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Seasonal Dynamics of Freshwater Bacterial Communities in Continental and Mediterranean Lakes

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ABSTRACT

Lakes are highly dynamic freshwater ecosystems where complex interactions between environmental factors and microbial communities regulate biogeochemical cycling and ecosystem stability. Seasonal dynamics of rare and dominant bacterial taxa, as well as the links between dominant taxa and environmental drivers in freshwater ecosystems across Croatia's contrasting climatic regions, remain insufficiently explored. To address this gap, this study investigated six deep karstic lakes across both regions. Amplicon-based 16S rRNA gene sequencing coupled with FAPROTAX-based functional predictions revealed that microbial community structure and inferred functional potential were strongly influenced by environmental variability. Dissolved organic carbon (DOC) was identified as the primary driver of community composition. Typical freshwater bacterial genera, including *hgcI*, *Cyanobium PCC-6307*, and *Luteolibacter*, dominated across all lakes. Mediterranean lakes exhibited greater heterogeneity in inferred functional potential across seasons, with variable enrichment in predicted pathways associated with carbon, nitrogen, and sulfur cycling. By contrast, continental lakes showed more stable inferred functional potential with less pronounced seasonal fluctuations. Overall, this study highlights how environmental and climatic variability shape the taxonomic composition and inferred functional potential of microbial communities in freshwater systems, emphasizing the ecological dynamics of dominant bacterial taxa, while also revealing seasonal and regional differences between dominant and rare bacterial taxa across contrasting climatic regions.

KEYWORDS

dominant taxa, rare taxa, inferred functional potential, environmental drivers, nutrient cycling, 16S rRNA gene sequencing

1. INTRODUCTION

Microbial communities play an important role in regulating water quality and driving biogeochemical cycling in freshwater ecosystems. Biodiversity, including microbial diversity [1] has been widely recognized for its crucial role in maintaining ecosystem functioning and stability [2]. It supports key ecological processes such as resource capture, biomass production, nutrient recycling, and the maintenance of ecosystem services that underpin ecosystem resilience and human well-being [3]. Environmental factors such as temperature [4], dissolved oxygen (DO) [5] and dissolved organic carbon (DOC) [6] alongside salinity [7] and nutrient loading [8] have been widely recognized as key drivers shaping microbial community composition and diversity in freshwater ecosystems. Climate change, particularly warming trends, may offset the mitigating effect of nutrient reduction through a major restructuring of both biotic and abiotic aquatic processes [9, 10]. Seasonal changes may play an important role in shaping lake microbial communities, potentially exerting a stronger influence than short-term nutrient pulses [11]. Collectively, hydrodynamic processes regulating nutrient transport [12–14], lake physicochemical conditions creating suitable habitats for microbial growth [15], and meteorological drivers such as temperature and precipitation affecting water-column stratification [16] interact to shape microbial community structure and regulate key ecosystem processes in freshwater systems [17]. Most communities are composed of a few dominant taxa and a great many rare ones [18, 19]. Dominant and rare bacterial taxa may have different ecological roles in natural ecosystems [20]. Recent studies have indicated that dominant and rare bacterial taxa may respond dissimilarly to changes of environmental conditions [19, 21–23]. Rare microbial taxa may be more sensitive to seasonal variation and deterministic assembly processes than dominant taxa [24]. Overall, these findings underscore the importance of evaluating seasonal differences between dominant and rare bacterial taxa and examining how environmentally associated shifts in dominant taxa contribute to community assembly patterns across environmental gradients in lake ecosystems.

To fill current knowledge gaps on microbial ecology in freshwater lake systems, this study investigated the seasonal distribution, environmental factors, and inferred the functional potential of bacterial communities in six lakes located in two different climatic regions of Croatia: the continental and Mediterranean regions. Two lakes situated in the continental region and four in the Mediterranean region were targeted, given their regional differences in climate, hydrology, nutrient inputs and physical characteristics, offering a unique natural gradient to explore how environmental variability shapes microbial communities. Accordingly, this study addressed the following research questions: (i) How do environmental parameters vary seasonally across lakes situated in contrasting climatic regions? (ii) How do dominant and rare bacterial taxa, along with overall community composition, change seasonally across continental and Mediterranean lakes? (iii) What is the inferred functional potential of dominant microbial communities across lakes and seasons?

By integrating taxonomic, environmental, and inferred functional potential data, this study provides additional insight into the seasonal variability and environmental sensitivity of microbial communities in different climatic regions.

2. METHODS

2.1. Study area and sampling

Research focused on six natural freshwater temperate lakes in Croatia, two continental and four Mediterranean (Fig.1, Table 1), where water samples were collected once a month from March 2019 to February 2020 at the deepest point of each lake. Samples were collected in 250 mL glass bottles and immediately filtered through 0.2 µm polycarbonate membrane filters (type GTTP; Whatman, UK) using a peristaltic pump. In the continental region, characterized by a continental climate with mild, sunny summers and long, snowy winters, two dimictic lakes within the Plitvice Lakes basin were sampled, including Lake Prošće, the uppermost and second-largest lake, and Lake Kozjak, the largest and deepest in the system. These oligotrophic lakes are interconnected by tufa barriers and cascades and are characterized by low nutrient, dissolved organic matter (DOM) concentrations [25] and high dissolved oxygen concentrations. The surrounding catchment is predominantly covered by beech and beech-fir forests, with smaller patches of pine and black alder [26]. Four monomictic lakes were sampled in the Mediterranean region, characterized by temperate climate with mild, wetter winters and hot, dry summers. Lake Vrana on Cres Island is an oligotrophic rain-fed cryptodepression lake with extended water retention time, surrounded by a southern Mediterranean vegetation zone, with mixed holm oak and black ash forests. Lake Visovac (VIS) is a barrage lake, receiving water from an upper flow, with the largest catchment area among the studied systems. The Baćina lakes are an interconnected complex of coastal cryptodepression lakes, including Oćusa (OCU) and Crniševo (CRN), both collecting precipitation from the surrounding region [27]. Although connected to Lake Crniševo by a narrow channel, Lake Očuša has significantly lower salinity, suggesting limited water exchange between the two lakes [28].

