Bacterial Diversity in the Hypersaline Lake Urmia Revealed by PCR-DGGE and Multivariate Ecological Analysis

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Abstract

Hypersaline lakes are extreme ecosystems that harbor specialized microbial communities adapted to high osmotic stress. Lake Urmia, one of the world's largest hypersaline lakes, has undergone pronounced salinity fluctuations (>340 g L^{-1}), providing a natural system for studying microbial community structure under extreme conditions. In this study, bacterial diversity in Lake Urmia was assessed using PCR-DGGE targeting the V3 region of the 16S rRNA gene. Water samples were collected from six sites in northern and central regions during spring and summer 2013. Community patterns were analyzed using DGGE fingerprinting combined with ecological indices and multivariate statistics, including UPGMA clustering and Bray–Curtis similarity analysis. Shannon diversity indices ranged from 1.21 to 2.08 and Simpson indices from 0.28 to 0.64, indicating moderate diversity with significant spatial heterogeneity (ANOVA, $p < 0.05$). Cluster analysis resolved two distinct community structures corresponding to northern and central lake regions, with >60% dissimilarity. Sequencing of representative DGGE bands revealed taxa affiliated with Proteobacteria, Bacteroidetes, and Actinobacteria. Several sequences showed <97% similarity to known organisms, suggesting the presence of potentially novel halophilic lineages. Notably, *Deefgea rivuli* and multiple uncultured bacterial groups were detected for the first time in Lake Urmia. These findings demonstrate strong spatial structuring of bacterial communities in response to environmental heterogeneity and highlight Lake Urmia as a reservoir of underexplored microbial diversity.

Keywords: Lake Urmia; hypersaline lake; bacterial diversity; 16S rRNA gene; PCR-DGGE; microbial community structure; multivariate analysis; halophilic bacteria

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Introduction

Biodiversity is a fundamental indicator of ecosystem health and stability and encompasses genetic, species, and ecosystem-level variation. Among these, genetic diversity forms the basis of adaptation, evolution, and ecological resilience (Cardinale *et al*., 2012). Microorganisms represent the largest and least explored component of global biodiversity, exhibiting remarkable metabolic and ecological versatility that enables survival across a wide range of environments, including extreme ecosystems (Coleine *et al*., 2024).

Extremophilic microorganisms, particularly those inhabiting hypersaline environments, occupy ecological niches previously considered uninhabitable (Acosta *et al*., 2013). These environments impose severe osmotic stress due to salt concentrations exceeding seawater salinity, selecting for organisms with specialized adaptive mechanisms. Halophilic bacteria require high salinity for growth, whereas halotolerant species can survive across broader salinity ranges. Their adaptation strategies include "salt-in" regulation, compatible solute accumulation, ion transport systems, and membrane modifications, enabling cellular homeostasis under extreme conditions (DasSarma *et al*., 2022). Extremophiles are able to maintain their internal homeostasis under extremely harsh conditions through a coordinated set of molecular, cellular, and genetic mechanisms. These characteristics are not only important for understanding the biology of life, but also have applications in biotechnology, enzyme industries, pharmaceuticals, and industrial biotechnology (Somayaji *et al*., 2022).

Hypersaline ecosystems are recognized as important reservoirs of microbial diversity and sources of novel taxa with biotechnological potential. Enzymes derived from halophilic microorganisms (extremozymes) are widely applied in industrial processes due to their stability under extreme salinity, temperature, and pH conditions (Dutta and Bandopadhyay, 2022). Consequently, these environments have become central to microbial ecology and biotechnology research.

Lake Urmia, located in northwestern Iran, is one of the largest hypersaline lakes in the world and has undergone severe ecological degradation in recent decades due to reduced freshwater inflow and climate change, leading to salinity levels exceeding 300–350 g L^{-1} (Eimanifar and Mohebbi, 2007). These extreme conditions make it an ideal natural laboratory for studying microbial adaptation and diversity in hypersaline environments. This study shows that Lake Urmia is a hypersaline ecosystem with exceptionally high microbial genomic diversity driven by strong environmental selection and extensive microdiversity among dominant halophilic populations (Kheiri *et al*., 2023).

