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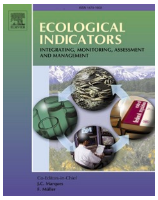

Original Articles

Environmental drivers of microbial assembly and stability in lakes across biogeographical regions

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ABSTRACT

Freshwater lake ecosystems play a crucial role in the global carbon cycle, acting as both carbon sinks and sources while providing essential ecosystem services. Within these ecosystems, planktonic microorganisms drive nutrient cycling and energy transfer in aquatic food webs. Due to their rapid response to environmental fluctuations, microbial communities serve as indicators of ecosystem change. In stratified lakes, thermal layering restricts energy and nutrient exchange, creating distinct microbial niches across depths. Microbial communities can be classified into free-living (FL) and particle-associated (PA) bacteria, which exhibit divergent metabolic strategies and responses to environmental change. Ecological theory suggests that PA bacteria are more influenced by deterministic factors (e.g., nutrient availability, oxygen gradients), whereas FL bacteria experience greater stochasticity (e.g., ecological drift). However, the stability and assembly mechanisms of these microbial fractions in dynamic lake environments remain poorly understood. In this study, we analyzed FL and PA bacterial communities in four karstic lakes in Croatia over two years, capturing both stratified and mixed conditions. Our results revealed that stratification and mixing events drove distinct microbial distribution patterns, with environmental gradients shaping niche partitioning between FL and PA bacteria. Contrary to the ecological theory, deterministic processes dominated community assembly in all lakes and fractions, though stochastic processes played a role, particularly in PA communities. Lakes with more stable conditions, such as Plitvice Lakes, exhibited resilient microbial networks, while more dynamic environments, like Baćina Lakes, supported less stable communities. In Lake Crniševo, seasonal salinity fluctuations created strong selection pressures, contributing to community divergence over time. These findings underscore the importance of environmental drivers in shaping microbial assembly and stability, highlighting the distinct ecological roles of FL and PA bacteria. Understanding these dynamics is crucial for predicting microbial responses to environmental change and enhancing biomonitoring strategies for freshwater ecosystem management.

1. Introduction

Karst freshwater lake ecosystems play a critical role in global carbon cycling, acting as both carbon sinks and sources (Cole et al., 2007; Zhang et al., 2022a). They also provide essential ecosystem services that sustain biodiversity and benefit human society (Diao et al., 2017). Within these ecosystems, planktonic microorganisms are fundamental to aquatic food webs, contributing to energy capture and nutrient cycling (Tranvik et al., 2009). Their rapid growth and adaptability make them ideal models for studying environmental fluctuations at fine spatial and temporal scales (Logares et al., 2013; Cram et al., 2015).

One of the key environmental drivers of microbial community dynamics is seasonal stratification, particularly in deeper lakes where temperature, oxygen, and nutrient gradients create distinct ecological niches (Winder and Sommer, 2012; Liu et al., 2024). During summer, many temperate lakes develop a thermally stratified water column, with a warm, oxygenated surface layer and a cooler, often hypoxic deeper layer (Diao et al., 2017). Global climate change is expected to prolong stratification periods, altering microbial diversity, abundance, and function (Huisman et al., 2004; North et al., 2014; Visser et al., 2016; Selak et al., 2022; Kajan et al., 2023). These stratification-induced shifts limit vertical mixing and nutrient exchange, influencing microbial

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composition at different depths (Yue et al., 2023). Given that seasonal anoxia in lakes is increasing due to rising temperatures (Ladwig et al., 2021; Jane et al., 2022; Zhang et al., 2024), understanding microbial responses to stratification and mixing events is critical. Despite evidence that stratification significantly alters microbial community structures (Yang et al., 2015; Yue et al., 2021), there remains a knowledge gap on how microbial communities transition between stratified and mixed conditions.

Microbial communities are typically categorized as free-living (FL) or particle-associated (PA) based on their relationship with particulate organic matter (Grossart, 2010). PA bacteria, which colonize particles, play a crucial role in the decomposition and remineralization of particulate organic carbon (POC) (Lapoussière et al., 2011; Baumas and Bizic, 2024). In contrast, FL bacteria remain suspended in the water column, relying primarily on dissolved organic carbon (DOC) as an energy source (Mestre et al., 2017). These two microbial fractions exhibit distinct metabolic trade-offs, community compositions, and assembly processes, reflecting their adaptation to environmental changes (Crespo et al., 2013; Polz and Cordero, 2016; Zhao et al., 2017; Sun et al., 2023). However, comparative studies on the stability and assembly of FL and PA bacterial communities under environmental fluctuations remain scarce.

Microbial community assembly is governed by deterministic (niche-based) and stochastic (neutral-based) processes (Stegen et al., 2013; Kajan et al., 2023). The niche-based model suggests that environmental selection and species interactions are the primary drivers of community composition (Zhang et al., 2021), whereas the neutral model attributes bacterial community shifts to random processes such as ecological drift (Chase and Myers, 2011). Studies suggest that PA bacteria are more strongly shaped by deterministic factors, while FL bacteria are more influenced by stochasticity (Yuan et al., 2021a; Liu et al., 2022a; Liu et al., 2022b). Seasonal fluctuations in microbial diversity have been linked to distinct environmental drivers, including temperature, salinity, and nutrient availability (Allgaier and Grossart, 2006; Zhang et al., 2021; Yu et al., 2023). However, the relative contributions of deterministic vs. stochastic processes in structuring microbial communities across different environmental compartments (FL vs. PA) remain underexplored.

This study investigates the spatiotemporal dynamics, community assembly mechanisms, and stability of FL and PA bacterial communities in four karstic deep-water lakes over a two-year period, capturing both stratified and mixed conditions. Specifically, we aim to: (1) Examine geographic distribution patterns of microbial communities across seasons and lakes, (2) identify the dominant assembly mechanisms shaping microbial community composition and (3) explore microbial co-occurrence patterns and functional diversity in response to environmental fluctuations. By addressing these objectives, our study provides critical insights into microbial ecosystem function in karst lakes, with implications for biogeochemical cycling, microbial resilience, and freshwater resource management.

2. Materials and methods

2.1. Study area

The seasonal dynamics of microbial communities were studied in four lakes across two distinct climatic regions in Croatia: the Plitvice Lakes in the Alpine biogeographical region and the Baćina Lakes in the Mediterranean biogeographical region (Fig. 1). Both systems comprise several interconnected lakes, with a sampling campaign focused on the largest ones. Specifically, samples were collected from Lake Kozjak (KOZ) and Lake Prošće (PRO) in the Plitvice Lakes system, and Lake

