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Ecological Assessment and Molecular Characterization of *Spirulina* in Freshwater Reservoirs of Kohat, Pakistan

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Abstract: This pioneering study uniquely identifies and characterizes the presence of algal species, mainly *Spirulina*, from water reservoirs in the Kohat district of Pakistan, a previously unstudied region for microalgae dynamics. Conducted over one year, from July 2022 to June 2023, the study examined 156 samples from 26 freshwater bodies in Kohat. Only one sample from Usterzai (pH 8.6 ± 0.08, TDSs 313 ± 0.81 mg/L, DO 4.50 ± 0.05 mg/L, EC 540 ± 0.81 µS/cm) contained *Spirulina* sp., highlighting its rarity and specific environmental preferences. The other 155 samples, with varying parameters, contained different microalgae. Microscopic analysis further confirmed the presence of *Spirulina* in only one sample. The morphological and molecular analyses of the isolated *Spirulina* culture showed variability within the population, with phylogenetic analysis illuminating closer relationship with *Arthrospira platensis*. While multivariate analyses identified key environmental parameters influencing algal species distribution, the selective presence of *Spirulina* was found less relevant, which requires further investigation in terms of nutrient availability, microbial interactions, or subtle variations in water chemistry for ecological preferences and adaptations.

Keywords: *Spirulina* isolation; phylogenetic analysis; Kohat water reservoirs; 16S rRNA sequencing; microbiological species identification

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1. Introduction

Algae constitute a diverse group of living organisms capable of colonizing nearly all types of habitats. Several species of algae are known to thrive in extreme environmental conditions, including volcanic waters, deserts, frozen soils, and environments with high acidity or alkalinity. Nonetheless, the majority of algae predominantly inhabit aquatic environments [1]. Algae play a crucial role in the Earth's ecosystems, particularly within the global food chain, by contributing approximately 40% of the planet's photosynthesis. This process results in the production of a substantial amount of oxygen, highlighting their significance in environmental health and sustainability [2–4].

Microalgae, while less studied than seaweed, have benefits such as quick growth, high photosynthetic efficiency, and the potential for growing under production conditions. Their biodiversity also provides a variety of biologically active compounds, including polysaccharides, lipids, proteins, and pigments [5]. One notable prokaryotic microalga is *Spirulina platensis*, commonly known as *Spirulina*. This photoautotrophic filamentous cyanobacterium has garnered global attention due to its abundance as a source of feed, valuable biomolecules, potential drugs, and its effectiveness in environmental remediation [6].

For the identification of cyanobacterial communities, understanding their habitat is crucial. Due to the diverse varieties of cyanobacteria, their habitat and growth requirements vary. Nutritional contents, particularly the amount and type of nutrients present

in the cyanobacterial habitat, are significant for their growth. Parameters such as temperature, pH, light intensity, and the amount of CO_2 are critical for their substantial growth [7]. According to data, most of the Earth's surface is covered by water bodies, indicating that photosynthetic entities are more abundant in water than on land [8]. Vincent and Quesada [9] suggested that high-latitude lakes, ponds, and streams are favorable sites for abundant cyanobacterial growth. Additionally, wetlands facilitate the growth of cyanobacteria. Freshwater bodies with optimal temperatures are ideal for sampling different varieties of blue-green algae [9]. Due to their short growth period, cyanobacteria can be cultivated rapidly even in nutrient-limited conditions when compared to other photosynthetic organisms [10].

Although the demand and applications of *Spirulina* are increasing, the identification of true strains of *Spirulina* and its precise taxonomic ranking has been a subject of regular questioning. This is because *Spirulina platensis* was previously classified as *Arthrospira platensis*. Some studies have referred to *Spirulina* as a dried product or a common name for *Arthrospira platensis* [11]. Furthermore, in earlier nomenclature categorization, the genera *Spirulina* and *Arthrospira* were considered synonymous [12]. However, detailed molecular examinations later distinguished *Spirulina* and *Arthrospira* as two separate genera within the *Oscillatoriaceae* [13]. Initially, the classification of *Spirulina* and *Arthrospira platensis* into two distinct groups was based on the absence or presence of septa in the spiral trichomes of *Spirulina* and *Arthrospira platensis*, respectively [14]. However, recent studies utilizing molecular tools for strain identification based on ribosomal RNA (rRNA) have largely invalidated this generic classification. Today, both *Spirulina* and *Arthrospira* are widely accepted as two distinct genera with minimal structural resemblances, each assigned to different orders [15]. However, there are still disagreements regarding this classification, with some studies proposing a new genus named *Limnospira* for the precise categorization of *Spirulina* and related species [13]. Besides taxonomic variations, differences in morphological attributes also impact the optimal growth conditions of each microalgal species [16].

In recent years, the exploration of *Spirulina* has gained considerable attention due to their versatile applications in nutrition, pharmaceuticals, and biofuels. However, research has predominantly focused on aquatic *Spirulina* strains, neglecting the potential of freshwater variants. This study presents the first comprehensive identification and molecular characterization of algal species, mainly *Spirulina* sp., from the Kohat district's water reservoirs, a previously unstudied region for this microalga. By integrating morphological, molecular, and phylogenetic analyses, the research confirms the presence of *Spirulina* sp. and elucidates its genetic relationship with *Arthrospira platensis*. These findings significantly enhance our understanding of *Spirulina*'s ecological preferences and genetic diversity in Kohat, laying the groundwork for future ecological assessments and conservation strategies.

2. Materials and Methods

2.1. Sample Collection and Enrichment

Sampling was conducted over one year, from July 2022 to June 2023. A total of 26 freshwater bodies were selected based on their size and historical background. The sites included a water reservoir with a minimum size of 5 m in length, 5 m in width, and 1 m in depth. It was ensured that the selected water bodies had been established for at least two years prior to sampling. The sites were categorized into four zones (South, North, West, East) based on their locations (Table S1) relative to the Kohat University of Science and Technology, Kohat, Pakistan (Figure 1). Meteorological parameters obtained from the Water and Sanitation Services Company Kohat are presented in Figure 2.

