative control (DMSO), were also applied, in parallel, with the tested algal extracts [8]. Following incubation, the inhibition zone surrounding the wells was measured and shown in millimeters as proof of antibacterial activity [15].

2.8 STATISTICAL ANALYSIS

Version 10.5.0 of GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used for the analysis. Over the course of six months, physical and chemical water parameters were assessed at four sites using a two-way ANOVA. Tukey's post-hoc test was then used for multiple comparisons. Statistical significance was defined as p-values less than 0.05, and all data are presented as the Mean± standard error (SE) [21].

3 RESULT AND DISCUSSION

3.1 PHYSICAL AND CHEMICAL PARAMETERS

3.1.1 WATER TEMPERATURE

The temperature of the water has a significant impact on the velocity of numerous chemical and biological reactions, as well as the quantity of oxygen gas that can dissolve in it [22]. Water temperature in the different springs varied throughout the monitoring period. Recorded water temperature ranged from (8.6 ºC -28.3 ºC). The statistical analysis for water temperature showed significant differences between the sites, except sites 2 and 3. Spatial and temporal variation in water temperature was investigated with significant differences between most months (Table 2 and Figure 2). Numerous environmental variables, including current velocity, water depth, bottom materials, inland water temperature, exposure to direct sunlight, degree of shade or vegetation cover, evaporation, and wind speed, can affect water temperature variation [23]. The monthly fluctuations in water temperature is influenced by air temperature, a phenomenon also observed in other aquatic systems in the Kurdistan Region of Iraq [24].

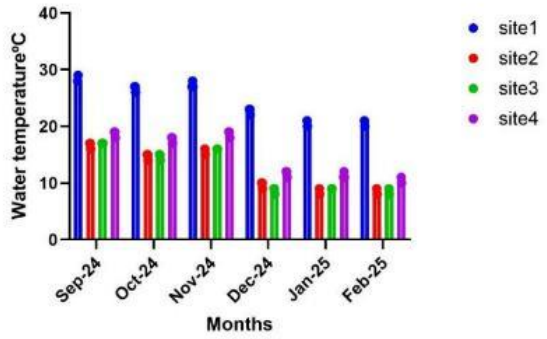
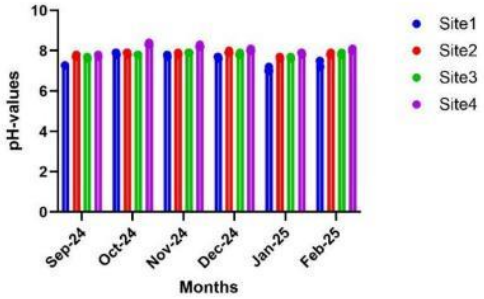


FIGURE2. Spatio-temporal variation of Water temperature (ºC) of khurmal different spring at ($p \leq 0.05$)

3.1.2 Hydrogen Ion Concentration (pH):

The ideal pH range for algal growth circumstances is 7.0–9.0; if the pH is not consistently maintained, it may cause abrupt cell death [25]. pH is a critical element in optimizing the rate of algae growth. According to WHO (2006), the pH value for all water and wastewater samples was within the ideal range of pH (6.6-9.5), making it a crucial quality criterion for both natural and wastewater [26]. Based on the data, it appears that the pH value moved toward the alkaline side of neutrality, as indicated in (Table 2 and Figure 3.) During the present study, significant differences were observed between most months and sites except sites 2 and 3. The minimum value of pH during the period of study was 7.06, measured at site 1 during January 2025 while the maximum value was measured during October 2024 at site 4, which was 8.33. Asimilar maximum value of 8.3 was recorded by [27]. The abundance or lack of free carbon dioxide, carbonate, and planktonic density can all contribute to variations in pH throughout the months [28].



