MOLECULAR INVESTIGATION OF CYANOBACTERIA IN THE CASPIAN SEA

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Abstract: This study examines the presence of cyanobacteria in the Caspian Sea and their potential for microcystin production. DNA from water samples collected across the sea was analysed using CC-CG PCR, revealing positive amplification in 9 of 14 samples, indicating active cyanobacterial presence. However, attempts to detect microcystin-producing genes (mcyB) were unsuccessful, likely due to low DNA concentrations and sample quality. Regular monitoring of physicochemical parameters highlighted the influence of nutrient changes on cyanobacterial communities. These findings underscore the need for systematic monitoring to prevent the proliferation of toxic species and inform strategies to mitigate their spread, contributing to understanding the Caspian Sea's ecosystem and conservation.

Keywords: Cyanobacteria, Microcystin, PCR, DNA extraction, Ecosystem monitoring

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Introduction:

Cyanobacteria, commonly known as blue-green algae, are prokaryotic organisms capable of photosynthesis due to the presence of chlorophyll-a pigment. These microorganisms are found across a wide range of environments, including aquatic ecosystems, soil surfaces, and in symbiotic relationships with certain plant roots (Berman-Frank & ets, 2003). As integral components of aquatic ecosystems, cyanobacteria play a crucial role in maintaining ecological balance. However, in recent decades, various environmental changes, particularly those driven by human activities, have led to the excessive proliferation ("blooming") of cyanobacteria. This phenomenon not only negatively impacts water quality and aquatic organisms but also results in the production of toxins that pose serious risks to human health (Nuriyeva, 2018; Nuriyeva, 2019).

The Caspian Sea is one of the regions significantly affected by this issue. Rapid growth of cyanobacteria in the sea has been accompanied by an increase in cyanotoxin levels. Industrial and domestic waste pollution creates favourable conditions for cyanobacterial blooms, with nutrient-rich discharges - especially nitrogen compounds - acting as primary drivers. The proliferation of cyanobacteria and their toxins has a detrimental impact on the survival of fish, molluscs, plants, and other aquatic organisms. Additionally, humans are exposed to these toxins directly or indirectly, jeopardizing public health (Akhundova, 1996; Berman-Frank & ets, 2003).

The cyanotoxins produced by excessive cyanobacterial blooms are biologically active and hazardous, capable of affecting human

health in various ways. Therefore, molecular-level studies of these toxins are critical to restoring the ecological balance of the Caspian Sea and safeguarding aquatic and human health. Advanced molecular techniques such as ELISA and PCR are widely used to analyze the composition and concentration of cyanotoxins, providing precise detection and forming the foundation for control strategies.

Comprehensive research is needed to identify the causes of cyanobacterial blooms in the Caspian Sea, assess the associated risks, and develop effective control measures. Such studies not only improve our understanding of cyanobacterial impacts but also help evaluate the effectiveness of physical, chemical, and biological methods for controlling blooms. By creating a robust knowledge base, it becomes possible to implement cost-effective and sustainable solutions for preventing cyanobacterial proliferation. These efforts are vital for improving the ecological condition of the Caspian Sea and ensuring environmental protection.

Materials and Methods:

This study utilized various methods to determine the presence of cyanobacteria and microcystin production in the aquatic environment of the Caspian Sea. A systematic approach, including water quality assessment, DNA extraction, and amplification procedures, was applied.

During the study, 14 water samples were collected from the Caspian Sea outlet on November 17, 2023, and March 5, 2024. The samples were collected in 1-litre glass containers and immediately transported to the laboratory. The samples were stored at 0-4 °C. Universal primers were used to determine the presence of oxyphotobacteria.

For DNA extraction, 0.5 L of the water sample was filtered. During filtration, nitrocellulose filters with a pore size of 0.45 µm were used. The filters were dissolved in 1 mL of PBS (phosphate-buffered saline) solution. The Qiagen DNeasy PowerWater Kit was used for DNA extraction. DNA quality and quantity were measured using a spectrophotometer (NanoDrop).

For PCR operations, CC-CG primer pairs were selected. Each PCR reaction was conducted with 12.5 µL of 2X PCR mixture, 1 µL of universal primer, 1 µL of DNA sample, and 9.5 µL of sterile water. The reactions included an initial denaturation step at 95 °C for 5 minutes, followed by 30 seconds of denaturation at 95 °C, 30 seconds of annealing at 55-65 °C, and a 1-minute extension step at 72 °C. The PCR products were separated using 1% agarose gel electrophoresis.