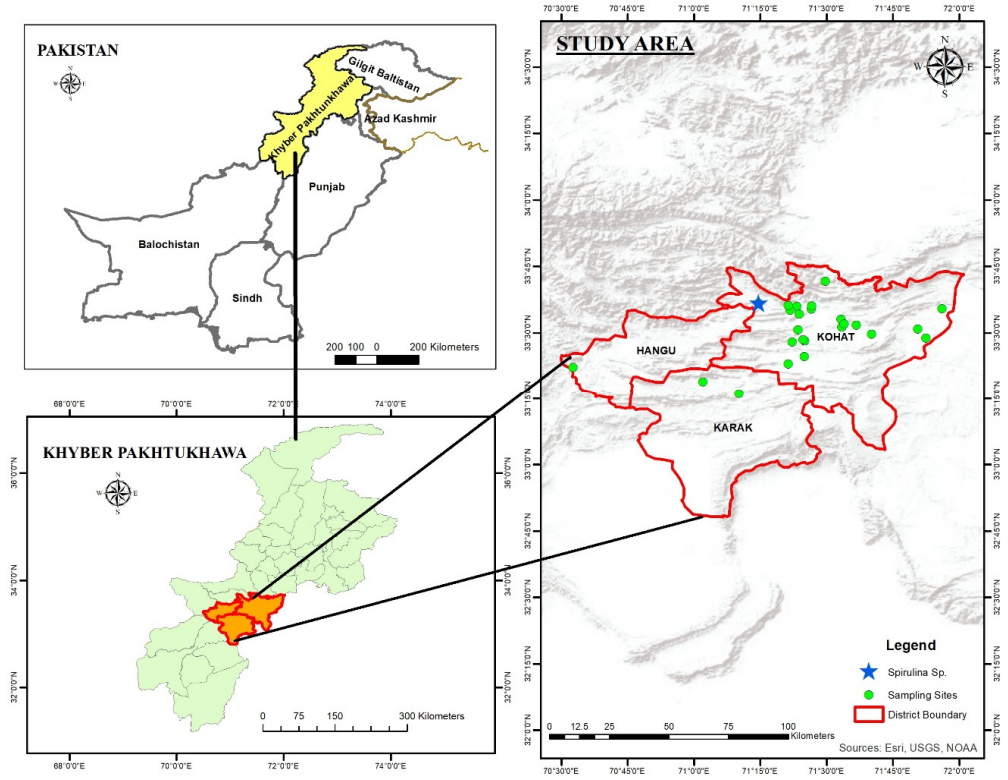

Figure 1. One-year sampling (July 2022 to June 2023) of 26 freshwater bodies in Khyber Pakhtunkhwa, Pakistan, with zones categorized by direction from Kohat University, indicating Usterzai as the source of *Spirulina*.

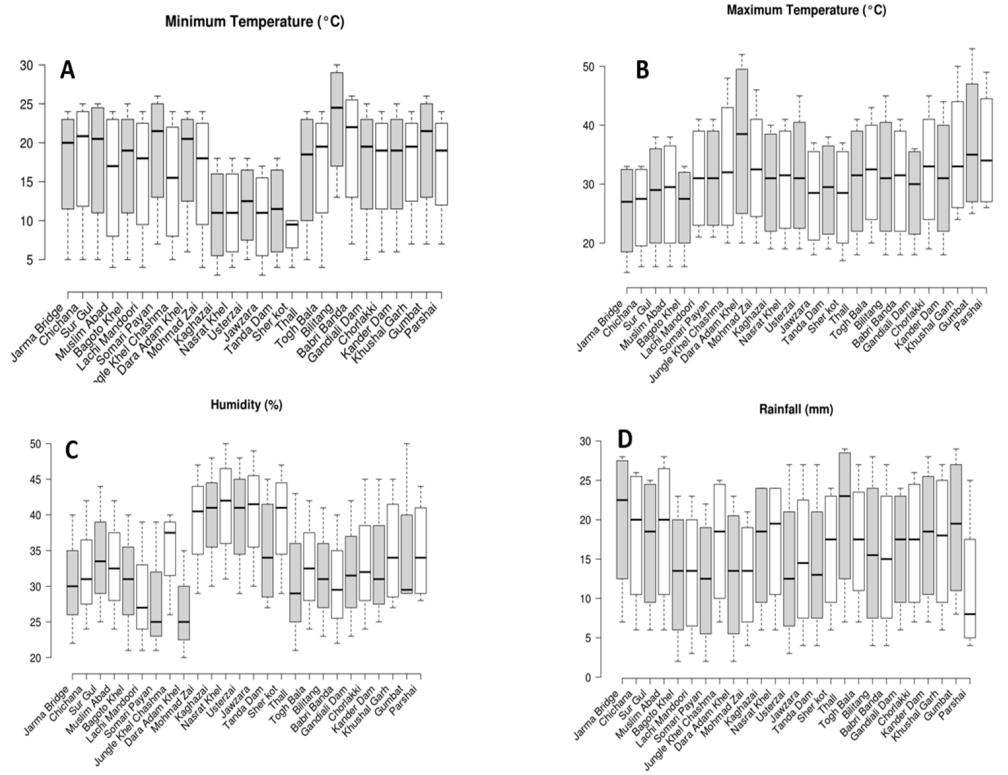

Figure 2. Box and Whisker plot representing fluctuations in meteorological parameters between July 2022 and June 2023; (**A**) minimum temperature, (**B**) maximum temperature, (**C**) humidity, and (**D**) rainfall.

All samples were collected from the top (water surface to a depth of ~30 cm) and bottom (below 30 cm) layers of freshwater reservoirs using a measurement-labeled snake catcher stick, with approximately 50 mL of water collected from each site. Samples were collected in triplicates using sterilized containers and transferred to the Molecular Ecology and Conservation Laboratory at the Kohat University of Science and Technology, Kohat. In the lab, BG11 enrichment medium was added, and the pH was maintained between 8.6 and 10 until processed microscopically [17].