FIGUREE3. Spatio-temporal variation of pH-values of khurmal different spring at ($p \leq 0.05$)

3.1.3 DISSOLVED OXYGEN (DO) MG/L

Since it is a measure of aquatic productivity, the dissolved oxygen (DO) of every body of water is a crucial one. Temperature, photosynthesis, and community respiration all affect the amount of oxygen in water [29]. The dissolved oxygen content for the present investigation varied between 1.66 and 8.38; the maximum value was obtained at Site 4 in November 2024, while the lowest value was reported at Site 1 in October 2024. There were substantial differences ($P \leq 0.05$) between each of the months that were analyzed, and there were noticeable changes among all the sites that were examined (Table 2), (Figure 4). This pattern is mainly due to temperature differences affecting Oxygen solubility: warmer water in the mineral spring holds less dissolved Oxygen, leading to lower DO levels, Conversely, higher DO is retained in colder water in the cold spring. This results from the inverse connection between oxygen concentration and temperature [30].

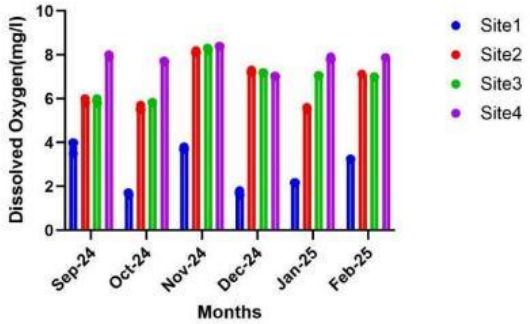


FIGURE 4. Spatio-temporal variation of Dissolved Oxygen(mg/l) of khurmal different spring at ($p \leq 0.05$)

3.1.4 SODIUM NA+ (MG/L)

Substances like NaSO4, NaF, NaHCO3, and NaOH may be the sources of the sodium found in water. However, the current conditions suggest that the lake is going through an ion exchange process, which could result in the production of additional chemicals [31]. Throughout this experiment, the sodium concentration ranged from 2-56.03 mg/L, the highest value of Sodium was recorded at Site1 during November 2024. Site 4 recorded the lowest value in October 2024.Statistical analysis demonstrated significant differences between sites (P≤0.05), with most months of the sites under investigation exhibiting a significant difference (P≤0.05) (Table 2), (Figure 5). The low sodium concentration of 2 mg/L in the cold fresh water spring is consistent with values from the cold spring in Barwari Bala, Duhok, which ranged from 0.9 to 15 mg/L [32]. Sodium concentrations in heavily mineralized waters are often high [33]. The World Health Organization (WHO) stated in 2006[26] that salt levels shouldn't be more than 200 mg/L.

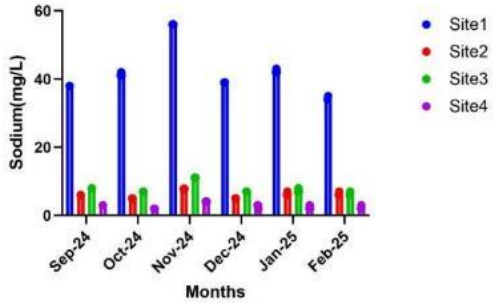


FIGURE 5. Spatio-temporal variation of Sodium (mg/L) of khurmal different spring at (p ≤ 0.05)

3.1.5 CALCIUM CA++ (MG/L)

The aquatic environment is impacted by the adsorption of calcium ions by metallic oxide since calcium is a necessary micronutrient. Additionally, bacteria that are essential to the circulation of calcium between the sediment and the water above it have an effect [34]. For both plants and animals, calcium is a vital mineral since it is required for the formation of bones, shells, and plant structures. The movement of calcium through gypsum, dolomite, limestone, and gypsiferous shale deposits results in the presence of calcium in water sources. [35].