Previous culture-dependent studies have reported halophilic genera such as *Halomonas*, *Salicola*, *Marinobacter*, and *Bacillus* in Lake Urmia (Arash Rad, 1999; Vahed *et al*., 2011; Salami *et al*.,

2025). However, such approaches capture only a minor fraction of total microbial diversity, often less than 1%. Culture-independent molecular techniques provide a more comprehensive assessment of microbial communities by eliminating cultivation bias. Among these methods, polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) has been widely used for microbial community profiling (Manaffar, 2012; Boon *et al*., 2002). This technique separates PCR-amplified DNA fragments based on sequence-dependent differences in melting behavior, enabling comparative ecological analysis of complex environmental samples.

Despite the ecological importance of Lake Urmia, comprehensive molecular and statistical investigations of its bacterial communities remain limited. Therefore, this study integrates PCR-DGGE with ecological diversity indices and multivariate statistical analyses to (i) characterize bacterial community composition, (ii) quantify diversity using Shannon and Simpson indices, (iii) evaluate spatial variation using clustering methods, and (iv) identify dominant and potentially novel bacterial taxa.

Materials and methods

Sample collection

Water and salt samples were collected from six locations within Lake Urmia during the spring and summer of 2013. Owing to the severe drought conditions and substantial reduction in lake water levels during the sampling period, access to many regions of the lake was restricted. Consequently, sampling was conducted at the northern part of the lake (Bari Station) and at both sides of the Lake Urmia causeway, where water recession was less pronounced. At all sampling sites, water salinity exceeded 340 g L^{-1} during both sampling seasons (Fig. 1).

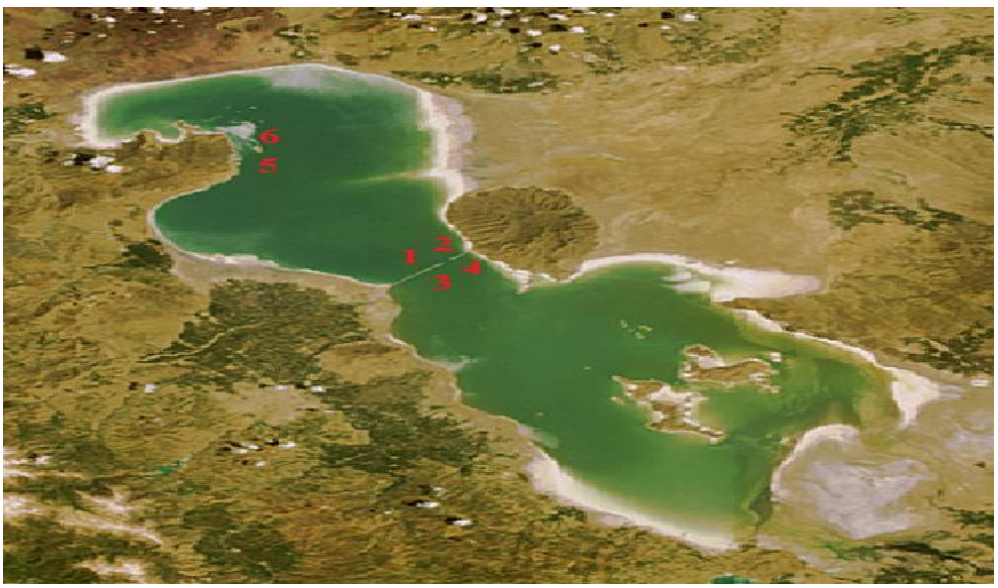

Figure 1: Satellite image of Lake Urmia indicating the sampling sites (Spring 2013).