2.2. Determination of Physiochemical Parameters

Before collecting water samples, the pH, electrical conductivity (EC), total dissolved solids (TDSs), and dissolved oxygen (DO) of water bodies located in the north, south, east, and west Kohat were analyzed to assess the potential availability of algal species. The pH was measured directly using a pH meter (PHS-3C POMETER), while the EC and TDSs were determined using a multifunctional meter by Hanna (model HI9811-51; HANNA Instruments, Woonsocket, RI, USA). The Hanna meter offers a measurement accuracy for TDS within ±2% of the reading. Dissolved oxygen (DO) and temperature were measured using a Micro-Processor Dissolved Oxygen meter [18]. The instruments were calibrated before each sampling regime to ensure accuracy.

2.3. Microscopic Analysis of Water Samples

The enriched samples were examined under a microscope at a magnification of 100× to assess morphological variations and to take measurements of each isolate using previously reported methods [19]. Among the microalgal species present in the samples, the *Spirulina* sp. were morphologically identified and photographed under a microscope (Olympus CX31 RBSF).

2.4. Microscopic Analysis of Spirulina Cell Sizes

In this investigation, *Spirulina* cells underwent meticulous size identification using the AXIO IMAGEN Z1 microscope, equipped with an EC plan-neofluar 10×/0.30M27 objective and employing bright-field microscopy. Illumination was provided by a TL Halogen lamp with a light source intensity of 4.23 volts, facilitating optimal imaging conditions. The imaging process involved the axiocam503 device with an exposure time of 13 ms and a depth of focus of 12.22 µm, ensuring precision in capturing cellular dimensions. Cultures of *Spirulina* were cultivated in standard growth media, allowing them to reach the logarithmic phase. Initially, single-cell visualization and analysis were conducted for size measurement using Zeiss software (version ZEN 3.8). Subsequently, a new slide was meticulously prepared, incorporating 100 µL of cell culture, and the entire slide was visualized. Measurements were performed on 100 images using ImageJ software (version 1.8.0) to ensure a representative sample. The quantification of the length, width, and area of *Spirulina* sp. cell contributed to the calculation of the mean cell size, providing valuable insights into the overall size distribution within the *Spirulina* population [20].

2.5. DNA Extraction, PCR Amplification, Sequencing, and Phylogenetic Analysis

The modified CTAB (cetyltrimethylammonium bromide) method was employed for the extraction of total genomic DNA [21]. The yield of the extracted DNA was determined by measuring its absorbance at 260 nm using a UV–visible spectrophotometer. The purity of the DNA and the presence of contaminant polysaccharides were assessed by measuring the absorbance ratios at A260/280 nm and A260/230 nm, respectively. Additionally, the extracted DNA was subjected to electrophoresis using a 1% agarose gel to visualize the DNA bands.

The molecular marker 16S rRNA was amplified using the primer pairs CYA106-F (5′-CGGACGGGTGAGTAACGCGTGA-3′) and CYA781-R (5′-GACTACTGGGGTATCTAATCCCATT-3′) [22] and 27F1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and 809R (5′-GCTTCGGCACGGCTCGGGTCGATA-3′) [23] through a polymerase chain reaction (PCR) (Table S1). The

amplification reaction was set up in a 25 µL mixture containing 10–50 ng of template, 20 mM of each primer, 1 mM dNTPs mix, 1.0 U of Taq DNA polymerase, 1× Taq buffer, and 2.5 mM $MgCl_2$. The PCR conditions involved 35 cycles with an initial denaturation at 94 °C for 90 s, denaturation at 95 °C for 60 s, annealing at 63.5 °C for 60 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min. The amplified PCR product was separated by electrophoresis using a 2% agarose gel stained with Midori Green Xtra, and the resulting bands were visualized on a transilluminator. After amplification, the PCR products were purified by precipitation using isopropanol and washing with EtOH. The samples were then sent to Macrogen Korea for sequencing. The acquired sequencing data were analyzed using offline BioEdit software (version 7.2). To identify and retrieve similar sequences available in databases, the Basic Local Alignment Search Tool for nucleotides (BLASTn) was employed.

We aligned the 16S sequences and constructed the phylogenetic relationships using MEGA7 (Version 7.0.14) with the integrated Tree Explorer. Multiple sequence alignment was performed using the MUSCLE algorithm. We inferred the evolutionary relationships of *Spirulina* samples and closely and distantly related species using the neighbor-joining algorithm with a bootstrap value based on 1000 replicates. Selected species from cyanobacteria, diatoms, and green algae were added as outgroups to our data.

3. Results

3.1. Spirulina Is Less Prevalent in Freshwater Reservoirs of District Kohat

For the sampling process, a total of 26 freshwater reservoirs within the district of Kohat were selected. Among the 156 samples examined, only one sample retrieved from the depth of a water reservoir in the "Usterzai" location exhibited a distinctive coiled spring-like structure with a blue-green coloration. This particular morphology strongly indicated the presence of *Spirulina* sp. (Figure 3). Conversely, the remaining 155 samples predominantly contained different microalgae, with no clear morphological resemblance to *Spirulina* being observed in these samples.

3.2. Determination of Physiochemical Parameters

The physicochemical factors of the water bodies in Kohat were analyzed, and the abundance of algal species from 2022 to 2023 was determined. Geographical locations and environmental conditions have a significant impact on the availability and survival of algal species. The physicochemical analysis of water samples revealed progressive changes in various parameters. The pH, a crucial environmental factor affecting algal abundance, ranged from 6.07 ± 0.22 in Bagoto to 8.90 ± 0.16 in Sherkot. EC, indicating the level of salt ions in water, ranged from 300 ± 1.29 µS/cm in Babri Banda to ~650 ± 0.81 µS/cm in Tanda Dam, Mohmad Zai, Kaghazai, and Nasrat Khel. DO levels, an indicator of water quality and oxygen availability for aquatic life, were also measured. Apparently, the abundance of algal communities increased with higher DO levels, which ranged from 3.00 ± 0.08 mg/L in Jarma Bridge and Somari Bayan to 8.72 ± 0.05mg/L in Gandiali Dam. Similarly, the TDSs were highest at 380 ± 1.70 mg/L in Muslimabad and lowest at 200 ± 1.07 mg/L in Gandiali Dam (Table 1). Water temperature was highest at 34 °C in Muslimabad and Bogoto, while it was lowest at 28 °C in Sherkot and 29 °C in Dara Adam Khel, Mohmad Zai, Kaghazai, Tanda Dam, Nasrat Khel, and also from Usterzai, the area from where *Spirulina* sp. was isolated (Figure 4).