The calcium Ca concentration experiments conducted in the current investigation yielded a range of values from 32.46 to 342.4 (mg/L). Significant variations were seen at every site in the study, with a significant difference (P≤0.05) observed in every month. (Table2 and Figure 6). This variation is likely due to elevated temperature in mineral springs and longer subsurface residence times which enhance the dissolution of calcium-bearing minerals such as calcite and dolomite. This finding is supported by [36], who reported that karst springs in the Makkok Anticline, Kurdistan region, Iraq are dominated by calcium -bicarbonate water type and exhibit calcite oversaturation due to active mineral dissolution under thermal conditions. Again, our cold spring site 4, calcium measured 32.46 mg/L- comparable to spring in duhok (Barwari bala) which yield 41-90 (mg/L) [32]. The typical calcium concentration in freshwater is below 10 mg/L, although water in close proximity to carbonate rocks and limestone can have calcium concentrations reaching up to 100 mg/L. This phenomenon occurs due to the solubility of calcium in rocks, particularly carbonate rocks and limestone, as well as its transportation from soils based on the geological properties of the surrounding research area [31], [33], and [34].

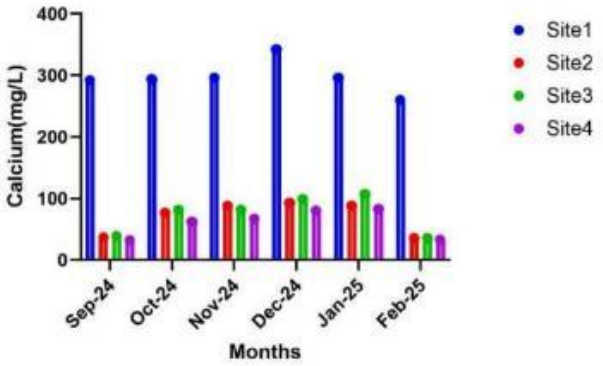


FIGURE 6. Spatio-temporal variation of Calcium (mg/L) of khurmal different spring at (p ≤ 0.05)

3.1.6 NITRATE NO3 (MG/L)

Nitrate is a vital nutrient in any body of water to indicating the trophic status (oligotrophic, mesotrophic, or eutrophic) [29]. It is the most readily available type of nitrogen that is chemically stable. Algal blooms are caused by an increase in the nitrate concentration in the water body [37]. Because nitrate fertilizes phytoplankton, high nitrate levels typically result in high phytoplankton productivity [33]. The nitrate concentration fluctuated between 0.83 and 22.34 mg/l during this investigation. Site 1 had the lowest nitrate concentration of 0.83 mg/l in October 2024, while Site 4 had the highest concentration of 22.34 mg/l in February 2025 (Figure 7). The statistical analysis revealed a significant difference ($P \leq 0.05$) between Sites and months during the studied period (Table 2). Normal levels of nitrate in surface water are typically low (8–10 mg/L), and [34] reported a different result, demonstrating that all of the tested springs had low nitrate concentrations and were thus good for drinking.

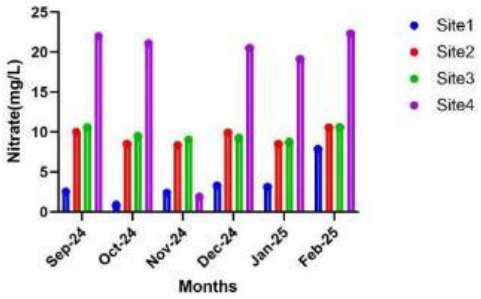


FIGURE7. Spatio-temporal variation of Nitrate NO3 (mg/l) of khurmal different spring at ($p \leq 0.05$)

3.1.7 SULFATE (SO4) 2- MG/L

The mineral sulfate is found naturally in water as barite ($BaSO_4$), epsomite ($MgSO_4.\ 7H_2O$), and gypsum ($CaSO_4.\ 2H_2O$) in many rocks. It is a frequent and abundant ion in the Earth's crust [38]. Between a few milligrams and several thousand milligrams per liter are its concentrations in water [34]. Sulfate concentration during this investigation ranged between 13.29 and 502.03 mg/L. The lowest value of sulfate concentrations was recorded at Site 4 during September. Comparable data from Barwari Bala Cold Springs in Duhok revealed sulfate concentration ranging from 27.27 – 116.4 mg/L. [32]. The highest value was recorded at Site 1 during February 2025. Statistical analysis revealed significant difference ($P \leq 0.05$) between Sites and months during studied period (Table 2 and Figure 8). These differences are mainly because higher temperature in mineral springs increases dissolution of sulfate-bearing mineral like gypsum due to deeper water circulation and longer contact with sulfate-rich rocks. Similar observations were reported by [39]. Who found that hot springs had elevated sulfate levels as a result of deeper water circulation and dissolution of subsurface sulfate minerals. Likewise, [40] demonstrated experimentally that sulfate-rich geothermal water significantly dissolves dolomite and related minerals, contributing to high sulfate concentrations in thermal environments.