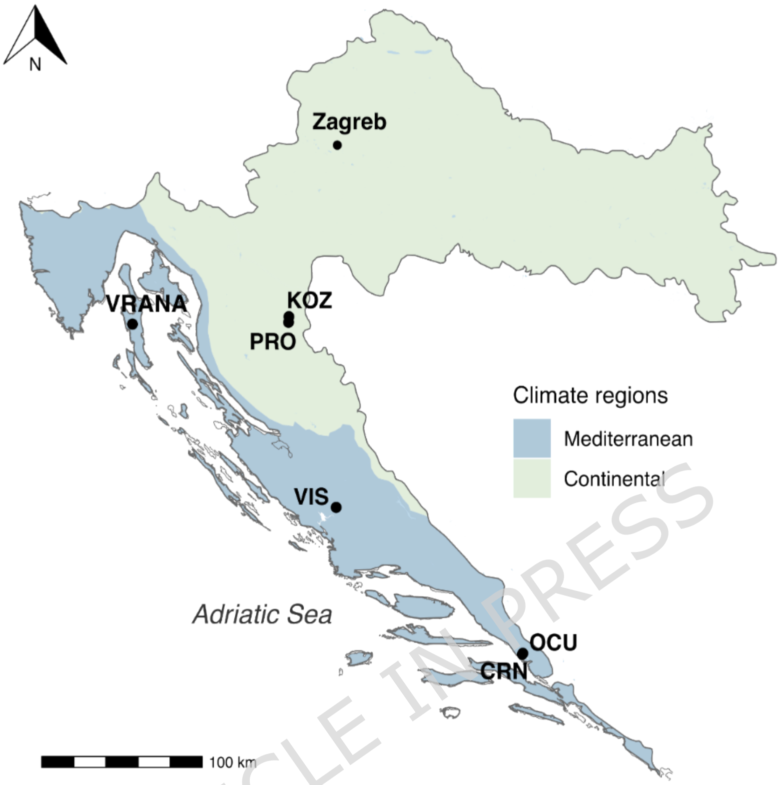

Fig. 1 Geographic location of the six studied karstic lakes in Croatia representing two climatic regions. Kozjak (KOZ) and Prošće (PRO) are situated in the continental region, while Vrana (VRANA), Visovac (VIS), Oćusa (OCU), and Crniševo (CRN) are located within the Mediterranean climatic zone along the Adriatic coast

Table 1. Location, regional distribution, geographic coordinates, and altitude of the study lakes

Lake	**Region**	**Longitude (°E)**	**Latitude (°N)**	**Altitude (m a.s.l.)**
Kozjak (KOZ)	Continental	15.604	44.890	534
Prošće (PRO)	Continental	15.601	44.860	639

Vrana (VRANA)	Mediterranean	14.383	44.850	14
Visovac (VIS)	Mediterranean	15.973	43.862	41
Očuša (OCU)	Mediterranean	17.430	43.070	0.8
Crniševo (CRN)	Mediterranean	17.426	43.075	0.8

2.2. Environmental characterization

Water temperature, conductivity, salinity, pH, total dissolved solids and dissolved oxygen were measured *in situ* with a Hach HQ40D Portable Multi Meter for Water (Hach Lange GmbH, Berlin, Germany). Samples for chemical analyses were taken simultaneously with samples for molecular analyses. Oxygen and total suspended particles were analyzed following the APHA [29]. Total nitrogen and total organic carbon were analyzed using a Shimadzu TOC-VCPH (Shimadzu, Germany).

2.3. DNA extraction and sequencing

Total genomic DNA was extracted using the DNeasy PowerWater kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The hypervariable V4 region of the prokaryotic 16S rRNA gene was amplified by PCR using primer pair 515F [30] and 806R [31]. All samples were amplified, barcoded, purified and prepared for sequencing on an Illumina MiSeq platform (v3 chemistry, 2 × 300 bp) at the Joint Microbiome Facility of the Medical University of Vienna and the University of Vienna, as described in [32, 33], under the JMF ID JMF-2206-01.

Individual amplicon pools were extracted from the raw sequencing data using the FASTQ workflow in BaseSpace (Illumina) with default parameters, allowing one mismatch for the 6-nucleotide (nt) library indexes. The input data were filtered for PhiX contamination with BBDuk (BBTools) [34]. Demultiplexing of each amplicon pool library into single amplicon libraries was performed using the demultiplex Python package [33] allowing one mismatch for barcodes and two mismatches for linkers and primers. Amplicon Sequence Variants (ASVs) were inferred using the DADA2 R package version 1.42 following the recommended workflow [35]. Primer sequences were removed prior to DADA2 using cutadapt (adapter/primer FASTA; minimum overlap 15 bp), and reads were truncated to 220 bp (forward) and 150 bp (reverse). Quality filtering was performed with filterAndTrim using trimLeft = c(0,0), maxEE = c(2,2), truncQ = 2 and minLen = 50. Filtered reads were dereplicated (derepFastq), error models were learned (learnErrors, self-consistency), ASVs were inferred (dada, pooled inference), paired reads were merged (mergePairs, minOverlap = 12, maxMismatch = 0), and chimeras were removed (removeBimeraDenovo, method = 'consensus'). FASTQ reads 1 and 2 were trimmed at 220 nt and 150 nt with allowed expected errors of 2. Taxonomy was assigned via the SILVA database (Ref NR 99 release 138.1) using the SINA version 1.7.2 classifier [36]. Prior to statistical analysis, ASVs classified as eukaryotes, mitochondria or chloroplasts, unassigned ASVs at the phylum level and singletons were removed.

2.4. Data analysis

All statistical analyses were conducted in R (v.4.2.2, R Core Team, 2022) using functions from the phyloseq [37], vegan [38], dplyr [39], UpSetR [40] and tidyr [41] packages. Environmental beta diversity was evaluated using Principal Component Analysis (PCA) on Z-score-standardized data. For estimation of alpha and beta diversity, rarefication was computed on the dataset to an equal sequencing depth (>1000 reads per sample) (Table S1). Alpha diversity was assessed using observed ASV richness, the Chao1 richness estimator, and the Shannon diversity index. Beta diversity of microbial communities was assessed using non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarities, with ordination goodness-of-fit evaluated using stress values. Additionally, to explore site-specific significant variations, a permutational multivariate analysis of variance (PERMANOVA) was carried out separately for every lake, evaluating the influence of sampling date, month and season on microbial beta diversity. Hierarchical clustering based on Bray–Curtis dissimilarity was also performed to further examine sample grouping. Taxonomic relative abundances at the phylum level were summarized by removing unassigned taxa and grouping phyla with a median relative abundance below 1% across all samples into a “remainder” category. Shared and unique ASVs of prokaryotic communities were visualized using UpSet plots as an alternative to traditional Venn diagrams. All ASVs with a relative abundance greater than 10% were classified as dominant, while those with a relative abundance below 10% were classified as rare. The relationship between environmental parameters and microbial community composition was explored using the Mantel test based on Pearson’s correlations. To infer the functional potential of prokaryotic communities, taxonomic assignments derived from 16S rRNA gene data were annotated using the FAPROTAX database, enabling indirect inference of potential metabolic processes associated with carbon, nitrogen, and sulfur cycling, as well as nutritional modes.