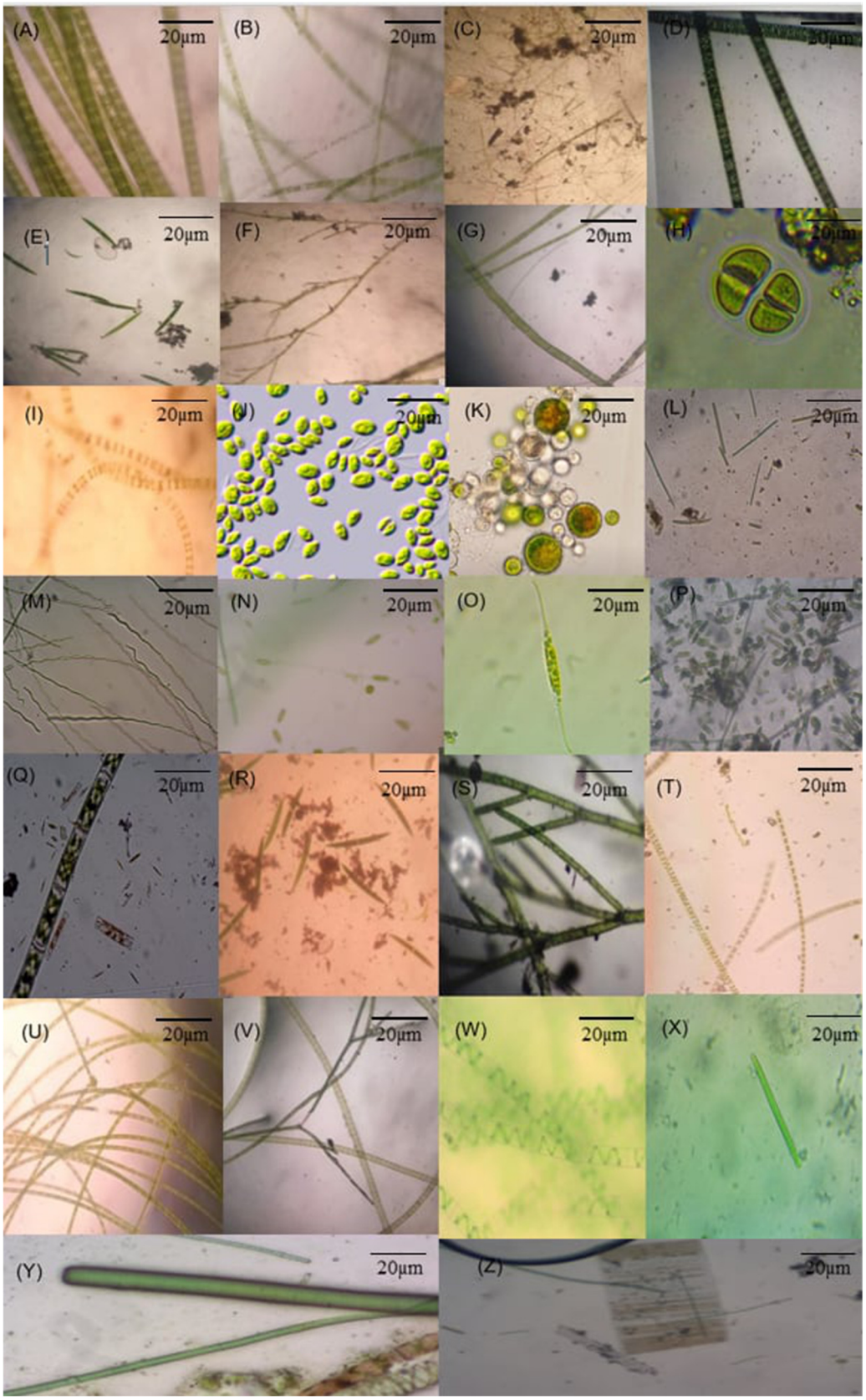

Figure 3. Microscopic images of different algal species prevalent in water bodies of the Kohat region. Representing *Spirogyra* sp. (**A**), *Spirogyra* sp. (**B**), *Spirogyra* sp., *Scenedesmus* sp. (**C**), *Spirogyra* sp. (**D**), *Scenedesmus* sp. (**E**), *Cladophora* sp. (**F**), *Cladophora* sp. (**G**), *Chroococcus turgidus* sp. (**H**), *Spirogyra* sp. (**I**), *Scenedesmus* sp. (**J**), *Hematococcus* sp. (**K**), *Oscillatoria* sp. (**L**), *Spirulina* sp. (**M**), *Scenedesmus* sp.,

Oscillatoria sp., *Chlorella* sp. (**N**), *Nitzschia acicularis* sp. (**O**), *Spirogyra* sp., *Scenedesmus* sp., *Oscillatoria* sp. (**P**), *Scenedesmus* sp., *Oscillatoria* sp., *Chlorella* sp. (**Q**), *Scenedesmus* sp. (**R**), *Cladophora* sp. (**S**), *Spirogyra* sp., *Ulothrix* sp. (**T**), *Spirogyra* sp. (**U**), *Cladophora* sp., *Spirogyra* sp. (**V**), *Spirogyra* sp. (**W**), *Oscillatoria* sp. (**X**), *Scenedesmus* sp., *Spirogyra* sp. (**Y**), *Fragelaria* sp. (**Z**).

Table 1. Physiochemical factors of water bodies analyzed for the isolation and identification of algal species during 2022 and 2023.