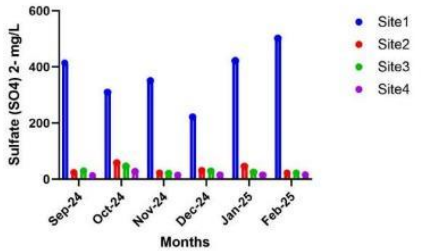


FIGURE 8. Spatio-temporal variation of Sulfate (SO4) 2- mg/L of khurmal different spring at ($p \leq 0.05$)

Table2. Physicochemical characteristics of the investigated region are shown as Mean ±S.E., Minimum, and Maximum for the period of September 2024–February 2025

Sites / Parameters	Mean±S.E Site1	Site2	Site3	Site4
Water temperature(ºC)	20.67-28.33[a] 24.39±1.41	8.66-16.67[b] 12.33±1.52	8.66-17[b] 12.33±1.61	10.67-18.67[c] 14.78±1.60
Hydrogen Ion Concentration(pH)	7.06-7.86[a] 7.50±0.12	7.66-7.93[b] 7.81±0.04	7.66-7.90[b] 7.78±0.03	7.76-8.33[c] 8.04±0.08
Dissolved Oxygen(mg/l)	1.66-3.82[a] 2.72±0.40	5.56-8.13[b] 6.59±0.43	5.83-8.26[c] 6.87±0.37	7.02-8.38[d] 7.80±0.18
Sodium Na++ (mg/L)	34.33-56.03[a] 41.84±3.06	5.00-7.83[b] 6.08±0.42	6.33-11.13[c] 7.80±0.70	2.00-4.13[d] 2.74±0.31
Calcium Ca ++ (mg/L)	260.3-342.4[a] 296.8±10.72	35.67-92.77[b] 69.81±10.76	35.67-107.2[c] 74.05±12.31	32.47-83.23[d] 59.81±9.03
Nitrate NO3- (mg/L)	0.83-7.85[a] 3.33±0.97	8.36-10.55[b] 9.32±0.38	8.73-10.59[c] 9.60±0.32	1.88-22.35[d] 17.84±3.22
Sulfate (SO4) 2- mg/L	222.2-502.0[a] 370.4±39.96	22.07-58.33[b] 34.07±6.12	21.37-46.33[c] 29.17±3.73	13.29-27.33[d] 16.95±2.11

3.2 MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF CYANOBACTERIA

Based on morphological characteristics observed under light microscopy and confirmed by 16S rRNA gene sequencing nine cyanobacterial species were identified from four different spring sites in the Khurmal District, Halabja Governorate. These included:

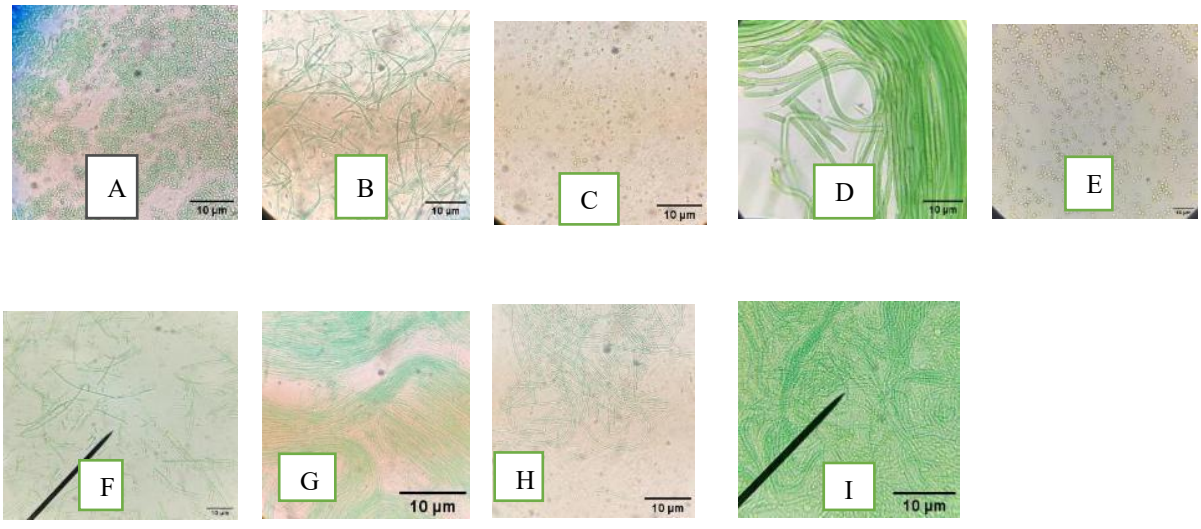


FIGURE 9. Morphology of Cyanobacteria species under the light microscope 40X: A. *Nostoc commune* B. *Phormidium autumnale* (*Microcoleus autumnalis*) , C *Aphanothece caldariorum* D. *Microcoleus vaginatus* E. *Gloeocapsa rupicola* , F. *Microcoleus pseudautumnalis* G. *Limnothrix planktonica* , H. *Thermocoleostomius sinensis,* I. *Pseudanabaena yagi*

3.3 PCR AMPLIFICATION OF PARTIAL GENES

For all cyanobacterial isolates, amplification of the 16S rRNA gene using the CYNO16S primer set resulted in distinct bands of the expected size (535bp). As shown in Figure 10, electrophoresis on 1.5% agarose gels verified that the amplification was successful.

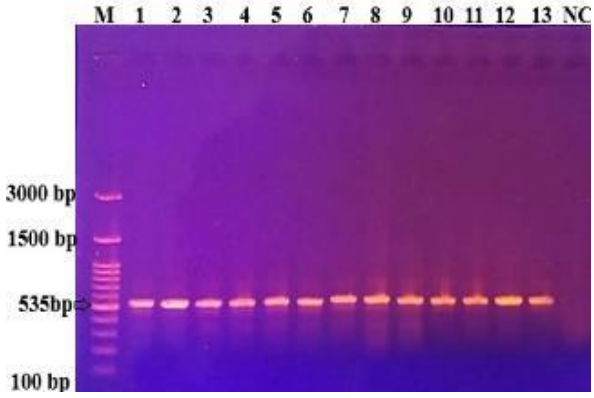


FIGURE 10. PCR amplification of partial 16S rRNA gene from cyanobacteria, M: DNA Ladder (1500-100 bp), Algae samples (lanes 1-13) gene bands with the size of 535 bp amplified.

3.4 PARTIAL GENES SEQUENCED: Z

Using only the forward primers of CYNO16S (5'-AGACTGCCGGTGACAAACC-3'), DNA sequencing was carried out independently using an ABI 3130X genetic analyzer (Applied Biosystem). All samples' PCR results served as a source of DNA templates for amplification using sequence-specific PCR.
Molecular identification of cyanobacterial genera and species Relieed on 16S rRNA The BLAST tool from Genbank (http://blast.ncbi.nlm.nih.gov/) provides the partial gene sequence, which is utilized to compare our amplified sequences with other species of sequences that are kept. According to the BLAST results, 100% identity was the highest query sequence (Table 3).