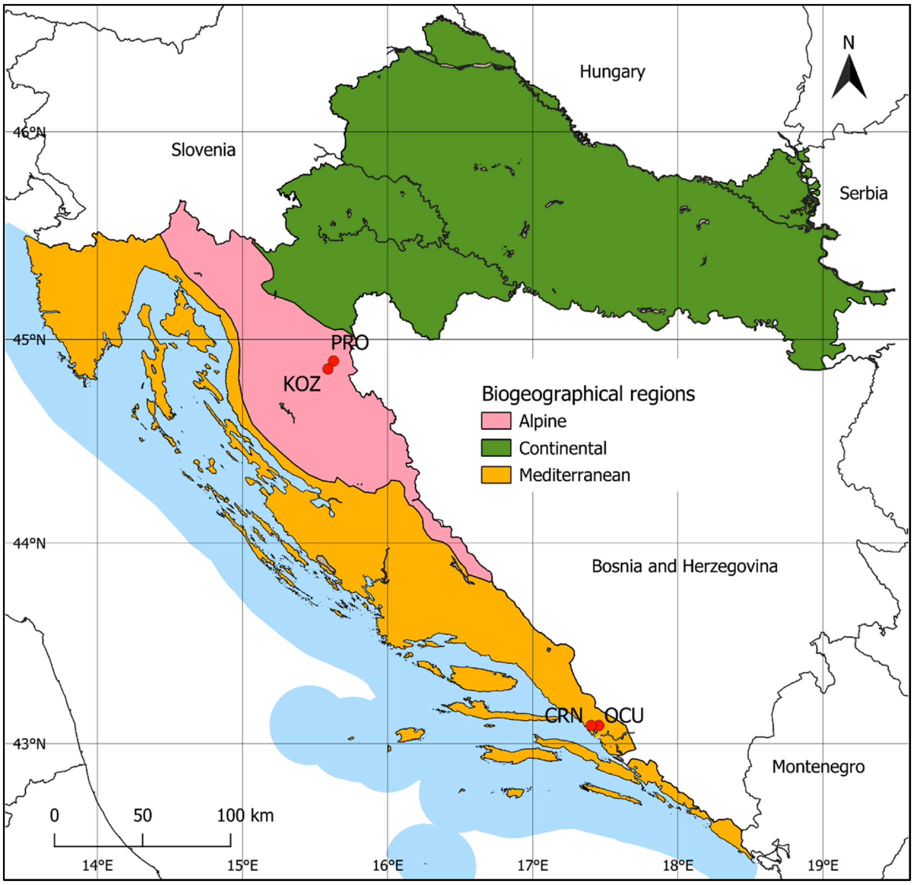

Fig. 1. Study area with locations of the sampled lakes in the Alpine and the Mediterranean biogeographical region.

Crniševo (CRN) and Lake Oćuša (OCU) in the Baćina Lakes system (Fig. S1).

According to the Köppen climate classification, Plitvice Lakes are located in a temperate continental climate with warm summers (Cfb). This classification is characterized by an average temperature below 0 °C in the coldest month, an average temperature below 22 °C in the warmest month and evenly distributed precipitation throughout the year (Šegota and Filipčić, 2003). Plitvice Lakes are oligotrophic and dimictic and are characterized by a low content of nutrients and dissolved organic matter (DOM) (Kajan et al., 2023) and high content of dissolved oxygen (Miliša and Ivković, 2023). In contrast, the Baćina lakes are located in a hot-summer Mediterranean climate (Csa), where the coldest month has an average temperature above 0 °C, the warmest month is above 22 °C and the driest month occurs in the warm season with less than 40 mm of precipitation (Šegota and Filipčić, 2003; Miko and Ilijanić, 2015) These lakes are mesotrophic and monomictic and exhibit summer stratification and winter mixing (Miko and Ilijanić, 2015; Hanžek et al., 2021). Additionally, the Baćina Lakes system is a cryptodepression, allowing seawater inflow due to sea level rise and the semipermeable bedrock connecting the aquifer to the Adriatic Sea (Miko and Ilijanić, 2015). Canalized limestone porosity and lower precipitation during summer months result in seasonal lower groundwater levels that enable higher density seawater intrusion deep within the aquifer (Alfarrah and Walraevens, 2018; Srzić et al., 2020). Salinization mainly affects Lake Crniševo, where seawater enters through a bottom sinkhole and the underwater salty spring Mindel (Miko and Ilijanić, 2015). Although connected to Lake Crniševo by a narrow channel, Lake Oćuša exhibits significantly lower salinity, indicating infrequent mixing of the waters between the two lakes (Bonacci and Roje-Bonacci, 2020).

2.2. *Sampling campaign and environmental parameters*

Water samples from four lakes were collected monthly over a two-year period, from May 2021 to February 2023. Sampling depths were determined based on the presence of thermocline or oxycline. When a thermocline was present, samples were collected at a minimum of three depths (above, within, and below the thermocline), while a minimum of two depths were sampled when water column was not stratified (approximately 5 m below the surface and 5 m above the bottom). Overall, 141 samples were collected during 23 sampling campaigns: 46 from Lake Crniševo, 35 from Lake Oćuša, 30 from Lake Kozjak and 30 from Lake Prošće. Dissolved oxygen (DO), temperature, pH, fluorescent dissolved organic matter (fDOM), turbidity, conductivity and salinity were measured in situ using the EXO3 Multiparameter Sonde (YSI, Yellow Springs, OH, USA). Two litre water samples were collected at each depth using UWITEC water sampler (GmbH, Mondsee, Austria), transported in sterile Nalgene PC bottles and immediately filtrated sequentially through polycarbonate filters (Whatman Nuclepore Track-Etch membrane, 47 mm diameter) with 3 μm pore size for particle-attached (PA) and 0.2 μm pore size for free-living (FL) microbial communities using a peristaltic pump (Masterflex, Cole-Palmer, Vernon Hills, IL, USA), resulting in a total of 282 filters. The filters were stored at −80 °C until DNA extraction. The filtrate was used for ion chromatography ($Ca2+$, $Mg2+$, $K+$, $Na+$, $NH3+$, $SO42-$, $Cl-$, $NO3–$, $PO43-$) using Dionex ICS-6000 DC ion chromatographer (Thermo Fisher Scientific, Waltham, MA, USA) and dissolved organic carbon (DOC) analysis using QBD1200 analyzer (Hach Company, Loveland, CO, USA).

2.3. *DNA extraction and sequencing*

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 polymerase chain reaction (PCR) with the 515F (Parada et al., 2016) and 806R (Apprill et al., 2015) primer pair, barcoded and sequenced on the 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 Pjevac et al. (2021), under the JMF ID JMF-2112-10. Demultiplexing was performed using the demultiplex Python package (Laros, 2023), allowing one mismatch for barcodes and two mismatches for linkers and primers. Amplicon sequence variants (ASVs) were inferred using the DADA2 R package v1.42 following the recommended workflow (Callahan et al., 2016). 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 (Pruesse et al., 2012) with a confidence threshold of 0.5. To minimize potential bias, singletons were excluded from downstream analyses as well as ASVs classified as eukaryotes, mitochondria, chloroplast and unclassified ASVs. Samples with fewer than 2000 reads per sample were excluded from the analysis. Amplicon sequencing datasets presented in this article have been deposited in the NCBI repository under BioProject accession number PRJNA1167227.

2.4. *Data analyses*

All statistical analyses were conducted in R (v.4.2.2, R Core Team, 2022) using functions from the phyloseq (McMurdie and Holmes, 2013), vegan (Oksanen et al., 2018), linkET (Huang, 2021), mgcv (Wood, 2011), plspm (Sanchez et al., 2024), dendextend (Galili, 2015) and ggplot2 (Wickham, 2016) packages. To assess microbial community diversity, both alpha and beta diversity indices were calculated at the ASV level using a dataset rarefied to an equal sequencing depth (>5000 reads per sample). Alpha diversity metrics, including observed, Chao1, and Shannon indices, were computed and compared across groups using the Kruskal-Wallis test. Statistical comparisons between sample groups were performed with pairwise significance testing. Beta diversity was estimated using Bray-Curtis dissimilarity, and non-metric multidimensional scaling (NMDS) was used to visualize differences in microbial community composition among samples. Statistical significance between groups was assessed using permutational multivariate analysis of variance (PERMANOVA, 999 permutations). Additionally, to explore site-specific variations, statistical analyses (PERMANOVA) were conducted separately for each lake, evaluating the influence of fraction type, oxygen concentration, temperature, sampling month, and season on microbial beta diversity. Hierarchical clustering was performed to explore relationships between samples based on the similarity or dissimilarity in ASV abundances. Hellinger transformation was applied to normalize abundances and pairwise dissimilarities were calculated using the Bray-Curtis metric. Clustering was conducted using the complete linkage method and the results were visualized as a dendrogram, with annotations representing sampling points and fractions. The relationship between environmental variables and microbial community composition was assessed using the Mantel test based on Pearson's correlations. To evaluate the contribution of environmental parameters to the composition of free-living (FL) and particle-associated (PA) microbial communities, Hellinger-transformed OTU tables were compared with standardized environmental variables (Z-score transformation) and Mantel test results were visualized using network-style correlation plots.