To determine microcystin production, genus-specific primer pairs (mcyB for Microcystis sp.) were used. The concentration of microcystin was assessed using the ELISA (Enzyme-Linked Immunosorbent Assay) test (Burrell & ets, 2016).

The results of the study provide monitoring data for cyanobacteria and microcystin production in the Caspian Sea's aquatic environment. These findings present important information for future research and are significant for assessing ecosystem health.

Results and discussions:

The first and crucial step in conducting molecular research on cyanobacteria is the isolation of pure cultures. For this purpose, the area where the samples will be collected must be determined initially, and the samples should be gathered in accordance with the required protocols. The cyanobacteria samples used in this study were collected from the shores of the Shirvan National Park on the Caspian Sea during the summer months of 2023.

To prevent contamination during the collection process, sterile equipment was used, and each sample was stored in specialized containers. These containers were maintained under proper conditions, with the appropriate temperature and lighting, until they were transported to the laboratory.

The samples were collected from the same source at various depths (5-10 cm from the surface of the shore) and taken multiple times. During the transportation of the collected material to the laboratory, all necessary requirements were adhered to. Upon arrival at the laboratory, the samples were processed

under suitable conditions to ensure the isolation of pure cultures (Figure 2).

Figure 1. Cyanobacteria collected from the shores of the Shirvan National Park area on the Caspian Sea.

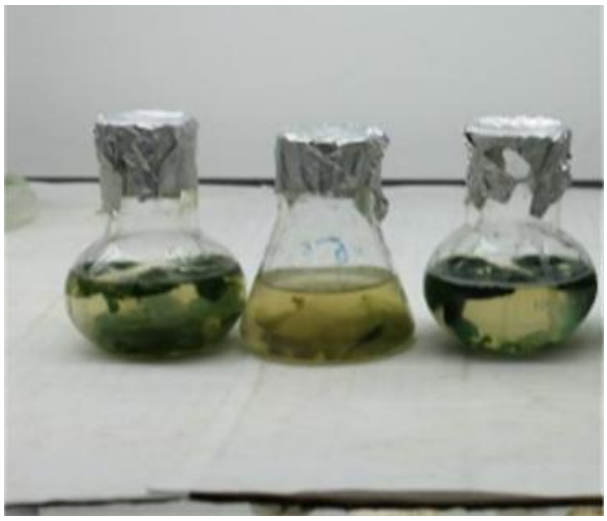

Figure 2. Enriched cultures of cyanobacteria.

The composition of the culture medium was prepared by the expected algological composition of the samples. The process of isolating pure cultures typically consists of three main stages: obtaining enriched (cumulative) culture (Figure), isolating the pure culture, and confirming the purity of the isolated culture. Individual pure cultures were isolated using the streak plate method. For this purpose, a small amount of the sample was taken using a microbiological loop and spread over the surface of the culture medium.

Initially, the streaks contained a large number of microalgal cells. However, with the movement of the loop, the number of these cells gradually decreased, eventually reaching a single-cell level. After the distribution of cyanobacteria in the culture medium, the plates were incubated to allow for colony growth (Figure 3).

The purity of all three isolated cyanobacteria cultures was verified using several methods: visual microscopic inspection and the use of

different culture media to confirm the culture's purity.

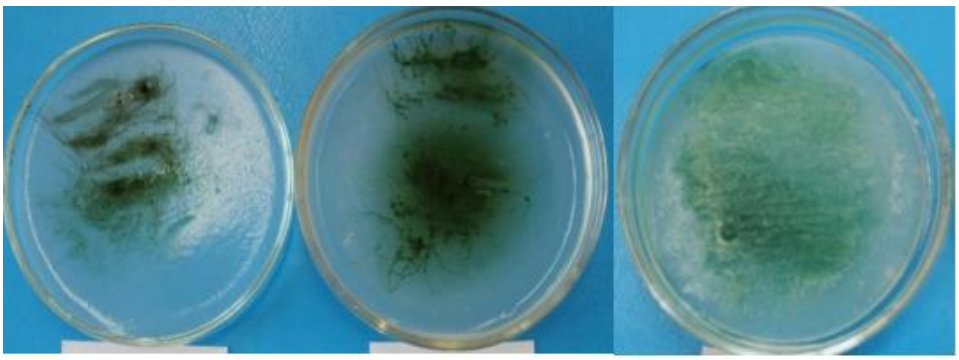

Figure 3. Growth of cyanobacteria on agar medium.