Sampling Sites		pH	TDSs (mg/L^{-1})	DO (mg/L^{-1})	EC (µS/cm^{-1})	Prevalent Algal Species *
South Kohat	Jarma Bridge	8.15 ± 0.05	230 ± 0.95	3.00 ± 0.08	450 ± 0.81	*Spirogyra* sp. (A)
	Chichana	7.80 ± 0.16	300 ± 1.70	7.07 ± 0.15	351 ± 0.95	*Spirogyra* sp. (B)
	Sur gul	7.60 ± 0.08	330 ± 1.63	5.00 ± 0.21	380 ± 0.81	*Spirogyra* sp., *Scenedesmus* sp. (C)
	Muslim Abad	6.90 ± 0.14	380 ± 1.70	5.00 ± 0.21	560 ± 1.63	*Spirogyra* sp. (D)
	Bagoto Khel	6.07 ± 0.22	276 ± 1.29	4.00 ± 0.21	380 ± 0.81	*Scenedesmus* sp. (E)
	Lachi (Mandoori)	8.6 2 ± 0.17	300 ± 0.81	6.05 ± 0.12	500 ± 1.70	*Cladophora* sp. (F)
	Somari Payan	8.10 ± 0.21	230 ± 1.29	3.00 ± 0.08	450 ± 1.29	*Cladophora* sp. (G)
North Kohat	Jungle Khel Chashma	7.42 ± 0.12	220 ± 1.70	6.20 ± 0.08	340 ± 0.81	*Chroococcus turgidus* sp. (H)
	Dara Adam Khel	7.60 ± 0.08	250 ± 0.50	6.60 ± 0.08	340 ± 1.63	*Spirogyra* sp. (I)
West Kohat	Mohmad Zai	8.20 ± 0.21	320 ± 0.81	3.20 ± 0.08	650 ± 2.16	*Scenedesmus* sp. (J)
	Kaghazai	8.70 ± 0.43	320 ± 1.63	3.19 ± 0.08	650 ± 1.29	*Hematococcus* sp. (K)
	Nasrat Khel	8.20 ± 0.08	320 ± 0.50	3.20 ± 0.08	650 ± 2.16	*Oscillatoria* sp. (L)
	Usterzai	8.60 ± 0.08	313 ± 0.81	4.50 ± 0.05	540 ± 0.81	*Spirulina* sp. (M)
	Jawzara	8.00 ± 0.08	318 ± 1.70	7.50 ± 0.14	490 ± 0.81	*Scenedesmus* sp. *Oscillatoria* sp. *Chlorella* sp.(N)
	Tanda Dam	8.20 ± 0.08	320 ± 0.81	3.10 ± 0.08	650 ± 0.81	*Nitzschia acicularis* sp. (O)
	Sher Kot	8.90 ± 0.16	302 ± 1.25	2.97 ± 0.15	580 ± 0.81	*Spirogyra* sp. *Scenedesmus* sp. *Oscillatoria* sp. (P)
	Thall	8.30 ± 0.08	318 ± 0.81	2.85 ± 0.05	609 ± 0.81	*Scenedesmus* sp. *Oscillatoria* sp. *Chlorella* sp. (Q)
East Kohat	Togh Bala	7.02 ± 0.12	230 ± 0.81	5.70 ± 0.08	376 ± 0.81	*Scenedesmus* sp. (R)
	Bilitang	7.50 ± 0.08	240 ± 0.81	5.00 ± 0.21	350 ± 0.81	*Cladophora* sp. (S)
	Babri Banda	8.07 ± 0.05	250 ± 1.25	6.00 ± 0.08	300 ± 1.29	*Spirogyra* sp. *Ulothrix* sp. (T)
	Gandiali Dam	7.15 ± 0.12	200 ± 1.70	8.72 ± 0.05	364 ± 0.81	*Spirogyra* sp. (U)
	Chorlakki Dam	7.30 ± 0.16	215 ± 0.81	8.60 ± 0.08	357 ± 0.95	*Cladophora* sp. *Spirogyra* sp. (V)
	Kander Dam	6.80 ± 0.24	320 ± 0.57	3.50 ± 0.08	550 ± 1.29	*Spirogyra* sp. (W)
	Khushal Garh	8.07 ± 0.09	250 ± 1.29	6.05 ± 0.12	530 ± 0.81	*Oscillatoria* sp. (X)
	Gumbat	7.90 ± 0.14	250 ± 1.29	5.00 ± 0.21	400 ± 0.95	*Scenedesmus* sp. *Spirogyra* sp. (Y)
	Parshai	7.90 ± 0.24	350 ± 1.25	5.15 ± 0.12	530 ± 0.81	*Fragelaria* sp. (Z)

* Alphabets (A–Z) are used to cross-reference the *Spirulina* sp. with Figure 3.

In western Kohat, *Oscillatoria* sp. was the most abundant species, found at pH levels ranging from 8.00 ± 0.08 (Jawzaro) to 8.90 ± 0.16 (Sherkot), followed by *Scenedesmus* sp., occurring at similar pH levels (Mohmad Zai, Jawzaro, Sherkot, and Thall). *Hematococcus* sp. and *Nitzschia acicularis* were the least abundant species, found in water bodies with a pH ranging from 8.70 ± 0.43 (Kaghzai) to 8.20 ± 0.08 (Tanda Dam), respectively. *Spirulina* sp. was only found in water bodies with a pH of 8.60 ± 0.08 (Usterzai).