Table 3. Basic local alignment search tool (blast) of the isolates that revealed 100% identity comparing with the source, partial 16S rRNA gene

Bacterial samples	Query Cover %	Identic Number %	Genbank Accession Number	Genbank bacterial Species Identification
1	100	100	KX638486	
	100	98.34	AP025732	*Nostoc commune*
	100	98.34	ON032965	
2	100	100	JQ769128	
	100	100	KM052847	*Phormidiumautumnale (Microcoleus autumnalis)*
	100	100	MK282267	
3	100	100	KC004019	*Aphanothece caldoriarum*
	100	100	MK478712	
4	100	100	MK478711	*Microcoleus vaginatus*
	100	100	MK47871	
5	100	100	OL310620	*Gloeocapsa rupicola*
6	100	100	LC486302	*Microcoleus pseudautumnalis*
	100	100	MG874711	
7	100	100	JQ004021	*Limnothrix planktonica*
	100	100	KP726241	
8	100	100	CP113797	*Thermocoleostomius sinensis*
	100	100	NR_177737	
9	100	100	LC314143	*Pseudanabaena yagii*
	100	100	LC506589	

3.5 PHYLOGENETIC INFERENCES

Based on the 16S rRNA nucleotide sequence, the MEGA 11 software of phylogenetic analysis showed that the Cyanobacterial species under investigation were grouped along predicted lines. It was discovered that species from the corresponding genera were closely

related to one another based on phylogeny and sequencing divergence similarity data. The Cyanobacteria species samples categorized each cluster according to how similar they were to one another. (Figure11).

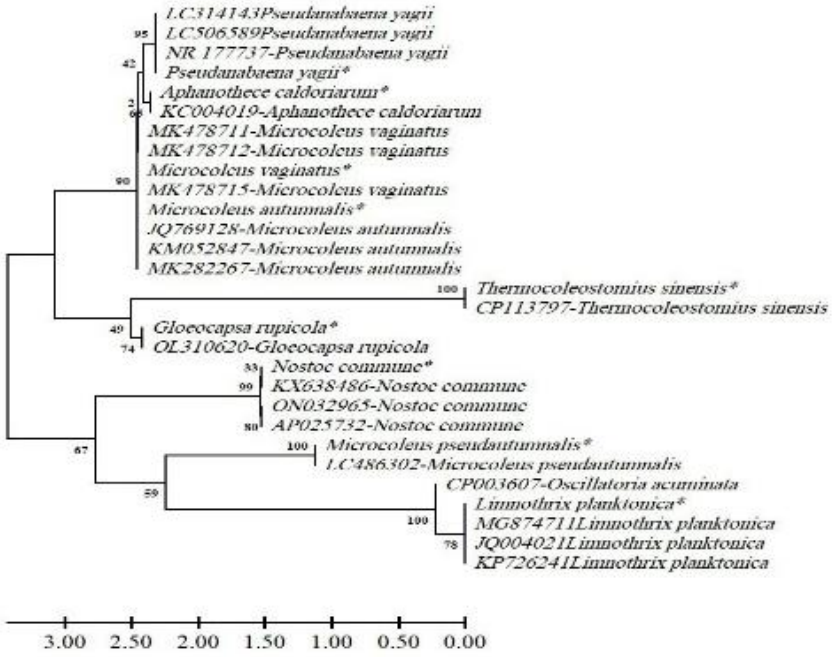


FIGURE 11. Employing Neighbor joining of Mega 11 program, show phylogenetic positioning of cyanobacterial samples (*) with similar gene Bank sequences of 16S rRNA that available in GenBank