The results of the study conducted to analyze the cyanotoxins of cyanobacteria collected from the shores of the Shirvan National Park on the Caspian Sea are presented in Table 1. In all samples, the concentration of microcystin in the Caspian Sea was 0.2 µg/L. In June and July, the concentrations of saxitoxin in two samples were near the detection limit of 0.03 µg/L, while the sample collected in August was approximately 0.02 µg/L.

Table 1. Concentrations of microcystin, saxitoxin, and anatoxin-a in samples from the Caspian Sea during June-August 2023:

	24.06.2023		15.07.2023	05.08.2023	Negativ
Microcystin µg/l	0.2	0.2	0.2		< 0.10 µg/l
Saxitoxin µg/l	0.01	0.03	0.02		< 0.02 µg/l
Anatoxin -a µg/l	53.6	9.5	25.1		< 10 µg/l

Results for anatoxin-a showed higher concentrations in the samples compared to microcystins and saxitoxin. In the sample collected from the Caspian Sea in June, the average concentration of anatoxin-a was 53.6 µg/L, 25.1 µg/L in August, and 9.5 µg/L in July.

ELISA kits are widely used by several laboratories for the detection of cyanotoxins. This method allows for rapid and relatively inexpensive detection of cyanotoxins in environmental samples. However, this method only estimates the total amount of toxin and has difficulty distinguishing between different analogs of the toxin. Additionally, due to high cross-reactivity, false positives may occur, and therefore, it is recommended to verify the results with other methods (Salmaso & Zignin, 2020). Since the analysis of saxitoxin and anatoxin-a with ELISA kits is relatively new, there is limited research available on this topic.

The risk of exposure to cyanotoxins in humans and animals is a cause for concern. Although the concentrations of microcystins and saxitoxin in the Caspian Sea are low, the concentration of anatoxin-a is sufficiently high. This exceeds the guideline value of 20.0 µg/L for recreational waters (Flombaum & ets, 2013). The high concentration of anatoxin-a is dangerous for both wild and domestic animals; in the United States, several dogs have died after drinking water contaminated with anatoxin-a (Flombaum & ets, 2013).

Fish are more susceptible to cyanobacterial toxins, affecting their embryonic development and growth rate. The composition of the fish community in the Caspian Sea has decreased with changes in the hydrological regime. The

Caspian Sea sustains the biodiversity of fish, which are a food source for the local population. However, the consumption of meat from organisms that accumulate microcystins is risky (Durai & ets, 2015).

The airborne transmission of microcystins and cyanotoxins could be problematic during recreational activities in lakes (Backer & ets, 2009). The fate of cyanotoxins in the aquatic environment is also of significant importance. Among biodegradation mechanisms, the biodegradation of these compounds appears to be the most effective. Heterotrophic bacteria use cyanotoxins as a carbon source (Nybom, 2013).

Molecular methods, particularly PCR (Polymerase Chain Reaction)-based techniques, are widely used in the study of cyanotoxins. These methods enable the identification of the presence of cyanobacteria and their potential for toxin production (Salmaso & Zignin, 2020). Spectrophotometric results of DNA samples from the Caspian Sea, when extracted by various methods, were characterized by low concentrations and purity indicators.

CC-CG PCR successfully amplified 606 bp fragments of cyanobacterial DNA. Positive amplification was observed in 9 out of the 17/11/2023 samples, providing significant information regarding the presence of cyanobacteria (Salmaso & Zignin, 2020).

Table 2. Concentration and purity of DNA extracted from the Caspian Sea (X) from June to August 2023. Extraction dates: 17/11/2023 and 05/03/2024, and positive samples (CC-CG) for Oxyphotobacteria-specific primers.

Sampling Location and Date		Extraction on 17/11/2023		CC-CG primers for positive samples	Extraction on 05/03/2024		CC-CG primers for positive samples
		Concentration ng/µl	**Purity A260/A280**		**Konsen-trasiya ng/µl**	**Saflıq A260/A280**	
X1	24.06.23	15.9	1.6		19.6	1.5	+
	15.07.23	18.7	2.1	+	34.2	2.4	+
	05.08.23	8.1	1.6	+	40.1	1.4	
X2	24.06.23	23.3	1.7		27.1	1.9	+
	15.07.23	13.8	3.9		58.9	2.1	
	05.08.23	4.4	-2.1	+	22.3	1.7	+