2.5. *Community assembly and network analysis*

To quantify the relative contributions of deterministic and stochastic processes on community assembly in each lake, the biodiversity ecological null model was used as described by Stegen et al. (2013). The β-nearest taxon index (βNTI) and the β-mean nearest taxon index (βMNTD) were calculated using the standardized abundance ASV table and the amplicon phylogenetic tree. The βNTI was computed as the difference between the observed βMNTD and the null distribution. Deterministic processes (variable or homogeneous selection) are indicated when βNTI is > 2 or <-2, whereas values [2, −2] suggest the predominance of stochastic processes (homogenizing dispersal,

dispersal limitation). Additionally, Raup-Crick (RC) beta diversity was calculated based on the sequence abundance to distinguish stochastic processes. Assemblages were considered structured by dispersal limitation for RC values >0.95, by homogenizing dispersal for RC values < −0.95, or by random processes without a dominant factor for RC values [−0.95, 0.95].

A co-occurrence network was constructed using the integrated network analysis pipeline (Feng et al., 2022). Spearman's rank correlation was used to assess pairwise associations among taxa, as microbial community data were non-normally distributed. Pairwise correlations with coefficients $|R| > 0.6$ and a p-value < 0.01 were considered strong and significant connections. Network modules were identified using fast greedy modularity optimization and nodes were categorized based on within-module (Zi) and among-module connectivity (Pi) identifying keystone taxa. To ensure that the established ecological network is nonrandom, 100 interconnected random networks were generated and compared using z-test. The networks were then visualized using Gephi v 0.10.1. The network's topological properties, including average degree, clustering coefficient, average path length and modularity were calculated.

2.6. Community stability analysis and PLS-PM modeling

The compositional stability of FL and PA bacterial communities in the four investigated lakes was calculated using the ASV table (Yuan et al., 2021b). The compositional stability was calculated for every sampling month to evaluate the change in community structure over time. A higher stability index indicates less change in community structure among time points. To visually compare the trends in FL and PA bacterial community compositional stability a generalized additive model for fitting was used (Wood, 2011), particularly suitable for ecological data enabling the capture of subtle variations in community stability.

Partial least squares path modeling (PLS-PM) was constructed to determine the effects of biotic and abiotic factors on the compositional stability of FL and PA microbial communities in the investigated lakes. The abiotic factors were categorized into six latent variables (precipitation, physico-chemical, salinity, particles, nutrients, and DOM), and biotic factors were represented by the Shannon diversity index. First, the structural model, illustrating the relationships between latent variables, was constructed. In the initial model, precipitation was expressed as 7 days' accumulated precipitation before the sampling day; physico-chemical factors included water temperature, pH and dissolved oxygen concentration; nutrients included NO3; salinity included salinity, SO42- and Cl-; particles included turbidity and DOM included fDOM. The measurement model, demonstrating the reflective relationships between each latent variable and its associated indicators was bootstrapped to obtain information about the variability of the parameter estimates (Sanchez, 2013). All variables with loadings <0.7 were removed to ensure model reliability. Next, the path coefficients, representing the strength and direction of the relationships between variables and predictors, were calculated along with their significance for each path. Finally, model performance was evaluated using the goodness of fit measure (GoF).

3. Results

3.1. Dynamics of thermal stratification and mixing period in the investigated lakes

The measured environmental parameters displayed spatial variability and seasonality across all four lakes (Fig. S2). In all lakes, the mixing of the water column occurred in winter, starting in December and ending in April, when stratification begins. The thermocline in Lake Crniševo occurred at 7 – 13 m depth (Fig. S2 1A), while in Lake Oćuša it was as deep as 17 m (Fig. S2 2A). In both Plitvice Lakes, the thermocline occurred at the 8 – 14 m depth interval (Fig. S2 3A and 4A). Water temperature ranged from 7.6 °C (February 2022) to 26.9 °C (July 2021) in Baćina Lakes (Fig. S2 1A and 2A) and from 3.9 °C (February 2022) to 21.4 °C (July 2022) in Plitvice Lakes (Fig. S2 3A and 4A). DO concentrations reached a maximum of around 16 mg/L in both systems above the thermocline, with the lowest DO recorded as 3 mg/L in Plitvice Lakes (Fig. S2 3B and 4B), and functional anoxia observed only in both Baćina Lakes (> 0.5 mg/L) (Fig. S2 1B and 2B). The deepest lake areas recorded the lowest DO concentrations during summer and autumn. Winter months exhibited well-mixed conditions with uniform temperature and DO values from surface to bottom. The fDOM values ranged from 0.3 to 7.8 RFU in Baćina Lakes (Fig. S2 1C and 2C) and from 0.04 to 2.5 RFU in Plitvice Lakes (Fig. S2 3C and 4C). The lowest DO concentrations and highest fDOM values occurred in summer and autumn across all four lakes. Freshwater Plitvice Lakes and Lake Oćuša exhibited low average salinity values (< 0.5 psu). Salinity was mostly stable across the water column and sampling period and was about tenfold higher in Crniševo than in other lakes (1.5 psu and 0.15 psu, respectively). Rise in salinity was recorded only in Lake Crniševo in the sediment–water interface in October in both sampling years, with a measured maximum of 13 psu, indicating the occurrence of brackish water and the formation of seasonal freshwater and seawater mixing zone (Fig. S2 1D). A summary statistics of the measured physico-chemical parameters for each lake is shown in Supplementary Table 1.

3.2. Composition and diversity of microbial communities

Sequencing of 16S rRNA gene amplicons generated 5716 unique ASVs with sequencing depth ranging from 26 to 33,463 reads per sample. After quality filtering and rarefaction, 3945 ASVs were retained for downstream analyses. A total of 5 samples were excluded due to insufficient sequencing depth. ASVs were taxonomically affiliated with a total of 58 phyla. The most dominant phyla were Proteobacteria (20.37 %), Cyanobacteria (17.11 %), Actinobacteriota (12.85 %), Verrucomicrobiota (12.76 %) and Bacteroidota (10.46 %).

The five most relatively abundant phyla alternated in maximum abundance throughout the sampling period (Fig. 2). In particular, the alternating dominance of Cyanobacteria and Proteobacteria in the Baćina Lakes, and Proteobacteria and Verrucomicrobiota in Lake Kozjak was reported. In Lake Prošće, a codominance of Proteobacteria and Verrucomicrobiota was observed throughout the entire sampling period. In Lake Crniševo, the first stratified period in spring and summer 2021 was dominated by Bacteroidota below and Proteobacteria above the thermocline, with Cyanobacteria present only in the surface and middle depths in the summer months (Fig. 2A). During the first mixing period, all five phyla stayed equally distributed throughout the water column (Fig. 2A). In the second stratified period, in spring and summer 2022, Cyanobacteria reached peak abundance in the upper parts of Lake Crniševo, followed by a resurgence of Bacteroidota during the second mixing period (Fig. 2A). In Lake Oćuša, a similar pattern was observed, with Proteobacteria dominating the entire water column during the first stratified period in spring and summer 2021. During the subsequent mixing period, Cyanobacteria became more prevalent in the upper layers, and this trend persisted throughout the second stratified period in spring and summer 2022, leading to Cyanobacteria dominance across all sampled depths (Fig. 2B). During the second mixing period, a Bacteroidota peak at the bottom of the lake was observed (Fig. 2B). In contrast, the Plitvice Lakes showed a higher abundance of Verrucomicrobiota compared to Baćina Lakes (maximum abundance being 50 % and 25 %, respectively). Lake mixing led to a switch from Verrucomicrobiota to Proteobacteria dominance in Lake Kozjak (Fig. 2C). Verrucomicrobiota dominated the entire column in the first part of the first stratified period, followed by Actinobacteriota dominance in the upper part (Fig. 2C). Proteobacteria then dominated the upper parts of the lake in the latter part of the first stratified period and continued to dominate the whole water column after lake mixing (Fig. 2C). In the second