3. RESULTS

3.1. Environmental parameters of lakes

Water temperature showed pronounced seasonal variation across all lakes, with minimum values in Winter and peaks in Summer. Continental lakes KOZ and PRO exhibited lower temperatures (4.1 –17.0 °C) than the Mediterranean lakes VRANA, VIS, OCU, and CRN (8.7 - 27.7 °C; Fig. 2a). Salinity remained low and stable in all lakes (0.1 – 0.3 ‰), except in CRN, which showed distinctly elevated and variable values (0.5 - 1.2 ‰) throughout the year (Fig. 2b). DOC concentrations varied between 0.4 and 1.6 mg C L^{-1}, with higher values observed in Spring and Autumn in CRN and during Summer in VRANA and VIS (Fig. 2c). Sulfate concentrations were near or below detection in KOZ and PRO (< 5 mg L^{-1}) and generally low in VRANA (≤ 10 mg L^{-1}), whereas VIS and OCU showed intermediate, seasonally variable levels (≈ 20 – 60 mg L^{-1}), with CRN exhibiting the highest and most variable concentrations (typically 80 – 120 mg L^{-1}) throughout the year (Fig. 2d). Total nitrogen (TN) ranged from 0.1 to 0.8 mg N L^{-1}, with higher concentrations recorded during Summer and Autumn, and consistently elevated values in the continental lakes KOZ and PRO (Fig. 2e). Dissolved oxygen (DO) fluctuated between 3.0 and 15.5 mg O_2 L^{-1}, peaking in Spring in PRO and CRN, and remaining elevated during Summer in both continental lakes (Fig. 2f).

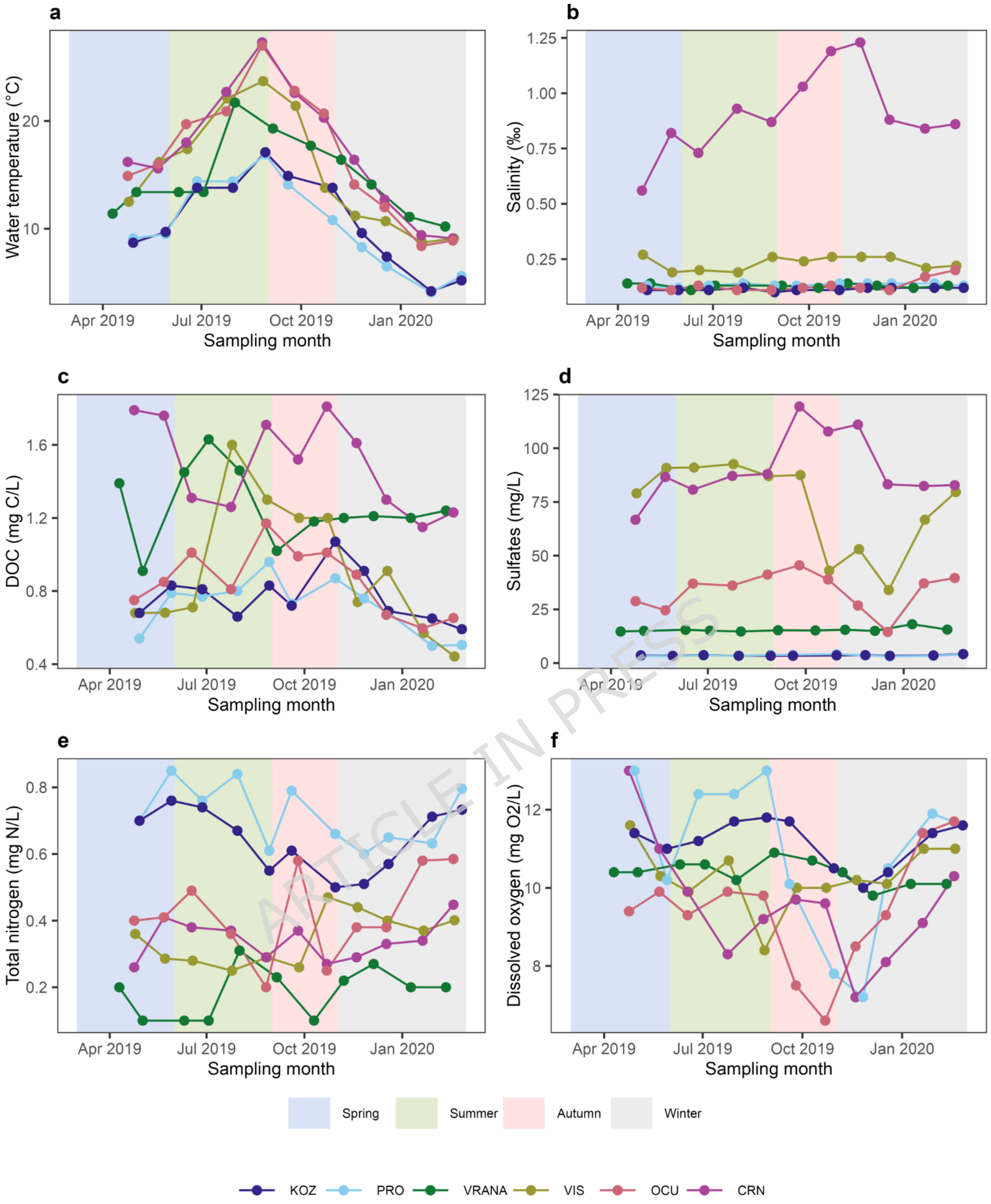

Fig. 2 Seasonal variation in environmental parameters across six deep karstic lakes. Temporal changes in (a) water temperature, (b) salinity, (c) dissolved organic carbon (DOC), (d) sulfates, (e) total nitrogen, and (f) dissolved oxygen were recorded from Spring 2019 to Winter 2020. Colored lines represent individual lakes (KOZ, PRO, VRANA, VIS, OCU, CRN) and shaded backgrounds indicate sampling seasons (Spring, Summer, Autumn, Winter)

PCA of standardized environmental parameters showed clear grouping of samples by lake and sampling season (Fig. 3a). Samples from CRN were clearly separated from all other lakes and formed a distinct cluster. KOZ and PRO clustered closely together, especially during Summer and Winter. VIS samples grouped separately along PC1, while VRANA and OCU showed partial overlap with other Mediterranean lakes. Summer samples from the Mediterranean lakes (VRANA, VIS, OCU, and CRN) were distinct from other seasons. PERMANOVA analysis showed that all tested environmental parameters significantly influenced microbial community composition across the studied lakes ($p < 0.001$ for all variables). Dissolved organic carbon (DOC) explained the highest proportion of variation ($R^2 = 13.8\%$), followed by total nitrogen ($R^2 = 10.7\%$), salinity ($R^2 = 8.6\%$), and temperature ($R^2 = 8.5\%$). Sulfates ($R^2 = 7.1\%$) and dissolved oxygen ($R^2 = 6.9\%$) also had significant, though weaker, effects.