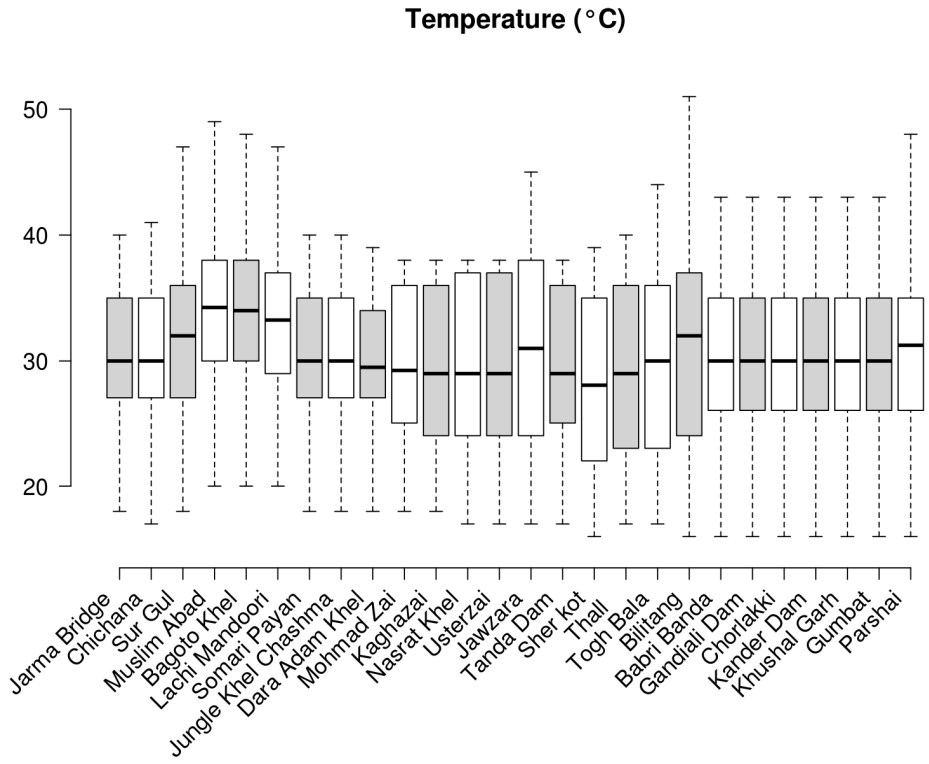

Figure 4. Box and Whisker plot representing temperature variations at different locations for the isolation and identification of algal species during 2022 and 2023.

Similarly, water from various localities in east Kohat was analyzed for the abundance of algal species, with a pH ranging from 6.80 ± 0.24 (Kander Dam) to 8.07 ± 0.05 (Babri Banda, Khushal Garh). *Spirogyra* sp. was the most abundant species, thriving in pH levels from 6.80 ± 0.24 (Kander Dam), 7.15 ± 0.12, 7.30 ± 0.16 (Gandiali Dam, Chorlakki) to 8.07 ± 0.05 (Babri Banda, Khushal Garh), respectively. While *Cladophora* sp., *Oscillatoria* sp., *Scendesmus* sp., and *Fragelaria* sp. were the least abundant found in water bodies with pH ranges of 7.50 ± 0.08 (Bilitang), 8.07 ± 0.09 (Khushal Garh), 7.90 ± 0.14 (Gumbat) and 7.90 ± 0.24 (Parshai), respectively.

The PCA reveals that the first principal component (PC1), explaining 41.83% of the variance, is primarily influenced by the TDSs, pH, and EC, with positive loadings indicating that sites with higher values of these variables score higher on PC1 (Figure 5). Conversely, DO and temperature show weak negative correlations with PC1. The second principal component (PC2), accounting for 28.18% of the variance, is positively correlated with the TDSs and temperature, suggesting that sites with higher values of these factors score higher on PC2, while pH and DO are negatively correlated with PC2. Sites such as Dara Adam, Usterzai, and Parshai, with high scores on PC1 and PC2, exhibit higher TDSs, pH, EC, and temperature, whereas sites like Babri Banda and Gumbat, with negative scores on these components, are characterized by lower values of these variables.

Redundancy analysis (RDA) revealed significant insights into the relationships between environmental variables and algal species (Figure 6). RDA 1, explaining 41.81% of the variance, is primarily influenced by the TDSs, pH, humidity, and temperature, while RDA 2, explaining 25.12%, is influenced by pH, EC, and maximum temperature. *Spirulina* sp. showed a preference for higher TDSs and pH levels, aligning positively with RDA 1. *Scenedesmus* sp. and *Oscillatoria* sp. are associated with higher pH, EC, and humidity levels, showing positive correlations with both RDA 1 and RDA 2. *Cladophora* sp. preferred lower DO levels, indicated by its negative correlation with RDA 1. Sites like Usterzai, Nasrat Khel, and Parshai, which have high scores on RDA 1, are characterized by higher TDSs, pH, and temperature. In contrast, sites like Chorlakki and Gandiali Dam positively aligned

with RDA 1, exhibiting higher humidity and rainfall levels. Sur Gul and Muslim Abad, associated with high maximum temperatures, show positive scores on RDA 2.

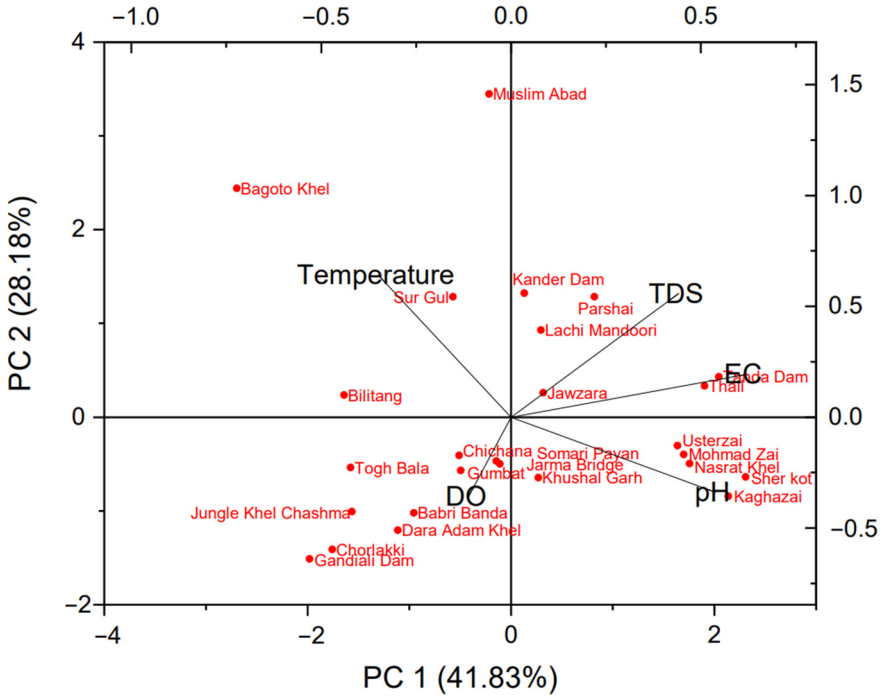

Figure 5. Principal component analysis (PCA) showing the trends in the variation of the physico-chemical parameters of the water bodies during 2022 and 2023, covering all seasons (summer, autumn, winter, and spring).