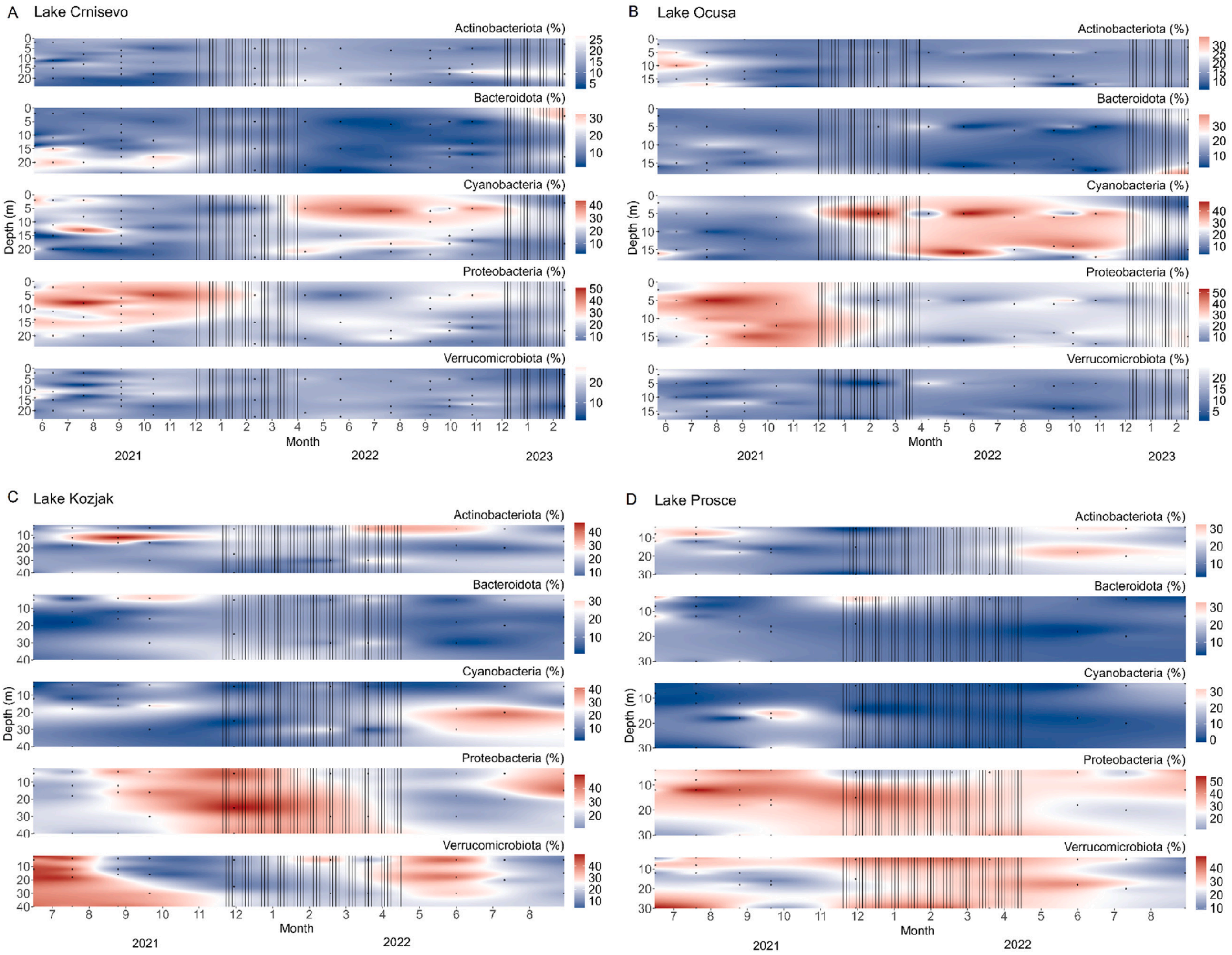

Fig. 2. Distribution of the five most abundant phyla throughout the sampling period in investigated lakes. (1) Crniševo; (2) Oćuša; (3) Kozjak; (4) Prošće. The lines represent mixing periods. Dots represent sampling points.

stratified period, there was alternating dominance between Verrucomicrobiota and Proteobacteria in the upper parts of the lake, and Cyanobacteria dominated the middle to bottom parts (Fig. 2C). In Lake Prošće, mixing had the least pronounced effect on microbial community composition. Proteobacteria and Verrucomicrobiota co-occurred at high relative abundances throughout the sampling period, with Proteobacteria prevailing during the stratified periods and Verrucomicrobiota during the mixed periods (Fig. 2D). Actinobacteriota were present in both stratified periods but at lower abundances compared to Lake Kozjak (maximum abundance being 30 % and 45 %, respectively) (Fig. 2D).

Hierarchical clustering of the samples showed that the samples could be divided into two clusters based on the lake system, within which further clustering based on the fraction was observed (Fig. S4). However, some exceptions were noted, with some samples from Baćina Lakes clustering with those from Plitvice Lakes. One possible explanation is that clustering was driven by shared core taxa, leading to an overlap in microbial community composition between the two lake systems. Higher microbial community richness (Chao1 and observed) and diversity (Shannon) was reported in Baćina Lakes compared to Plitvice Lakes (Fig. S5). Observed and estimated richness measures between FL and PA communities were similar, while Shannon diversity differed between the fractions (Fig. S5). Non-metric multidimensional scaling (NMDS) showed the grouping of microbial communities by lake ecosystem and fraction (Fig. S6), and PERMANOVA analysis showed that differences between lakes explained more variation in the data (14.37 %) compared to the differences between fractions (10.98 %). Regarding site-specific variations, PERMANOVA analysis showed that differences in sampling months explained the highest variation across all lakes (R^2 = 24–35 %). Fraction was a major driver in lakes Crniševo, Kozjak, and Prošće (R^2 = 17–23 %), but in Lake Oćusa, its influence was lower (R^2 = 9 %). Temperature had a similar effect across all lakes (R^2 = ~5%–9%), while oxygen concentrations were only relevant in Lake Crniševo (R^2 = 7.2 %).

3.3. The environmental drivers of microbial community variation between two fractions

Ion composition in the studied lakes exhibited variations based on depth and time, as well as differences between the lakes, highlighting seasonality and changes during stratification (Fig. S3). Chloride and sulfate concentrations, indicative of salinity, were notably higher in the Baćina Lakes compared to the Plitvice Lakes. Also, chloride concentrations in Lake Crniševo were thirtyfold higher than in Lake Oćuša, while sulfate concentration were similar in both lakes. Chloride concentration in Lake Crniševo increased with depth and was higher, particularly during the summer months, peaking in the autumn of 2021. The Baćina Lakes exhibited a twentyfold higher sulfate concentration, ranging from 13.5 mg/L to 141 mg/L compared to the Plitvice Lakes (from 2.5 mg/L to 6.3 mg/L). The highest nitrate concentration in all investigated lakes was approximately 2.5 mg/L, but in the majority of Baćina Lakes samples, nitrates were below the detection limit.