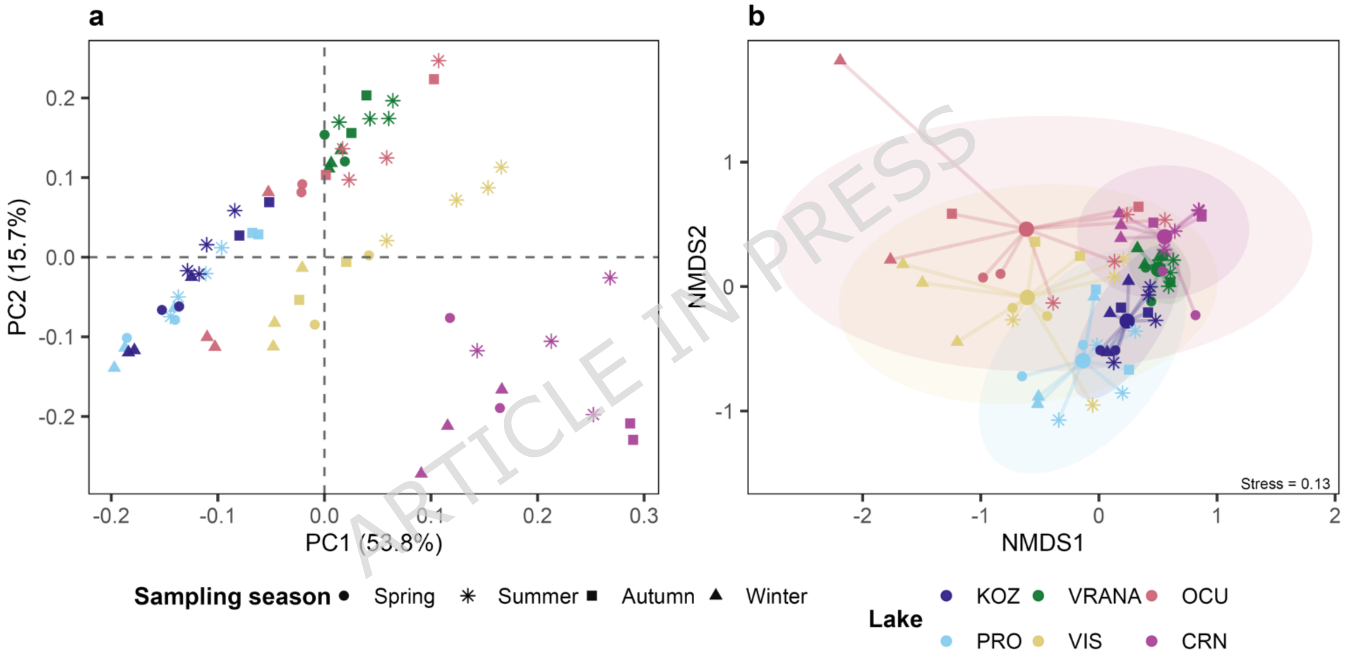

Fig. 3 PCA of environmental parameters (a) and NMDS ordination of microbial community composition (b) across six karstic lakes and sampling seasons

3.2. Seasonal microbial community composition

Alpha diversity metrics, including observed ASV richness, the Chao1 richness estimator, and the Shannon index, were used to assess microbial richness and overall diversity across the six lakes (Fig. S1). VIS and OCU exhibited the highest richness based on the Chao1 estimator, whereas PRO, KOZ, and VRANA showed intermediate values, and CRN displayed lower richness. Similarly, VIS and OCU showed the highest Shannon diversity, continental lakes exhibited intermediate diversity, whereas VRANA and CRN showed lower diversity.

NMDS ordination based on Bray-Curtis dissimilarities indicated partial grouping of samples by lake, with some overlap among lakes and seasons (Fig. 3b). Continental lakes (KOZ and PRO) tended to form more compact clusters, whereas Mediterranean lakes (VRANA, VIS, OCU, and CRN) were more dispersed, with OCU and VIS differing most from the other lakes. Seasonal patterns were evident, with Autumn and Winter samples showing clear separation, particularly within VIS and OCU. These visual patterns were supported by PERMANOVA, which revealed significant differences in microbial community composition among lakes ($R^2 = 0.36$, $p = 0.001$) and across sampling seasons ($R^2 = 0.10$, $p = 0.001$).

Samples from the same lake showed clustering at low dissimilarity levels based on Bray-Curtis dissimilarity (Fig S2). Within individual lakes, seasonal samples generally grouped together, whereas Winter samples from lake OCU merged with other samples at higher dissimilarity levels compared to OCU samples from other seasons (Fig. S2).

Fig. 4 Heatmap showing the relative abundance of dominant bacterial genera across six karstic lakes (KOZ, PRO, VRANA, VIS, OCU and CRN) and sampling seasons

Overall community composition at the phylum level showed a consistent dominance of *Proteobacteria* (PRO and OCU in Winter 18.3%, KOZ in Spring 16.0%, and VIS in Winter 10.0%) and *Actinobacteriota* (PRO in Summer 18.3%, KOZ in Autumn and OCU in Summer 16.3%, VRANA in Spring 22.9%, VIS in Spring 25.1%, and CRN in Winter 17.2%). (Fig. S3). *Bacteroidota* were also highly abundant, particularly in the Mediterranean lakes, with peak relative abundances observed in VIS during Winter (21.1%) and in OCU during Autumn and Winter (16.0%) (Fig. S2). *Cyanobacteria* abundance peaked in VRANA and CRN in Autumn (22.7%), VIS in Spring (16.4%) and OCU in Summer (27.5%). A distinct increase in *Firmicutes* was observed in Winter in OCU (Fig. S3). *Planctomycetota* and *Verrucomicrobiota* contributed to the remaining variability among lakes.

Seasonal variation in dominant bacterial genera was observed across all lakes (Fig. 4). The genus *CL500-29 marine group* showed clear seasonal fluctuations across all lakes, with the highest relative abundance observed in Winter in Lake PRO, reaching approximately 9.1% relative abundance. The genus *hgcl clade* was consistently dominant across all lakes and seasons, except in OCU, where its abundance decreased during Autumn and Winter. *Candidatus Planktophila* was more dominant in the continental lakes during Spring and Summer with maximum abundances recorded in Summer in KOZ (10.4%) and PRO (8.9%). In Mediterranean lakes, *Candidatus Planktophila* also showed increased abundance, mainly during spring in VRANA (4.9%) and VIS (7.9%). *Armatimonas* dominated in Spring in PRO (12.3%) and in Winter in VIS (21.3%). *Bacteroidia* represented primarily by *Flavobacterium* and *Pseudarcicella*, was dominant in the Mediterranean lakes VIS and OCU (10.9% in Autumn) and 21.2% and 15.9% in Winter. The genus *Cyanobium PCC-6307* exhibited high relative abundance across all seasons in KOZ, VRANA, and CRN, reaching up to approximately 20.0–36.0%, while in VIS and OCU elevated abundances were mainly observed during Spring and Summer, with maxima of approximately 14.0–27.0%. *Pirellula* was detected in PRO (6.7%), VIS (3.5%), and OCU (2.0%) during Summer. Among *Proteobacteria* (class *Alphaproteobacteria*), *Sphingorhabdus* appeared in Spring in PRO (5.2%) and in Winter in VIS (7.1%), *Roseomonas* in Summer in PRO (5.1%), OCU (2.3%), and VRANA (1.8%) and *Rhodobacter* in Winter in VIS (33.0%).

Within *Gammaproteobacteria*, *Limnohabitans* was present throughout the year in all lakes except CRN, with the highest abundance in Winter PRO (18.4%) and VIS (10.0%)

while *Polynucleobacter* dominated in the continental lakes during Spring in KOZ (2.2 - 6.6%) and PRO (3.5 - 8.2%). *Luteolibacter* (class *Verrucomicrobiae*) was present year-round in the continental lakes, with peak abundances recorded in Autumn in PRO (19.0%) and in Spring in KOZ (15.5%). In the Mediterranean lakes, elevated abundances were observed in VRANA during Autumn (4.6–11.7%), in

VIS during Autumn (16.3%) and Summer (14.6%), in OCU (2.8–5.9%), and a pronounced Spring maximum was detected in CRN (20.7%).