The use of 16S rRNA sequencing enabled accurate identification of cyanobacteria that were morphologically difficult to distinguish. This method is considered a reliable tool for cyanobacterial taxonomy, as it overcomes limitations of morphology-based classification according to [41]. A polyphasic approach that includes 16S rRNA gene analysis significantly improves species-level identification and is now essential for modern cyanobacterial classification [41]. The detection of *Nostoc commune* a heterocyst-forming, nitrogen-fixing cyanobacterium – indicates the presence of species capable of contributing to nitrogen input in Khurmal aquatic ecosystems. Nostoc is known to inhabit soils, springs, and crusted surfaces in moist and mineral-rich environments. A study by [42] demonstrated that the nitrogen-fixing ability of dried *Nostoc commune* under rehydrated conditions, highlighting its ecological resilience and role in nutrient cycling. Filamentous non-hetercystous cyanobacteria such as *Phormidium autumnale, Microcoleus vaginatus,* and *Microcoleus pseudautumnalis* are commonly found in microbial mats in mineral-rich and hot spring environments. These genera are morphologically similar, making molecular identification critical for distinguishing between them. Planktonic genera such as *Aphanothece caldariorum, Limnothrix planktonica, and Pseudanabaena yagii* were identified in Khurmal springs. These cyanobacteria are well-adapted to low-nutrient freshwater systems, as described by [43] in their taxonomic review of cyanobacterial genera, which highlights these taxa as common components of oligotrophic lakes and springs globally. The identification of *Gloeocapsa rupicola* from the Khurmal springs was achieved using 16S rRNA gene sequencing, which allows for accurate differentiation of morphologically similar coccoid cyanobacteria. Molecular tools have enhanced taxonomic resolution within the genus Gloeocapsa, aiding in ecological and biodiversity studies of freshwater habitats [43]. The identification of *Thermocoleostomius sinensis* was confirmed through 16S rRNA and ITS sequence analysis, which placed it as a distinct lineage within the family Oculatellaceae. This finding may support its classification as a novel genus and species adapted to hot-spring environments [44].

3.6 ANTIMICROBIAL ACTIVITY

The ongoing rise in antimicrobial resistance is one of the biggest risks to public health, making antibiotics ineffective in treating microbial illnesses and raising global mortality rates. In order to find new possible antimicrobial drugs, intense efforts are needed [45]. The antibacterial properties of cyanobacteria extracts have been thoroughly studied and are interesting sources for a new novel bioactive agent [46] and [47].

The present investigation examined the antimicrobial activity of cyanobacterial species against both Gram-positive and Gram-negative bacteria (Table 4). The degree of microbial activity varied depending on the microorganism type, the type of solvent used for extraction, and the cyanobacterial species. Using the agar well diffusion method, the antimicrobial activity of crude extracts was assessed for bacterial strains in relation to the reference medication antibiotic (cefuroxime 30µg).

Table 4. Antimicrobial activity of Cyanobacteria ethanol extract's

	Inhibition zone against		
Cyanobacteria species	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Escherichia coli*
Aphanothece caldariorum	-	12 mm	-
Nostoc commune	-	11 mm	9 mm
Microcoleus vaginatus	15 mm	13 mm	10 mm
Microcoleus pseudoautumnalis	16 mm	11 mm	-
Cefuroxim(30µg, positive control)	26mm	22mm	25mm
Negative control (DMSO)	-	-	-