In Lake Crniševo, both PA and FL communities were positively correlated with calcium ion concentration, and the PA community also positively correlated with chloride (Fig. 3A). Regarding the environmental parameters, salinity showed a positive correlation with magnesium ion concentration, chloride, sulfate, and nitrate and a negative correlation with oxygen, while calcium ion concentration showed a negative correlation with temperature and chloride (Fig. 3A). In Lake Oćuša, fDOM negatively correlated with oxygen (Fig. 3B). Also, sulfate and nitrate positively correlated with the PA community, while chloride positively correlated with the FL community (Fig. 3B). In Lake Kozjak, sulfate and nitrate negatively correlated with calcium and magnesium ion concentration while both PA and FL communities correlated positively with calcium ion concentration, magnesium ion concentration, nitrate and sulfate (Fig. 3C). In Lake Prošće, the PA community correlated positively with calcium ion concentration, magnesium ion concentration and sulfate, while the FL community correlated positively only with sulfate (Fig. 3D). Regarding environmental parameters, calcium ion concentration correlated negatively with sulfate and temperature, and positively with magnesium ion concentration (Fig. 3D).

3.4. *Assembly patterns of microbial communities*

The βNTI values showed that deterministic processes dominated most of the microbial community assembly across lakes and fractions, as most of the values were below −2, indicating the dominance of homogenous selection (Fig. 4B and D). Only Lake Crnisevo had a mean βNTI value between 2 and −2, indicating the dominance of stochastic processes (Fig. 4B). In Baćina Lakes assembly patterns were dominated by homogenous selection and dispersal limitation, while Plitvice Lakes were under the influence of undominated processes and homogeneous selection (Fig. 4A). The variable selection had more influence on community assembly only in Lake Crniševo (Fig. 4A). Similar to the lakes, homogeneous selection dominated the microbial community assembly of both FL and PA fraction (Fig. 4C). However, dispersal limitation played a larger role in PA than in FL where undominated processes were more pronounced (Fig. 4C).

3.5. *Community stability of FL and PA bacterial communities*

Average microbial community stability in all four lakes was higher for FL compared to PA communities (Table S2). Lake Crniševo had the lowest average stability value (0.658), while the highest value was reported in Lake Prošće, where both fractions showed similar mean values (0.737 for FL and 0.725 for PA) (Table S2).

Compositional stability in Lake Crniševo was higher for FL than PA bacterial communities in the first sampling year (Fig. 5A). Afterward, both FL and PA communities had similar values and followed similar trends (Fig. 5A). In Lake Oćuša, both fractions exhibited high temporal variability in stability, with clear non-alignment in stability peaks between FL and PA communities (Fig. 5B). FL communities were generally more stable than PA communities, but fluctuations were evident throughout both mixing and stratified periods (Fig. 5B). In Lake Kozjak, the stability of FL and PA bacterial communities followed opposite trends. FL communities were the most stable during the mixed period,

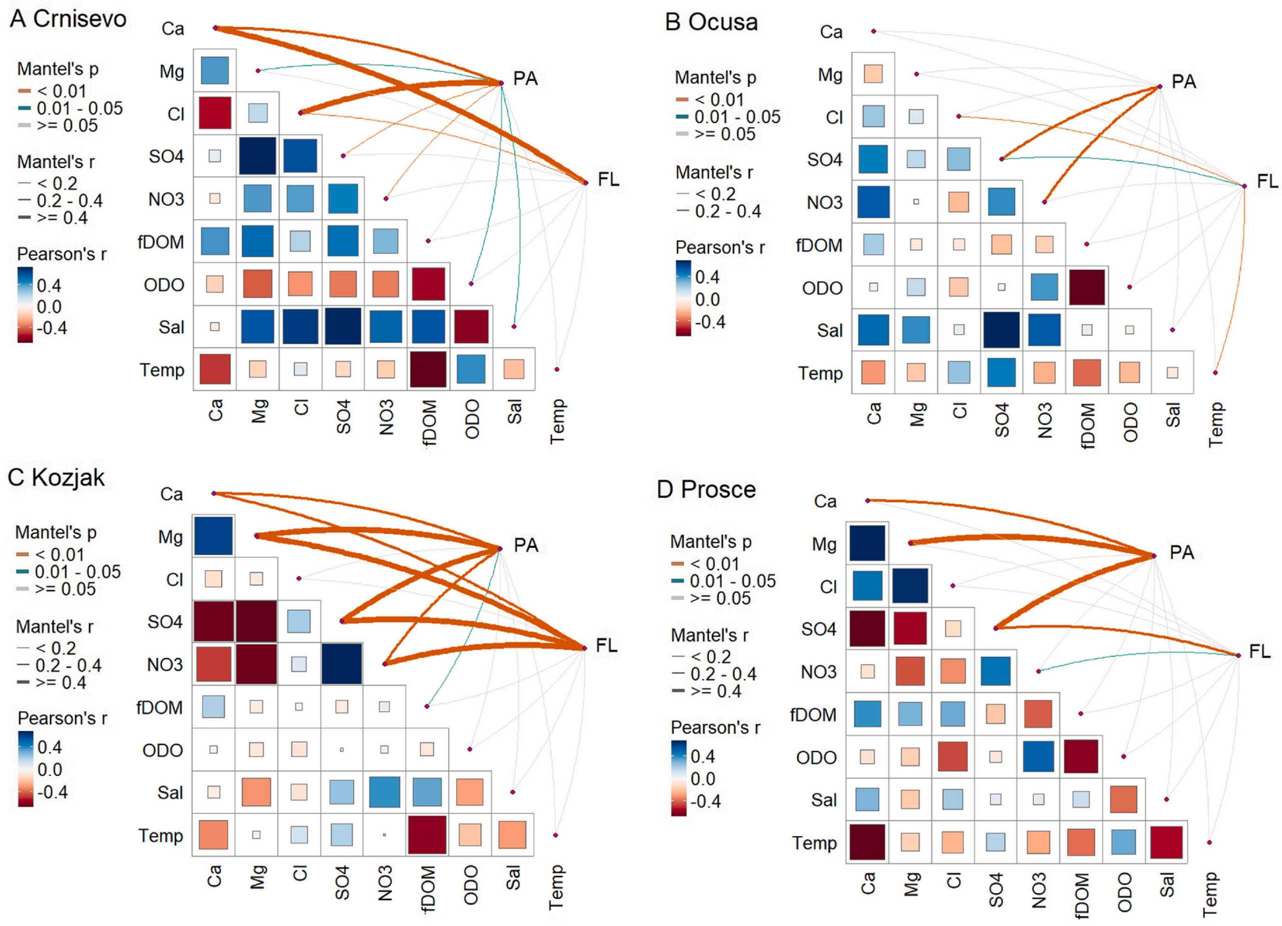

Fig. 3. Pairwise comparison between environmental factors with a color gradient representing Pearson's correlation coefficients for each lake: (1) Crniševo, (2) Oćuša, (3) Kozjak, (4) Prošće. Partial Mantel test was performed for FL and PA communities and each environmental factor, respectively. Line width indicates the partial Mantel's r statistic for corresponding correlations, with the larger values indicating stronger correlations and line color indicating significance.