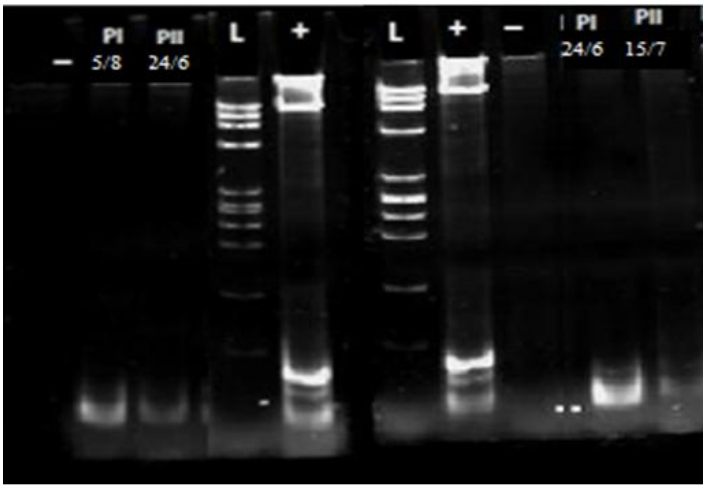

Figure 4. PCR results for the detection of toxic Microcystis in the Caspian Sea using primers specific to the Microcystis sp. mcyB gene. (+) Positive control, (-) Negative control, (L) Ladder.

At the PI sample collection station located at the mouth of the Caspian Sea, using universal primers for oxyphotobacteria, two positive amplifications were observed in PCR for the months of June, July, and August. This sampling station is characterized by the highest salinity throughout the study period. The sample taken on 05/08/23 tested positive after the PCR operation for both extractions. A sample collected at the same station in June showed a positive band, but no amplification was observed in the July extracts.

PCR reactions were performed several times using genus-specific primer pairs to amplify the regions of the gene responsible for the synthesis of microcystin (mcyB in Microcystis sp.). A nested PCR reaction was carried out with 1 µl of PCR product, but no amplification was detected. Microcystis sp., used as a positive control, showed good amplification in all tests, indicating that the PCR reaction was well optimized. The failure of genus-specific primers to amplify bands in the PCR is related to the low quantity and quality of the DNA extracted on 17/11/23.

Negative PCR results with genus-specific primers can be consistent with the low microcystin concentration measured by ELISA in the Caspian Sea. This may indicate that the cyanobacterial cells detected in microscopic analyses and CC-CG PCR do not produce microcystin. It is thought that blooms in the same water column may show variations in toxicity from year to year.

Further research on the cyanobacterial profiles of the Caspian Sea may be needed in the future. PCR optimization could also be effective. The multilayered cell wall of cyanobacteria and the mucous layer around the cells hinder effective DNA extraction. Additionally, environmental samples are often associated with humic acids, sands, and other materials that accompany DNA. Spectrophotometric results have detected protein contamination in several samples with a purity of 2 A260/A280 or higher.

Long-term analyses may help explain the nature of cyanobacterial communities in these lakes. Low nutrient concentration, especially nitrogen, promotes the growth of non-toxic Microcystis cells. Similarly, the low concentration of nitrogen compounds in the Caspian Sea corresponds to the characteristics of cyanobacteria in these waters.

Microscopic identification and PCR amplification detected the presence of cyanobacterial cells. The absence of PCR bands corresponding to mcy gene regions may indicate that the cyanobacteria in the Caspian Sea do not produce microcystin. Toxin analyses demonstrated low concentrations of microcystin and saxitoxin in the Caspian Sea, but a high concentration of anatoxin-a.

The continuous monitoring of physical-chemical parameters, along with the abundance of blue-green algae and cyanotoxin concentration in the Caspian Sea, can help improve the current state of the lakes. Since there is frequent displacement of toxic and non-toxic cyanobacteria in the water environment, restoration strategies should be implemented in the future to prevent the development of toxic cyanobacteria species.

Conclusıon:

This study investigated the presence of cyanobacteria and microcystin production in the Caspian Sea water environment. DNA analysis amplified by CC-CG PCR method yielded positive amplification results in 9 out of 14 samples. This indicates that cyanobacteria are active in this area and could potentially produce toxins. At the same time, PCR reactions conducted using genus-specific primers (mcyB) to detect microcystin production did not yield positive results. This was associated with the possible low DNA concentration and the quality of the samples.

Monitoring the environmental conditions in the Caspian Sea revealed that the cyanobacterial flora may show changes. Physical-chemical parameters, nutrient concentrations, and ecosystem health should be regularly assessed. Furthermore, the study results are crucial for developing strategic plans to prevent the future development of toxic cyanobacteria species. Systematic monitoring and research are necessary to protect the

Caspian Sea's ecology, which will help to better understand the factors affecting human health and local ecosystems. The results provide an important foundation for the implementation of scientifically and ecologically relevant measures.

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