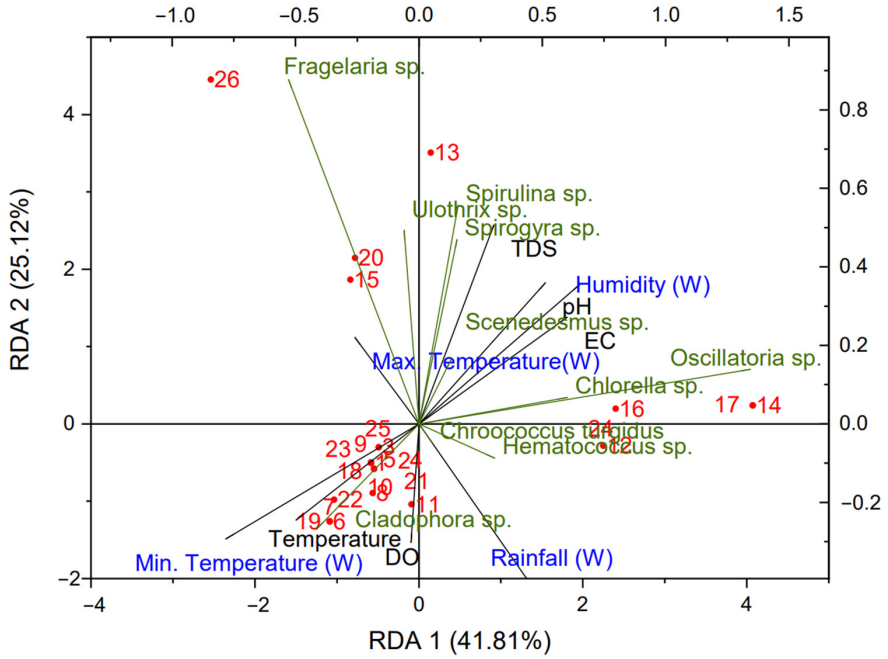

Figure 6. Redundancy analysis (RDA) showing the relationship between species composition and environmental variables. Localities are numbered as follows: Jarma Bridge (1), Chichana (2), Sur gul (3), Muslim Abad (4), Bagoto Khel (5), Lachi (Mandoori) (6), Somari Payan (7), Jungle Khel Chashma (8), Dara Adam Khel (9), Mohmad Zai (10), Kaghazai (11), Nasrat Khel (12), Usterzai (13), Jawzara (14), Tanda Dam (15), Sher Kot (16), Thall (17), Togh Bala (18), Bilitang (19), Babri Banda (20), Gandiali Dam (21), Chorlakki Dam (22), Kander Dam (23), Khushal Garh (24), Gumbat (25), Parshai (26).

3.3. Spirulina Cell Sizes

The reference single *Spirulina* sp. cell, analyzed using Zeiss software (Figure S1A,B), exhibited a length of 405.4 µm, a width of 15.4 µm, and an area of 5918.0 µm^2. Subsequent measurements on 100 images using ImageJ software revealed the mean cell size for the *Spirulina* sp. to be approximately 599.5 µm in length, 18.6 µm in width, and 11,324.7 µm^2 in area (Figure S2).

3.4. Molecular Analysis

The morphologically identified *Spirulina* sp. isolate was successfully obtained as a pure culture from the enriched culture. Following the successful completion of the DNA extraction procedure, the DNA was visualized on agarose gel to confirm its quantity and quality. The visibility of DNA on agarose gel electrophoresis allowed for the estimation of both its quantity and quality. The 16S primers (CYA106F/CYA781R and 27F1/809R) were used to amplify the *Spirulina* sp. samples, and the amplicon sizes were compared with a 100 bp DNA ladder, resulting in bands of 700 bp and 800 bp, respectively (Figure S3).

3.5. Phylogenetic Analysis

We submitted our 16S sequences to the nucleotide BLAST (BLASTn) search http://www.ncbi.nlm.nih.gov/BLAST (accessed on 18 February 2024), as shown in Table S4, and the top hits resulted in 100% similarity to *Arthrospira platensis* species. Phylogenetic analysis for 16S gene sequences, including our *Spirulina* sp. isolate, revealed clustering according to systematic groups. Our isolate (indicated by black circles in Figure S1) clustered with *Arthrospira platensis* sequences from GenBank and was clearly delineated from their sister taxa A. maxima, as supported by significant bootstrap values (Figure 7).

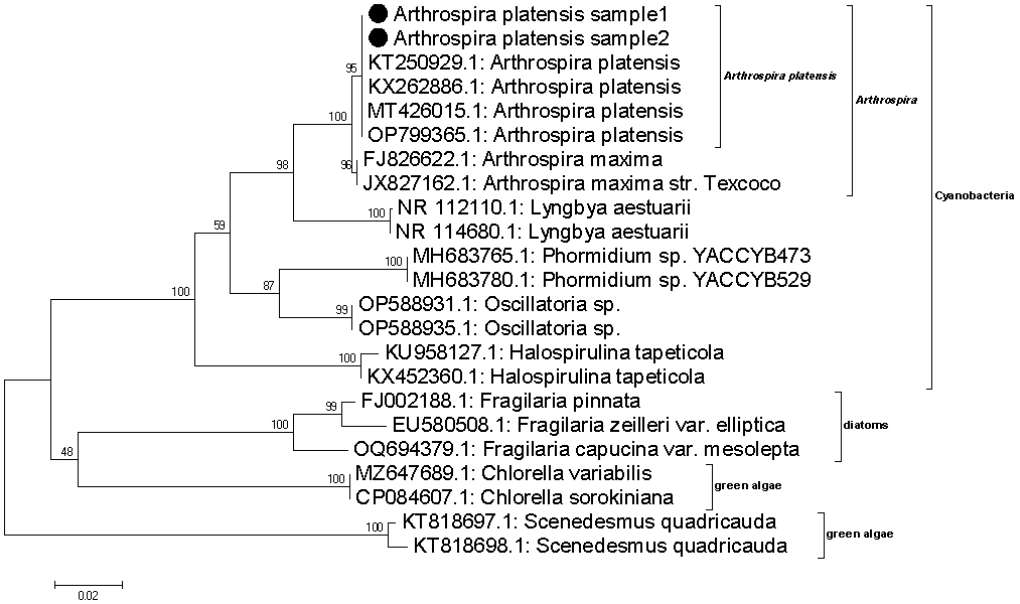

Figure 7. Phylogenetic tree of *Spirulina* sp. using the Neighbor-joining method. Systematic groups are highlighted. Samples of our study are indicated by black circles. Significance values are presented at the nodes.