Sample processing and DNA extraction

Bacterial cells were concentrated according to a previously published protocol. Briefly, samples were thoroughly vortexed and transferred into Falcon tubes, followed by centrifugation at 3,000 × g for 15 min. The supernatant was discarded, and the pellet was retained. This procedure was repeated until the supernatant became completely clear. To separate bacterial cells from suspended particles and other contaminants, samples were filtered through sterile 0.22-µm membrane filters. Genomic DNA was extracted from the concentrated bacterial biomass. Briefly, 400 µL of each sample was incubated at 95°C for 5 min and immediately transferred to ice for an additional 5 min to facilitate bacterial cell lysis. DNA quantity and purity were subsequently evaluated using a spectrophotometer.

PCR amplification of the 16S rRNA gene

Amplification of bacterial 16S rRNA gene fragments was performed using primers previously described in the literature. The primer pair consisted of 338F (5′- actcctacgggaggcagcag -3′) and 518R (5′- attaccgcggctgctgg -3′) (Boon *et al*., 2002; Ghyselinck *et al*., 2013). All thermal cycling conditions and amplification protocols were conducted according to the referenced study (Manaffar, 2024). PCR reactions were performed in optimized reaction mixtures containing PCR buffer, $MgCl_2$, dNTPs, Taq DNA polymerase, primers, and template DNA. Amplification was carried out in a thermal cycler under the specified cycling conditions. The presence and size of PCR products were verified by electrophoresis on 1.5% agarose gels. Electrophoresis was performed in 0.5× TBE buffer. For each sample, 10 µL of PCR product was mixed with 2 µL of loading buffer and 1 µL of nucleic acid staining dye prior to loading onto the gel. A 1,500-bp DNA ladder was used as a molecular size marker. DNA fragment sizes were estimated by comparison with the migration pattern of the DNA ladder.

DGGE analysis and band sequencing

DGGE analysis was performed using primers containing a GC-clamp sequence. The GC-rich clamp prevents complete denaturation of double-stranded DNA fragments during electrophoresis and ensures separation based on sequence-specific melting behavior in the presence of denaturing agents such as urea and formamide. After polymerization, the DGGE gel was assembled in the electrophoresis apparatus and the wells were thoroughly rinsed with running buffer. For each sample, 6 µL of PCR product was mixed with 24 µL of loading dye and loaded into the wells. Empty wells were filled with loading dye to ensure uniform electrophoretic conditions. Electrophoresis was conducted at 120 V for 16 h as previously described (Manaffar, 2012; Najdegerami and Manaffar, 2024).

Following electrophoresis, gels were removed from the glass plates and stained in a solution containing ethidium

bromide. The stained gels were visualized under UV illumination and photographed using a gel documentation system. DNA bands were excised from DGGE gels under UV illumination using sterile scalpels while appropriate protective measures were observed. Prior to excision, sterile microcentrifuge tubes were weighed individually. Each distinct band was carefully removed from the gel and transferred to a separate tube, after which the net weight of the gel fragment was determined. DNA was purified from excised gel fragments using a commercial purification kit (Roche, Germany) according to the manufacturer's instructions.

Distinct DGGE bands were subsequently excised, purified, and re-amplified by PCR (re-PCR). This step improved amplification specificity and yielded DNA fragments corresponding to individual bacterial taxa. The resulting PCR products were submitted to Sina Clon Company for DNA sequencing, which was performed by a sequencing facility in the United Kingdom.

Statistical and ecological analysis (Shannon, 1948; Shannon, 1948)

Band intensity was used as a proxy for relative abundance. The following indices were calculated:

- Shannon–Wiener diversity (H')
- Simpson diversity (1–D)
- Species richness (S)

Formulas:

$H' = -\Sigma\, pi \ln pi$

$D = \Sigma\, pi^2$

Multivariate analysis:

- UPGMA clustering
- Bray–Curtis similarity index

One-way ANOVA for spatial comparison ($p<0.05$)

Results

PCR amplification and DGGE profiling

PCR amplification followed by re-PCR generated clear and distinct DNA fragments with no evidence of nonspecific amplification or smearing. Amplified products of approximately 150 bp were successfully obtained from the analyzed samples. Representative PCR products are shown in Figures 2 and 3.