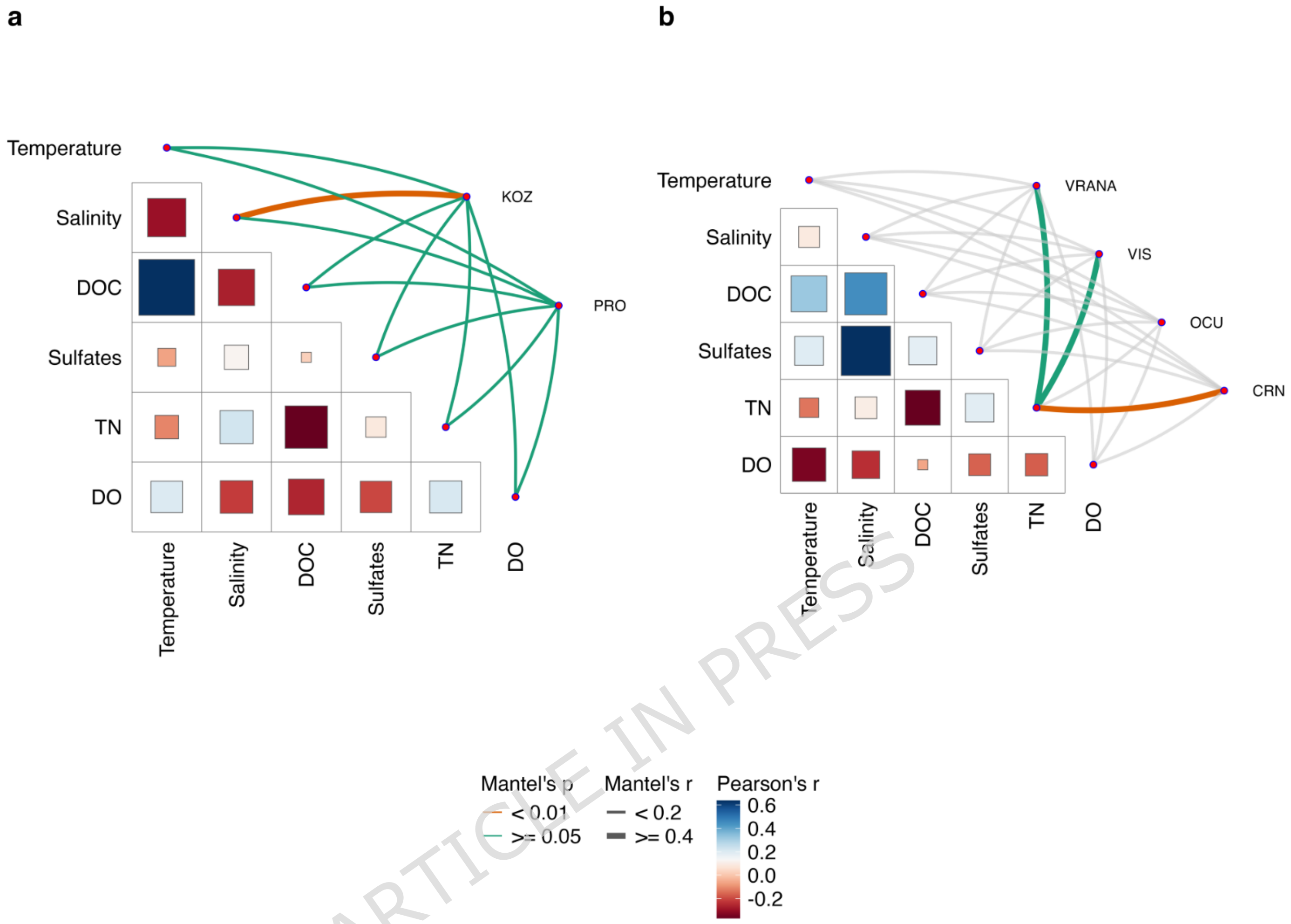

Fig. 5 Correlation network showing relationships between environmental parameters (temperature, salinity, DOC, sulfates, total nitrogen (TN), and dissolved oxygen (DO)) and microbial community composition across lakes. Panel (a) depicts continental lakes, and panel (b) displays Mediterranean lakes. Colors indicate Pearson's correlation coefficients (r), while edge thickness and color represent Mantel's r and significance (p)

Among continental lakes, KOZ showed stronger Mantel's *p* value (greater than 0.01) with salinity, while PRO showed Mantel's *p* values greater than or equal to 0.05 for all selected environmental parameters. Mantel's *r* for KOZ is higher than or equal to 0.4, whereas for PRO it is less than 0.2 (Fig. 5a). Pearson's *r* showed the strongest positive correlation between DOC and temperature, and strong negative correlations between salinity and temperature, DOC and salinity, TN and DOC, as well as DO and DOC. In Mediterranean lakes, CRN showed a Mantel's *p* value greater than 0.01 and an *r* value between 0.2 and 0.4. VRANA and VIS exhibited Mantel's *p* values between 0.01 and 0.05, and Mantel's *r* values between 0.2 and 0.4 (Fig. 5b). Pearson's *r* revealed strong positive correlations between sulfates and salinity, as well as between DOC and salinity, and strong negative correlations between total nitrogen and DOC, and between DO and temperature.

BioENV analysis identified DOC and TN as environmental parameters significantly associated with differences in microbial communities(Spearman's ρ = 0.31, *p* = 0.001).