4. Discussion

Algae are integral to global ecosystems, primarily due to their significant role in photosynthesis and oxygen production. Our study of the algal population in Kohat's freshwater reservoirs reveals critical insights into the ecological preferences and adaptability of *Spirulina*, which, despite its notable applications in various industries, remains underrepresented in natural freshwater systems.

The limited detection of *Spirulina* sp. in our study is consistent with Freeman et al. (2020), who also reported its scarcity in similar environments [24]. This aligns with the hypothesis that *Spirulina* sp. has specific habitat requirements and distinct morphological traits that limit its widespread occurrence in freshwater ecosystems. Our findings underscore the importance of physicochemical parameters [25], particularly pH, in determining algal distribution, echoing the conclusions of Braga et al. [25]. The observed pH variability in Kohat's water bodies suggests that slight changes in the environmental conditions can significantly influence the presence of *Spirulina* sp., corroborating the species' sensitivity to pH as a critical environmental factor.

The physicochemical analysis of water samples from Kohat's reservoirs highlighted significant variations in pH, EC, DO, TDSs, and temperature. Notably, *Spirulina* sp. was isolated from the Usterzai location, where water parameters showed a pH of 8.60 $\pm$ 0.08, an EC of 540 $\pm$ 0.81 µS/cm, a DO of 4.50 $\pm$ 0.05 mg/L, and TDSs of 313 $\pm$ 0.81 mg/L. These findings indicate that *Spirulina* sp. thrives in specific environmental conditions, underscoring the importance of monitoring these parameters for successful cultivation and conservation efforts, supported by the work of [26,27]. Overall, this study reveals that the TDSs, pH, and EC are critical determinants of water quality and the distribution of *Spirulina* sp. species in the Kohat district. PCA and RDA analyses demonstrated that higher levels of these parameters are closely associated with the prevalence of *Spirulina* sp., suggesting its preference for nutrient-rich and alkaline environments. While PCA and RDA analyses indicated that the TDSs, pH, and EC are significant factors influencing the distribution of different forms of algae, including *Spirulina* sp., these parameters alone do not fully account for the selective presence of *Spirulina* sp., suggesting that additional microenvironmental factors such as specific nutrient availability, microbial interactions, or subtle variations in water chemistry, which were not measured in this study, must be investigated in future research.

The morphological variability within *Spirulina* sp. isolates, as noted in this study, parallels the earlier findings which documented similar variability in cultured environments [28]. This highlights the adaptability of *Spirulina* sp. to different environmental conditions, which may manifest in morphological diversity. The precise measurement of *Spirulina* sp. cell dimensions in our study adds to the body of knowledge by providing detailed morphological data that can be referenced in future research.

The successful application of molecular techniques for identifying *Spirulina* sp., as demonstrated in our study, validates the methodologies used by Nübel et al. [22] who have employed similar approaches for cyanobacterial identification. Our phylogenetic analysis, which confirmed a close relationship between our *Spirulina* sp. isolate and *Arthrospira platensis*, adds to the existing literature by providing updated genetic information that supports the use of molecular markers in distinguishing closely related cyanobacterial species.

In conclusion, our study contributes valuable data on the occurrence and ecological preferences of *Spirulina* sp. in natural freshwater reservoirs. The critical comparison with earlier studies highlights the specificity of *Spirulina*'s habitat requirements and its sensitivity to environmental conditions. These insights emphasize the need for targeted conservation efforts to preserve algal biodiversity and the ecological integrity of freshwater ecosystems. Future research should continue to explore the interactions between physicochemical parameters and algal distribution to develop comprehensive strategies for the management and conservation of these vital resources.

5. Conclusions

This research provides a comprehensive examination of *Spirulina* sp. presence in the freshwater reservoirs of Kohat, Pakistan, revealing a sparse distribution across the sampled locations. Among 156 samples from diverse depths across 26 reservoirs, only a single instance of *Spirulina* sp. was confirmed, underscoring the species' rare occurrence in this geographical area. Physiochemical analyses revealed a range of environmental conditions across the sites, with the presence and abundance of algal species correlating variably with

factors such as pH, dissolved oxygen, and other parameters. Consistent early morning sampling minimized the impact of diurnal DO fluctuations. The isolation and molecular characterization of *Spirulina* sp., along with phylogenetic analysis, not only confirm its rare presence but also contribute significantly to understanding its ecological preferences and genetic relationships within Kohat's water bodies. While PCA and RDA analyses identified the TDSs, pH, and EC as significant factors influencing algae distribution, including *Spirulina* sp., additional microenvironmental factors such as specific nutrient availability, microbial interactions, or subtle water chemistry variations must be investigated in future research in order to fully account for *Spirulina*'s selective presence. Similarly, future research may explore additional morphological and physiological characteristics, in addition to cell size, and the potential mechanisms of *Spirulina* adaptation to these conditions.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/su16156400/s1. Table S1: List of freshwater bodies identified within the district Kohat, Khyber Pakhtunkhwa, Pakistan, and selected for sampling; Table S2: Primer details for amplification of 16S rRNA (CYA106F/CYA781R) and (27F1/809R) in Spirulina, including primer sequences, product size, melting temperature ™, and GC content; Table S3: NCBI BLAST analysis of Spirulina sp. using 16S rRNA (CYA106F/CYA781R) and (27F1/809R) primers, highlighting sequence similarity and coverage with Arthrospira platensis. Figure S1: (**A**,**B**). Cell Size Analysis: Single Image Measurements Using Axio Imager. Z1; Figure S2: Pictorial Analysis: Cell Size Measurements Using ImageJ Software (100 Images); Figure S3: Molecular identification of Spirulina isolates using 16S at position No.1 (CYA106F/CYA781R) and at Position No. 2 (27F1/809R) molecular marker, shown alongside a 100 bp DNA ladder.

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