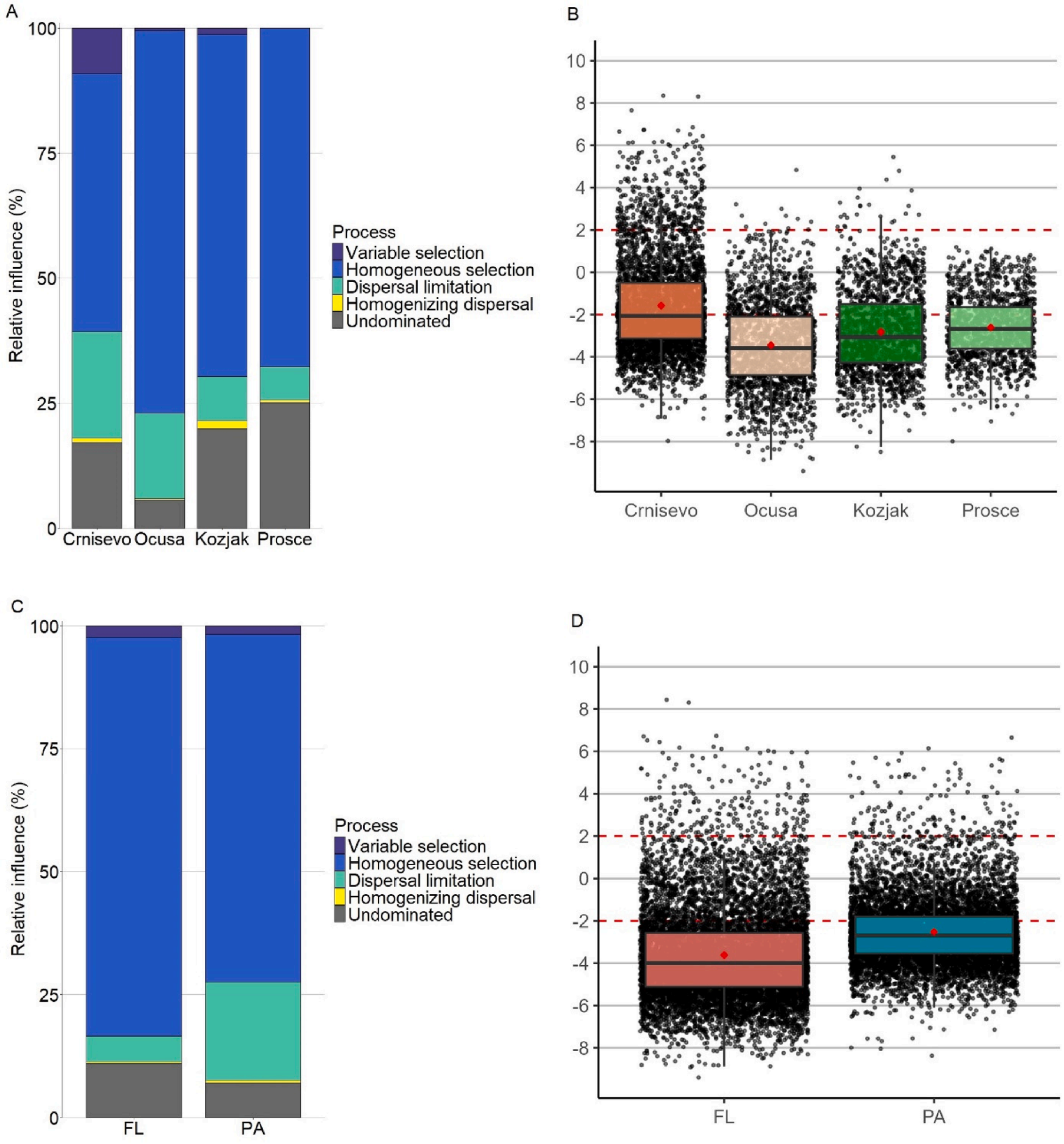

Fig. 4. Community assembly mechanisms of microbial communities (A) in four investigated lakes with (B) their βNTI values, and (C) between fractions with (D) their βNTI values.

while PA communities exhibited lower stability. During the stratified period, their stability patterns converged, showing similar values (Fig. 5C). Over time, FL communities remained more stable, whereas PA communities showed greater variability. In Lake Prošće, both fractions showed increasing trends in stability, but the peaks occurred at different times (Fig. 5D). FL communities were generally more stable, but stability declined during the mixing period from November to February. In contrast, PA communities peaked in stability after this period, particularly during the stratified phase from late winter to summer (Fig. 5D).

3.6. Modelling of microbial communities and environmental variables using the partial least squares path modeling (PLS-PM)

The partial least square path modeling revealed direct and indirect effects of abiotic and biotic factors on the compositional stability of FL and PA bacterial communities. In Lake Crniševo, physico-chemical properties had an indirect positive effect on the stability of the FL community and salinity and particles had an indirect negative effect, and diversity had a direct negative effect (correlation coefficient = -0.4402) (Fig. 6A). For the PA community, the particles had a significant negative effect (correlation coefficient = -0.4993), and DOM had a significant positive effect (correlation coefficient = 0.3846) on the compositional stability, while physico-chemical properties had an indirect negative effect (Fig. 6A). For both FL and PA communities in Lake Oćuša, nutrients had a direct positive effect (correlation coefficient = 0.546 and 0.4973, respectively) on compositional stability, while physico-chemical properties had an indirect positive effect (Fig. 6B). In Lake Kozjak, particles had a direct positive effect (correlation coefficient = 0.3258) on the compositional stability of the FL community, and precipitation and physico-chemical properties had indirect negative and positive effects, respectively (Fig. 6C). Stability of the PA community in Lake Kozjak was influenced positively by nutrients (correlation coefficient = 0.5953) (Fig. 6C). In Lake Prošće, DOM and nutrients had a direct negative effect (correlation coefficient = -0.5777 and −0.598, respectively) on the compositional stability of the FL community and precipitation and physico-chemical properties had an indirect positive effect (Fig. 6D). For the PA community, physico-chemical properties had a direct positive effect (correlation coefficient = 0.6737) on

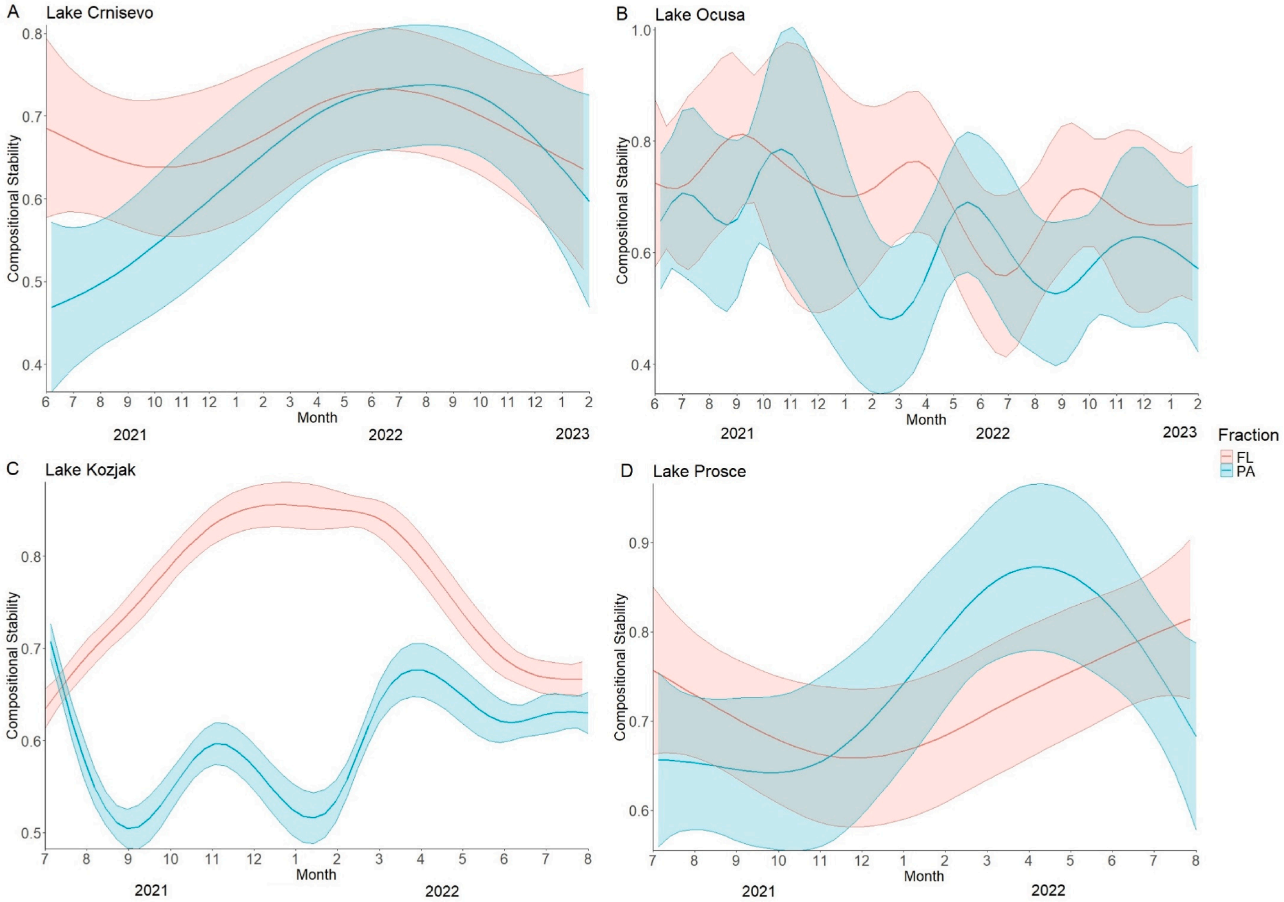

Fig. 5. Dynamics of the community stability of free-living (FL) and particle-associated (PA) bacterial communities in four investigated lakes. (A) Crniševo; (B) Oćuša; (C) Kozjak; (D) Prošće.