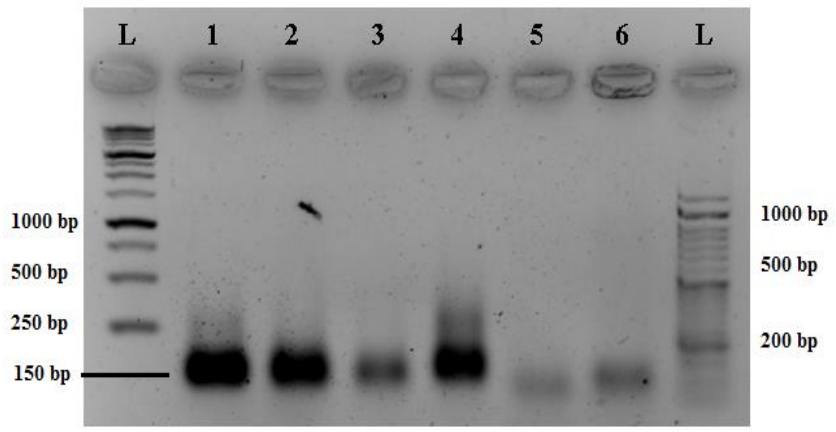

Figure 2. Agarose gel electrophoresis of bacterial PCR products from six sampling sites in Lake Urmia. Lane L: DNA ladder; lanes 1–6: sampling sites.

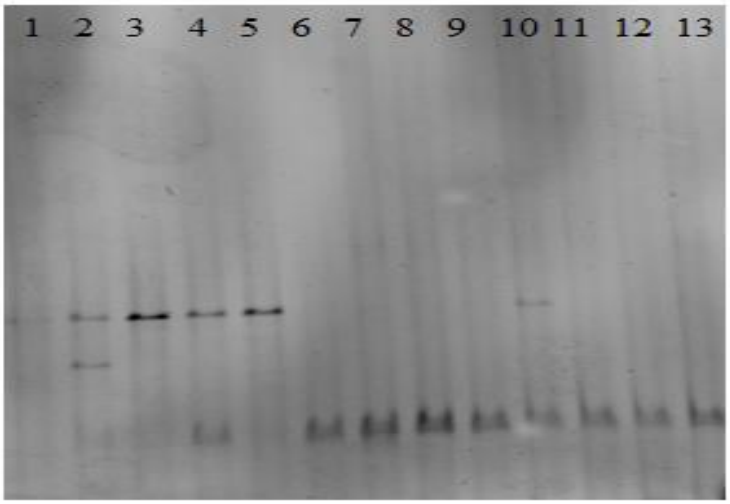

Figure 3: DGGE fingerprint of bacterial communities from six sampling sites in Lake Urmia. Lane L: DNA marker; lanes 1–5: northern region; remaining lanes: causeway region.

Discussion

To the best of our knowledge, this study represents the first culture-independent investigation of bacterial diversity in Lake Urmia using PCR-DGGE analysis. Despite the severe drought conditions and exceptionally high salinity of the lake during the sampling period, which may have reduced microbial richness, the applied molecular approach enabled the detection of several bacterial taxa that had not previously been reported from this hypersaline ecosystem.

Previous investigations of hypersaline lakes and saline habitats have documented diverse communities of halophilic and halotolerant bacteria. Similarly, a study conducted on Lake Urmia in 2011 reported the occurrence of microorganisms affiliated with the γ-Proteobacteria and Firmicutes, with the closest phylogenetic relationships to members of the genera *Halomonas*, *Salicola*, *Pseudomonas*, *Idiomarina*, *Marinobacter*, *Bacillus*, and *Halobacillus*. The differences observed between the findings of the present study and those reported previously are likely attributable to methodological differences in microbial detection and identification approaches (Arash Rad, 1999) which these cases have also been confirmed in other lakes (Acosta *et al*., 2013).