3.3. Seasonal distribution of dominant and rare bacterial ASVs

In Spring (Fig. 6a), a total of 36 dominant ASVs were shared among all six lakes, representing the core microbial community for this season. A smaller number of ASVs (10–1) were shared among subsets of lakes. These 36 ASVs were taxonomically classified into the following phyla: *Proteobacteria* (12 ASVs), *Actinobacteriota* (11 ASVs), *Bacteroidota* (6 ASVs), *Planctomycetota* (2 ASVs), and *Verrucomicrobiota* (2 ASVs), with *Proteobacteria* dominated by the genera *Limnohabitans* (3 ASVs), the *GKS98 freshwater group* (2 ASVs), and *Polynucleobacter* (2 ASVs), and *Actinobacteriota* dominated by the genera *hgcI clade* (5 ASVs) and *Candidatus Planktophila* (2 ASVs), while the remaining ASVs were assigned to other phyla and genera. The total number of detected ASVs per lake was similar, with slightly higher values in VRANA and PRO. During Summer (Fig. 6b), the highest number of shared ASVs (41) was detected across all six lakes. These 41 ASVs were taxonomically classified into the following phyla: *Bacteroidota* (12 ASVs), *Actinobacteriota* (11 ASVs), *Proteobacteria* (10 ASVs), *Planctomycetota* (3 ASVs), *Verrucomicrobiota* (3 ASVs) and *Cyanobacteria* (2 ASVs) with *Bacteroidota* dominated by the genera *Flavobacterium* (3 ASVs), *Pseudarcicella* (2 ASVs) and *Sediminibacterium* (2 ASVs) and with *Actinobacteriota* dominated by the genera *hgcI clade* (5 ASVs) and *Candidatus Planktophila* (2 ASVs). Mediterranean lakes (VRANA, VIS, OCU, and CRN) exhibited strong overlap in ASV composition, while continental lakes (KOZ and PRO) shared fewer ASVs with the others. Several unique ASVs were also present in individual lakes. In Autumn (Fig. 6c), 28 ASVs were shared among all six lakes. These 28 ASVs were taxonomically classified into the following phyla: *Bacteroidota* (9 ASVs), *Actinobacteriota* (8 ASVs), *Proteobacteria* (8 ASVs), and *Planctomycetota* (2 ASVs) with *Bacteroidota* dominated by the genera *Flavobacterium* (2 ASVs) and *Pseudarcicella* (2 ASVs) and *Actinobacteriota* dominated by the genera *hgcI clade* (4 ASVs) and *Candidatus Planktophila* (2 ASVs). The Mediterranean lakes (VRANA, VIS, OCU, and CRN) displayed stronger connectivity, while the continental lakes (KOZ and PRO) contributed fewer shared ASVs. Several smaller intersections indicate ASVs shared among subsets of lakes, and a few unique ASVs appeared in individual systems. During Winter (Fig. 6d), 17 ASVs were shared among all six lakes and 16 ASVs were shared among all lakes except OCU, representing the lowest overall overlap compared to other seasons. These 17 ASVs were taxonomically classified into the following phyla: *Proteobacteria* (6 ASVs), *Actinobacteriota* (4 ASVs) and *Bacteroidota* (4 ASVs) with *Proteobacteria* dominated by the genera *Limnohabitans* (2 ASVs) and *Actinobacteriota* dominated by the genera *hgcI clade* (2 ASVs). Throughout the year, rare ASVs exhibited pronounced seasonal variation, with the highest overlap among all six lakes observed in Summer (87 shared ASVs) and the lowest in Spring (40 shared ASVs) (Fig. S3). The 87 Summer rare ASVs were taxonomically classified into the following class: *Alphaproteobacteria* (24 ASVs), *Cyanobacteriia* (15 ASVs), *Gammaproteobacteri*a and *Bacteroidia* (10 ASVs), *Actinobacteria* (9 ASVs), *Verrucomicrobiae* (6 ASVs), *Acidimicrobiia* and *Planctomycetes* (4 ASVs) with *Alphaproteobacteria* by the order *Rickettsiales* (9 ASVs), *Sphingomonadales* (4 ASVs), *Caulobacterales*, *Rhodobacterales* and *SAR11 clade* (3 ASVs). The 40 Spring rare ASVS were taxonomically classified into the following class: *Cyanobacteriia* (9ASVs), *Alphaproteobacteria* and *Gammaproteobacteria* (8 ASVs), *Actinobacteria* (4 ASVs), *Acidimicrobiia* and *Bacteroidia* (3 ASVs) (Fig. S3). A strong association was observed between the two Mediterranean lakes, OCU and VIS, which shared rare ASVs in all seasons, with the highest overlap observed in Autumn (118 ASVs) and Winter (162 ASVs) (Fig. S3). The 118 Autumn rare ASVs were primarily classified into the following orders: *Burkholderiales* (48 ASVs), *Rickettsiales* (43 ASVs), *Flavobacteriales* (32 ASVs), *Sphingobacteriales* (30 ASVs), *Chloroplast* (26 ASVs), *Woesearchaeales* (17 ASVs), and *Sphingomonadales* (15 ASVs). Similarly, the 162 Winter rare ASVs were mainly assigned to *Burkholderiales* (60 ASVs), *Chloroplast* (51 ASVs), *Rickettsiales* (49 ASVs), *Flavobacteriales* (38 ASVs), *Woesearchaeales* (31 ASVs), *Sphingobacteriales* (26 ASVs), *Sphingomonadales* (22 ASVs), and *Pseudomonadales* (20 ASVs). (Fig. S3). In contrast, the continental lakes PRO and KOZ shared fewer rare ASVs, with the highest overlap observed in Summer (37 ASVs), which were primarily classified into the following orders: *Rickettsiales* (12 ASVs), *Chloroplast* (5 ASVs), and *Burkholderiales* and *Flavobacteriales* (3 ASVs) (Fig. S3).