compositional stability, and precipitation had an indirect negative effect (Fig. 6D). A detailed description of the PLS-PM total effects is shown in Supplementary Table 3.

3.7. Co-occurrence network analysis of microbial communities

Microbial co-occurrence networks provide insights into species interactions, potential niche differentiation, and community stability. Nodes represent ASVs, with larger nodes indicating highly connected taxa (hubs) that may play important ecological roles. Edges indicate significant co-occurrence relationships, reflecting potential ecological associations. Modularity quantifies network clustering, where higher modularity suggests a more compartmentalized community structure, potentially driven by environmental selection or functional specialization. Degree centrality highlights ASVs with many connections, which may indicate keystone taxa that influence network stability.

Microbial network in Lake Crniševo was well-connected and dense with several distinct clusters (Fig. 7A). The network in Lake Oćuša exhibited a relatively high degree of connectivity with several dense clusters, but with slightly fewer isolated nodes compared to Crniševo (Fig. 7A). Both lakes' networks showed distinct modularity, with clear clusters of similarly colored nodes (Fig. 7A). Baćina Lakes also had complex interaction patterns with more nodes involved in multiple connections. Lakes Kozjak and Prošće had a more fragmented structure with multiple small clusters and fewer connections between them (Fig. 7A). The networks of Plitvice Lakes had less distinct modularity with less pronounced separation between clusters and more isolated nodes (Fig. 7A).

All computed topological properties of co-occurrence networks are depicted in Supplementary Table 4. Microbial networks of Baćina Lakes exhibited more nodes and edges (132 nodes with 431 edges in Lake Crniševo and 148 nodes with 387 edges in Lake Oćuša) compared to Plitvice Lakes (113 nodes with 255 edges in Lake Kozjak and 112 nodes with 264 edges in Lake Prošće) (Fig. 7B). In all investigated lakes, the bacterial network displayed a very high percentage of positive correlations (~80 %), indicating mutualistic interactions between communities (Table S4). The average clustering coefficient (avgCC) values were similar for all four investigated lakes ranging from 0.625 for Lake Oćuša to 0.596 for Lake Prošće (Table S4). The average path length (APL) value was highest for Lake Crniševo, followed by Lake Prošće, then Lake Oćuša and Lake Kozjak (Table S4). Modularity analysis showed that Baćina Lakes had greater modularity values compared to Plitvice Lakes, as well as the average degree values (avgK) (Table S4). The higher avgK suggests a stronger average interaction intensity among bacteria (Deng et al., 2018). In contrast, the shorter APL implies higher efficiency in the transmission of information and energy, which may influence network stability (Zhang et al., 2021). Lake Crniševo, exhibits high modularity and clustering combined with a longer APL, reflecting a network with strong within-module connections but limited interaction across modules (Liu et al., 2022c). Lake Crnisevo had the highest number of edges as well as the highest avgK, suggesting a denser network that may facilitate robust nutrient exchange, cross-feeding or cooperative interactions (Liu et al., 2022c). Lake Crniševo displays simpler and more modular networks, with most isolated and modular structure. These results suggest that the network might be less efficient in terms of information or resource flow across the network, implying lower resilience. For the identification of keystone taxa within the networks, the topological roles of ASVs were depicted in Z-P plots (Fig. 8). Lake

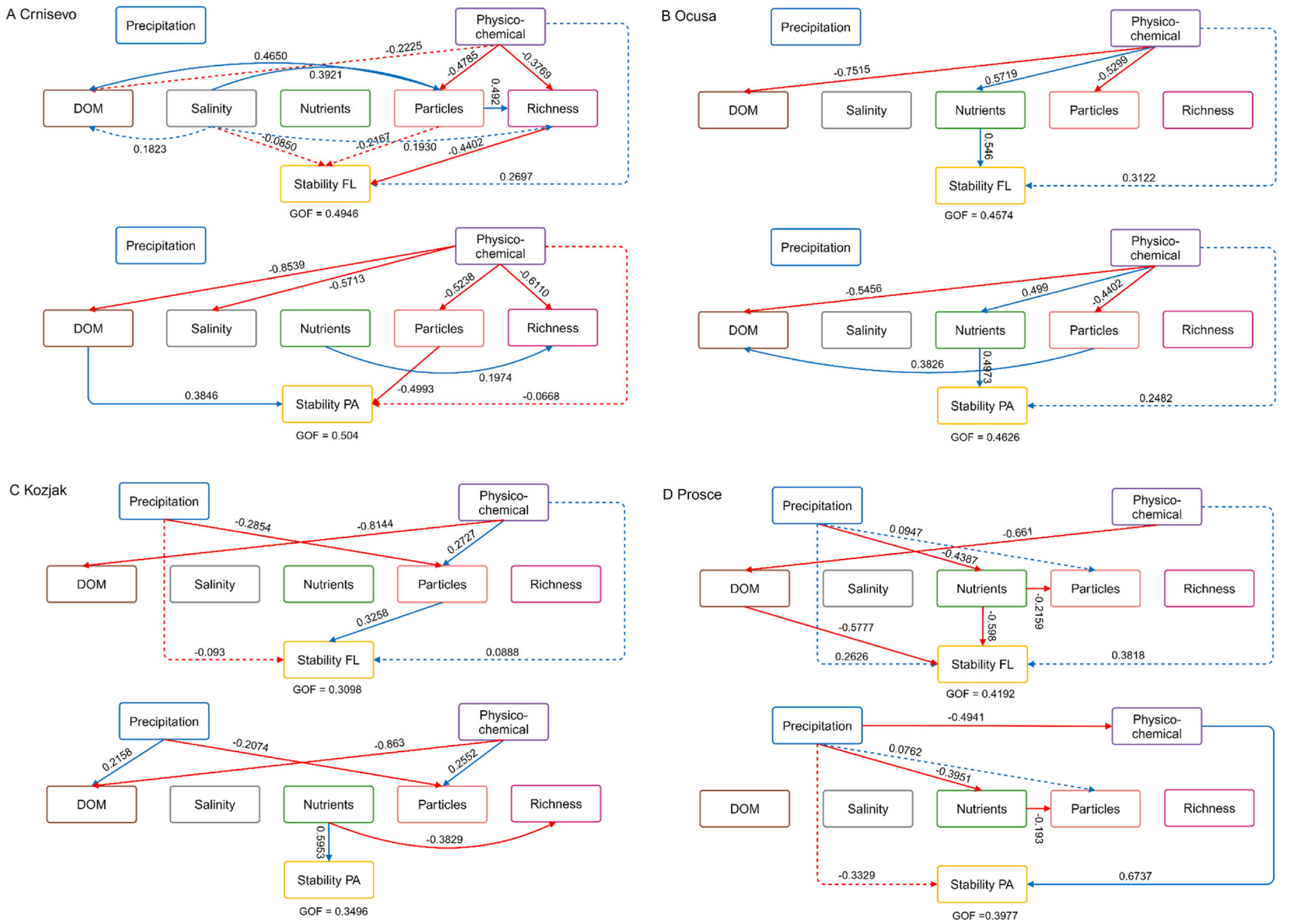

Fig. 6. Partial least squares path models (PLS-PM) showing the relationship between environmental drivers and the community stability of free-living (FL) and particle-associated (PA) bacteria in four investigated lakes. Blue and red lines represent positive and negative effects, respectively, and only significant paths ($p < 0.1$) are shown. Dashed lines represent indirect effect and full lines represent direct effect.