Traditional cultivation-based approaches provide access to only a small fraction of environmental microorganisms, often estimated at less than 1% of the total microbial community. Furthermore, culture conditions selectively favor the growth of particular microbial groups, potentially resulting in biased representations of natural microbial assemblages. In contrast, culture-independent molecular methods eliminate the requirement for laboratory cultivation and allow direct examination of microbial communities from environmental samples. Nevertheless, these approaches are not without limitations, as DNA extraction efficiency, PCR amplification bias, and

primer specificity may influence the representation of microbial taxa.

The absence of substantial overlap between taxa identified through culture-dependent and culture-independent approaches has also been reported in previous microbial ecology studies. This discrepancy may reflect both methodological biases and the limited number of isolates characterized through conventional cloning and sequencing procedures. Consequently, a polyphasic approach combining cultivation-based techniques with molecular analyses is likely to provide the most comprehensive assessment of microbial diversity. Similar conclusions have been reported in investigations of other Iranian hypersaline ecosystems, including Aran-Bidgol Salt Lake.

Sequence analysis indicated that a considerable proportion of the detected bacterial community was affiliated with the phylum Bacteroidetes. Within this group, sequences closely related to Salinibacter ruber were particularly abundant, accounting for a substantial proportion of the identified sequences. Similar observations have been reported from other hypersaline environments, where members of Bacteroidetes frequently constitute dominant components of microbial communities.

Notably, a significant fraction of the obtained sequences exhibited less than 97% similarity to reference sequences available in public databases. Such levels of divergence may indicate the presence of previously uncharacterized bacterial lineages or poorly represented taxa within existing sequence repositories. Sequences affiliated with the genera Salinibacter, Adhaeribacter, and Cesiribacter, all belonging to the phylum Bacteroidetes, were detected among the analyzed samples.

One of the most noteworthy findings of the present study was the detection of sequences closely related to *Deefgea rivuli,* a member of the class Betaproteobacteria. Members of the phylum Proteobacteria represent one of the most diverse bacterial lineages and include organisms occupying a wide range of ecological niches, from free-living environmental species to bacteria involved in nutrient cycling and other important ecological processes. The occurrence of *D. rivuli*-related sequences in Lake Urmia suggests that this taxon, or closely related microorganisms, may possess adaptive mechanisms enabling survival under hypersaline conditions.

In addition, sequence analysis identified an uncultured Actinobacteria-related taxon. Members of the phylum Actinobacteria are Gram-positive bacteria characterized by high genomic G+C content and are widely distributed in terrestrial and aquatic environments. This group includes numerous ecologically and biotechnologically important microorganisms, many of which are recognized for their ability to produce bioactive compounds and secondary metabolites. For instance, in a review study the diversity and applications of extracellular proteases produced by halophiles was tested. These enzymes are of significant biotechnological interest due to their

high stability and activity under extreme conditions, such as high salinity and alkaline pH, and therefore exhibit considerable potential for industrial applications in areas such as detergents, food processing, and bioprocesses. However, their large-scale industrial utilization is still limited by challenges including difficulties in mass production, high purification costs, and the need for optimization of operating conditions (Nwankwo *et al*., 2023).

According to the researches halophilic microorganisms in hypersaline environments exhibit high diversity and rely on complex genetic and metabolic adaptations to maintain survival, thereby playing a key role in ecosystem stability and functioning (Oren, 2024).

Overall, the results of this study demonstrate the utility of PCR-DGGE as an effective culture-independent approach for investigating bacterial diversity in hypersaline ecosystems. The detection of previously unreported taxa highlights the considerable microbial diversity of Lake Urmia and suggests that this unique environment may serve as a valuable reservoir of microorganisms with potential ecological, industrial, and biotechnological significance.

Conclusion

Lake Urmia harbors structured and diverse halophilic bacterial communities shaped by extreme salinity gradients. Statistical analysis confirmed significant spatial variation, while molecular data revealed potentially novel taxa. PCR-DGGE combined with ecological indices provides an effective framework for microbial ecological assessment in hypersaline environments.

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