The ethanol extract of *Aphanothece caldariorum* showed antimicrobial activity against *Staphylococcus epidemides,* with an inhibition zone of 12 mm, while *Staphylococcus aureus* and *E. coli* were resistant to *Aphanothece caldariorum*. Ethanol extract of *Nostoc commune* had antibacterial action against *Staphylococcus aureus*, but it was ineffective against *Staphylococcus epidermidis* and *Staphylococcus coli*, whose inhibition zones measured 11 mm and 9 mm in diameter, respectively. For every pathogen utilized in this investigation, the ethanol extract of *Microcoleus vaginatus* demonstrated antimicrobial activity, with inhibition zones of 15 mm for *Staphylococcus aureus*, 13 mm for *Staphylococcus epidermidis*, and 10 mm for *Escherichia coli*. *Microcoleus pseudoautumnalis* ethanol extract have also showed antibacterial efficacy against *Staphylococcus aureus* and *Staphylococcus epidermidis*, with inhibition zones measuring 16 mm and 11 mm in diameter, respectively. *Escherichia coli*, on the other hand, showed resistant to the *Microcoleus pseudoautumnalis* extract. Gram-positive bacteria were generally more susceptible to the extract than Gram-negative bacteria. The highest inhibition zone, measuring 16 mm in diameter, was observed against *Staphylococcus aureus* by *Microcoleus pseudoautumnalis* crude extract, while the lowest inhibition zone, measuring 9 mm in diameter, was observed against *Escherichia coli* by *Nostoc commune*. These variations are caused by the microbe's genetic or chemical makeup [48]. As opposed to gram negative bacteria, whose cell membrane is made up of double layers that separate due to periplasmic space, gram positive bacteria may also have cell membranes that are composed of high levels (90–95%) of peptidoglycan, lipopolysaccharides, and phospholipids (5–10%) in order to find a suitable medium for reaction and the entry of antimicrobial agents (bactericidal and bacteriostatic agents) into gram positive bacteria and disrupt the cell membrane or protein biosynthesis unit (DNA and RNA). Peptidoglycan (5–10%) is present in the inner membrane, whereas phospholipids, lipoproteins, and mucopolysaccharides are present in the outer membrane. That is to say, the Gram-negative cell membrane is composed of a large percentage of lipids (90–95%), which prevents the antimicrobial drugs from entering the cell and diminishes their impact on the harmful microbes [49]. The antibacterial activity of *Nostoc commune's* ethanol extract against *Escherichia coli*, whose inhibition zone measured 9 mm in diameter, was comparable to the outcome obtained by [50]. The ethanol extract of *Aphanothece caldariorum* showed no inhibition zone against *Escherichia coli*. This result disagrees with the finding of [51], who reported inhibition zones of 16.9 mm and 23.3 mm for acetone and methanol extracts of *Aphanothece sp.* The difference in results may be due to the type of solvent used, as solvent polarity can influence the extraction of active compounds. Ethanol extract (70%) of *Microcoleus vaginatus* showed antibacterial activity against *Escherichia coli* with a 10 mm inhibition zone. This result agrees with the findings of [52], who reported inhibition zones ranging from (7-10 mm) for *Microcoleus sp*. at different extract concentrations using 80% ethanol. In contrast, *Microcoleus pseudoautumnalis* showed no inhibitory effect against *Escherichia coli* which disagrees with the same study that demonstrated antimicrobial activity of *Microcoleus sp.* This variation may be due to differences in species, solvent concentration, and extract composition. The antimicrobial activity of cyanobacterial extracts was attributed to the presence of many compounds characterized by their antibacterial and antifungal activity such as saturated and polyunsaturated fatty acids [53]. The discrepancy between our results and those from previous studies may be due to variations in the assay techniques and extracted metabolites.

CONCLUSION

This study offers the first thorough examination of the antimicrobial potential and diversity of cyanobacteria in Khurmal springs in Halabja, Kurdistan region, Iraq. By combining 16S rRNA gene sequencing with morphological observation, we indicate a diverse community that includes both thermophilic and mesophilic taxa. The ecological uniqueness of the habitats was highlighted by the significant effects of variations in water temperature, pH, and mineral content across the four spring sites on the distribution and composition of cyanobacterial species. Ethanol extracts from specific cyanobacteria were tested for their antimicrobial properties against *Escherichia coli, Staphylococcus aureus,* and *Staphylococcus epidermidis. Microcoleus vaginatus* exhibited activity against all three strains, but *Microcoleus pseudoautumnalis* had the strongest inhibitory effect on *Staphylococcus aureus,* among the taxa that were studied, with an inhibition zone of 16 mm. *Aphanothece caldariorum* exhibited weak antibacterial activity against Gram-negative *Escherichia coli,* while *Nostoc commune* showed weak to moderate inhibition of the Gram-positive strains. Due to structural differences in their cell walls, the Gram-positive bacteria were generally more susceptible than the Gram-negative *Escherichia coli.* These findings show that specific pathogenic strains are targeted by the selective antibacterial activity of some cyanobacteria from Khurmal springs.

CONFLICT OF INTERESTS

The authors declare that there is no conflicts of Interest.

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