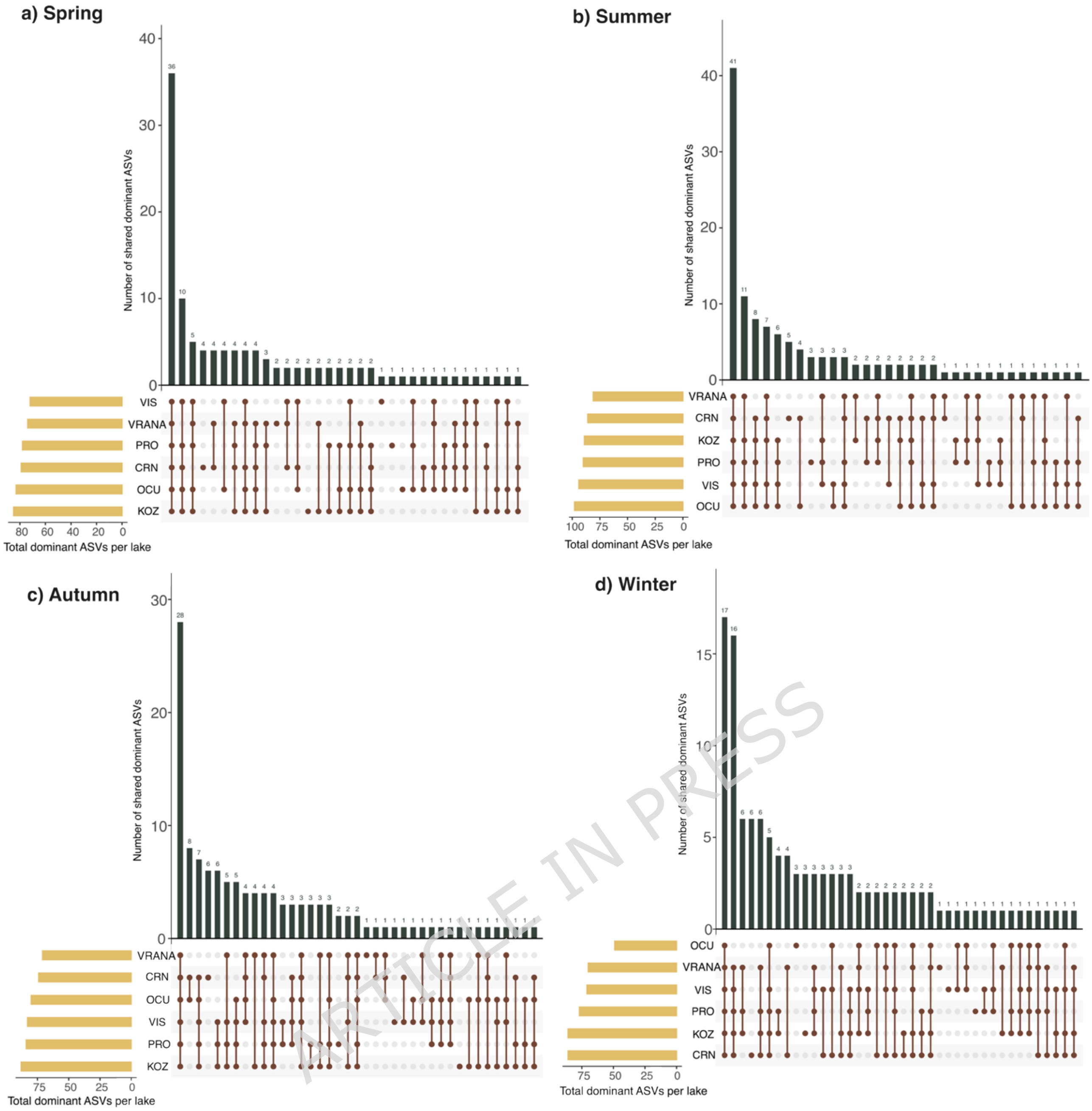

Fig. 6 UpSet plots showing the seasonal distribution of total and shared dominant ASVs among six karstic lakes for (a) Spring, (b) Summer, (c) Autumn, and (d) Winter

3.4 Seasonal and regional patterns in inferred functional potential

FAPROTAX-based functional predictions were performed on the 16S rRNA gene data to examine the *inferred functional potential* of dominant bacterial communities across seasons and regions (Fig. 7). Across all lakes, the most dominant predicted functions were related to energy acquisition, particularly aerobic chemoheterotrophy and photoautotrophy, reflecting the prevalence of heterotrophic and light-utilizing bacteria in both regions. However, distinct regional and seasonal patterns were observed. In the continental lakes (KOZ and PRO), functional profiles were more consistent across time, showing less pronounced seasonal fluctuation. These lakes were primarily characterized by stable contributions from sulfur-cycle functions (*e.g.*, sulfate respiration, respiration of sulfur compounds) and a narrower spectrum of carbon and nitrogen cycle-associated functions. By contrast, the Mediterranean lakes (VRANA, VIS, OCU, and CRN) exhibited greater functional heterogeneity across seasons. Energy-related functions remained dominant, but the C-, N-, and S-cycle functions were more variable and sporadically enriched, especially in VIS and OCU.

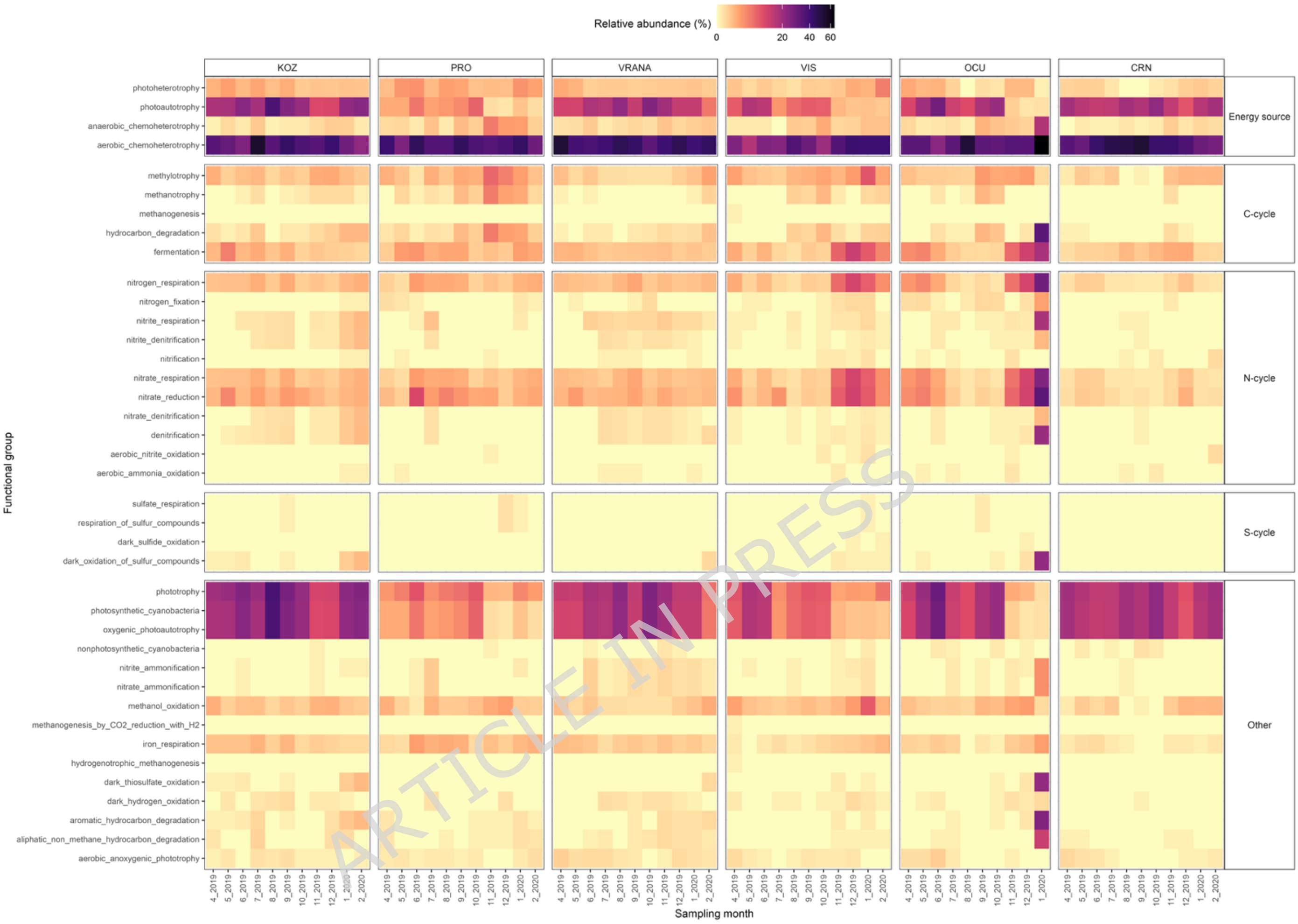

Fig. 7 Predicted functional potential of microbial communities in six karstic lakes. Heatmap showing the relative abundance of *inferred microbial functional categories* related to energy metabolism, and carbon (C), nitrogen (N), sulfur (S), and other metabolic pathways across samples from lakes KOZ, PRO, VRANA, VIS, OCU, and CRN

4. DISCUSSION

Our study provides comprehensive insights into the seasonal dynamics and environmental drivers of bacterial communities in six freshwater lakes situated in two climatically distinct regions of Croatia. By examining both continental and Mediterranean lakes which differ in temperature regimes, nutrient inputs, salinity and hydrological characteristics we were able to evaluate how seasonal variability shapes microbial community structure and patterns of inferred functional potential.

4.1. Environmental influences on microbial community structure

The Mantel test indicated that dissolved organic carbon (DOC) was the most important environmental factor associated with microbial community structure, while salinity, sulfate, and temperature showed somewhat weaker but still significant effects. These results suggest that the availability of dissolved organic carbon may play a key role in shaping the composition and dynamics of microbial communities in lake ecosystems. Consistently, bioENV analysis identified the combination of DOC and total nitrogen (TN) as the set of variables most strongly associated with Bray-Curtis community dissimilarities (Spearman's $\rho = 0.31$, $p = 0.001$). This relationship highlights the importance of nutrients and organic matter as major drivers of microbial differentiation among samples. DOC is often considered one of the primary energy sources for heterotrophic microorganisms, which may explain its strong influence on microbial community structure under varying seasonal and spatial conditions, in agreement with previous studies [42]. In continental lakes, the strong positive correlation between DOC and temperature suggests that warmer conditions are associated with shifts in bacterial community composition along DOC gradients and may influence patterns of inferred functional potential. Such an effect is consistent with previous research showing that at colder temperatures, bacteria begin to lose substrate affinity, leading to carbon limitation despite available sources in the environment, possibly due to stiffening lipid membranes [43]. DOC concentrations were higher during Spring and Summer, but some previous studies have shown that DOC concentrations in lakes can be affected by temperature over long time scales, while DOC dynamics are driven by the interaction of multiple climatic and catchment factors, not just temperature [44]. Moreover, the importance of DOC in this context aligns with earlier studies emphasizing its role in regulating both biotic and abiotic aquatic processes [45]. In Mediterranean lakes, salinity and sulfate were positively correlated, reflecting the accumulation of sulfate as a major inorganic sulfur component under increasingly saline conditions. This relationship is ecologically relevant, as elevated sulfate concentrations can promote sulfur-based microbial metabolisms and influence biogeochemical cycling in hypersaline environment [46]. In our study, lake CRN, which exhibited the highest salinity among the sampled lakes, also consistently showed the highest sulfate levels. This pattern supports the idea that ongoing salinization may enhance sulfur availability, potentially shaping microbial community composition and functional processes through shifts toward sulfur-adapted taxa.

4.2. Inferred functional potential of microbial communities

Under natural conditions, microbial communities are typically composed of a few dominant taxa and a large number of rare ones [47]. While dominant taxa are known to drive key ecological functions such as carbon metabolism and nutrient cycling due to their numerical dominance, rare taxa are thought to contribute to multifunctionality and community adaptability [47]. In our study, rare taxa, although representing a small fraction of the microbial community in terms of relative abundance, displayed clear seasonal structuring across all lakes (Fig. S4). The greater overlap of both rare and dominant ASVs during Summer may reflect increased connectivity among lakes or more homogeneous environmental conditions during this period. In contrast, the reduced overlap of rare taxa in Spring and of dominant taxa in Winter indicates that transitional and colder periods may enhance community differentiation [48]. Notably, Mediterranean lakes, shared a substantial number of rare ASVs across seasons, with the highest overlap observed in Autumn and Winter. This pattern may indicate stronger regional connectivity [49] or more similar environmental filtering within Mediterranean systems. Conversely, continental lakes showed limited sharing of rare ASVs and weaker seasonal overlap, suggesting stronger local environmental constraints [49].

Consistent with previous studies, we detected typical freshwater bacterial phyla such as *Bacteroidota*, *Proteobacteria*, *Actinobacteriota*, and *Cyanobacteria* (Fig. 4., [50]). The genera *hgcI clade*, *Cyanobium PCC-6307*, and *Luteolibacter* were consistently present and highly dominant across all seasons and lakes, regardless of climatic region. These taxa are associated with key ecological functions. For example, the *hgcI clade* contributes to nitrate and phosphate reduction [51], supporting nutrient cycling in freshwater ecosystems. *Cyanobium PCC-6307* was the most dominant genus overall, with significantly higher relative abundance in low-salinity environments [52]. FAPROTAX-based functional annotation indicated that this genus was predominantly associated with inferred functional potential related to phototrophy, oxygenic photoautotrophy, and cyanobacterial metabolic pathways. Although several genera of *Cyanobacteria* (*Geitlerinema*, *Leptolyngbya, Oscillatoria Planktothrix* etc.) discovered in freshwater systems are known or suspected to produce cyanotoxins, indicating a potential health risk to aquatic organisms, *Cyanobium PCC-6307* is not among the genera commonly associated with cyanotoxin [53]. *Luteolibacter* was more dominant across seasons in continental lakes, and its presence is considered an indicator of good quality lakes [54].

The inferred functional potential of microbial communities was particularly heterogeneous in the Mediterranean lakes (VRANA, VIS, OCU and CRN), which exhibited greater seasonal shifts in predicted metabolic profiles. While energy-related pathways such as aerobic chemoheterotrophy and photoautotrophy were consistently dominant, pathways associated with carbon, nitrogen, and sulfur cycling showed greater temporal variability and sporadic enrichment, particularly in lakes VIS and OCU. In the Mediterranean lakes, *Flavobacterium* (phylum *Bacteroidota*) was particularly dominant during Autumn and Winter, consistent with its description as psychrophilic or psychrotolerant and its activity under colder environmental conditions [55]. As observed in earlier studies, *Flavobacterium* was associated with elevated dissolved oxygen (DO) levels [56], as shown by its dominance in OCU during Winter, where DO reached seasonal maxima (Fig. 2.). Pearson correlation analysis further supported this pattern, revealing a strong negative correlation between DO and temperature (Fig. 5). FAPROTAX-based annotation suggested that fermentation-related functional potential was prominent in the Winter microbial community of OCU, with *Firmicutes* representing the primary contributors, followed by *Bacteroidota*, which also exhibited high relative abundance during this period (Fig. S2). These findings are consistent with studies showing that saline lake microbial communities support anaerobic metabolism [57]. In addition to fermentation, the inferred functional profiles of OCU during Winter indicated enrichment in pathways associated with methanol oxidation and hydrocarbon degradation. These patterns suggest a shift toward the utilization of simple organic substrates under colder conditions, when reduced inputs of phytoplankton-derived organic matter create environments that favor methanotrophs over other bacterial groups [58]. Elevated salinity, total nitrogen, and dissolved oxygen levels during this period may have contributed to shaping these predicted functional signatures.

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Author Contributions

I.V. and A.Č. conceptualized and designed the study and developed the methodology. I.V. analyzed the data. I.V. and A.R.S. prepared data visualizations. I.V. wrote the original draft of the manuscript. A.Č., A.R.S., A.J.P., and S.O. contributed to manuscript revision and editing and provided critical feedback. S.O. acquired funding, supervised the work, and revised the manuscript. All authors reviewed and approved the final version of the manuscript.

Data Availability

The 16S rRNA gene amplicon sequencing data generated in this study have been deposited in the Sequence Read Archive (SRA) under the BioProject accession number PRJNA1375786.

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