Crniševo and Lake Kozjak contained one module hub (Fig. 8A and C), Lake Oćuša had three module hubs and two connectors were identified (Fig. 8B) while Lake Prošće had one module hub and two connectors (Fig. 8D). The taxonomic information of keystone taxa is listed in Supplementary Table 5.

4. Discussion

4.1. Distribution patterns of bacterial phyla driven by thermal stratification and turnover events

Seasonal stratification and mixing events in the lakes strongly influenced the alternating dominance of bacterial phyla such as Cyanobacteria, Proteobacteria, and Verrucomicrobiota (Fig. 2). During stratified periods, distinct thermal layers formed within the lakes, creating varying environmental conditions that favored specific microbial groups. Bacteroidota exhibited varied responses to oxic and anoxic conditions depending on their species and environmental context (Lee et al., 2024). In Lake Crniševo, the dominance of Bacteroidota below the thermocline during stratification in the first sampling year suggests their adaptation conditions with lower oxygen levels, as reported in Diao et al. (2017). Sun et al. (2023) and Lee et al. (2024) showed that Bacteroidota are involved in various biogeochemical cycles, including those related to sulfur cycling. The higher abundance of Bacteroidota during the stratified period may be attributed to increased sulfate availability and seasonal hypoxia. In contrast, Proteobacteria and Cyanobacteria, which dominate the upper layers, favor warmer, light-exposed, nutrient-rich surface waters. This was particularly evident during the summer months, when Cyanobacteria can form blooms due to increased light availability and higher temperatures (Feuchtmayr et al., 2019; Erratt et al., 2023). Alternating patterns in Proteobacteria and Cyanobacteria abundances suggest competition between these groups, likely driven by competition for essential nutrients (Xie et al., 2024b), and potentially by the production of algicidal compounds by some Proteobacteria species (Coyne et al., 2022; Ren et al., 2023).

Cyanobacteria, known for their ability to thrive in high-light and nutrient-rich conditions, particularly in the upper parts of the water column, often dominate during periods of high productivity (Smith, 2003), such as the summer stratified period observed in Lake Oćuša. The appearance of Cyanobacteria in the deeper parts of the lake during second stratification period may be due to their redistribution in the previous mixing period when they reached and dominated the whole water column (Dasauni et al., 2022; Xie et al., 2024b). The subsequent dominance of Bacteroidota in deeper layers during mixing periods might indicate a shift in available nutrients or organic matter, favoring bacteria that can degrade complex organic compounds or thrive under different oxygen conditions (Lee et al., 2024).

The observed alternating dominance between Proteobacteria, Verrucomicrobiota, and Actinobacteriota in Plitvice Lakes reflects niche partitioning among these groups. In oligotrophic lakes, such as Plitvice Lakes, Verrucomicrobiota often adapt to more specialized niches and metabolic processes that suit low-nutrient conditions. Findings suggest that Proteobacteria and Verrucomicrobiota can coexist, as reported in Lake Prošće (Yang et al., 2024a), but may also compete for resources such as space and nutrients, leading to one phylum dominating the niche at a specific time (Shen et al., 2019; Zhao et al., 2024), as observed in

Fig. 7. Co-occurrence network of bacterial communities. (A) Visualization of microbial network patterns in four investigated lakes with the top 8 modules represented by different colors. (B) Topological properties of the constructed co-occurrence networks; avgK, average degree; avgCC, average clustering coefficient; APL, average path length.

Lake Kozjak.

The distinct microbial community structures observed between Baćina Lakes and Plitvice Lakes demonstrate that local factors have a significant influence on shaping these communities. The increased abundance of Verrucomicrobiota and reduced abundance of Cyanobacteria in Plitvice Lakes, compared to the opposite trend in Baćina Lakes, may reflect the differences in trophic status between the two lake systems (Shen et al., 2019). In Baćina Lakes, the distribution of bacterial phyla relative to depth and stratification also indicates the influence of oxygen gradients and redox conditions. The more uniform distribution of all five phyla during mixing periods reflects the homogenization of the water column, where nutrients and oxygen are redistributed, enabling different bacterial groups to coexist throughout the water column. In contrast, the less pronounced influence of mixing on microbial community composition in Plitvice Lakes suggests that these lakes maintain more stable environmental conditions, which limit significant shifts in the dominant phyla during mixing and stratification periods.

4.2. *Factors affecting stability of FL and PA bacterial communities*

The results of this study reveal clear seasonal patterns of compositional stability in FL and PA bacterial communities across four lakes, each influenced by distinct environmental factors. FL communities were influenced by nutrients and physico-chemical properties, while PA communities were influenced by factors such as particle dynamics, as these components directly affect their habitat structure and resource availability.

Out of all investigated lakes and fractions, salinity only had an effect on FL bacterial communities in Lake Crniševo. Higher salinity levels impose significant selective pressures on bacterial communities, influencing the composition and assembly mechanisms and negatively impacting stability (Li et al., 2021; Wei et al., 2024). The distinct responses of the two fractions can be attributed to their differing ecological niches, dispersal capacities and functional strategies (Wang et al., 2020). As FL communities are more directly exposed to the saline environment compared to the PA communities, less-adapted species tend to be filtered out which may lead to lower stability (Zhang et al., 2022b). In Lake Crniševo, the negative effect of diversity index on the stability of FL communities can be explained by the fact that higher diversity can lead to increased competition and complexity within the community, thereby reducing stability under certain conditions. In a diverse community, the presence of many different species can result in niche overlap and competition for resources. This competition may destabilize the community, especially if environmental conditions fluctuate or if certain species begin to dominate (Fujita et al., 2023; Babajanyan et al., 2024). Particles in aquatic ecosystems are highly heterogeneous; thus, an increase in particle abundance creates diverse niches, leading to competition and shifts in community structure and reducing overall community stability (Hu et al., 2020; Wang et al., 2024). This has been observed in the PA communities of Lake Crniševo. Conversely, particles in Lake Kozjak positively influenced the stability of the FL communities by acting as nutrient hotspots that release DOM and nutrients into the surrounding water, particularly in an oligotrophic lake like Kozjak (Hu et al., 2020; Van Le et al., 2024). In contrast to particle dynamics, DOM serves as an easily available and more evenly distributed carbon source throughout the water column (Wang et al., 2024), especially in the mesotrophic lake like Crniševo. Higher DOM values positively influenced the stability of PA communities, indicating that these communities utilize DOM through diffusion around particles (Yang et al., 2024b). Additionally, PA communities effectively break down complex particulate organic matter (POM), which increases the local DOM concentration near the particles, thereby benefiting from the available carbon sources in their environment (D'Andrilli et al., 2019). On the other hand, in an oligotrophic Lake Prošće, DOM had a negative influence on stability of FL communities due to the limited availability of easily degradable organic material, leading to competition and destabilization of the community structure (Xie et al., 2024a). The significant positive effect of nutrients on the stability of both FL and PA communities in Lake Oćuša suggests that nutrient availability is a key driver of microbial stability in this lake. High nutrient levels can promote microbial growth and activity, leading to a more resilient and stable

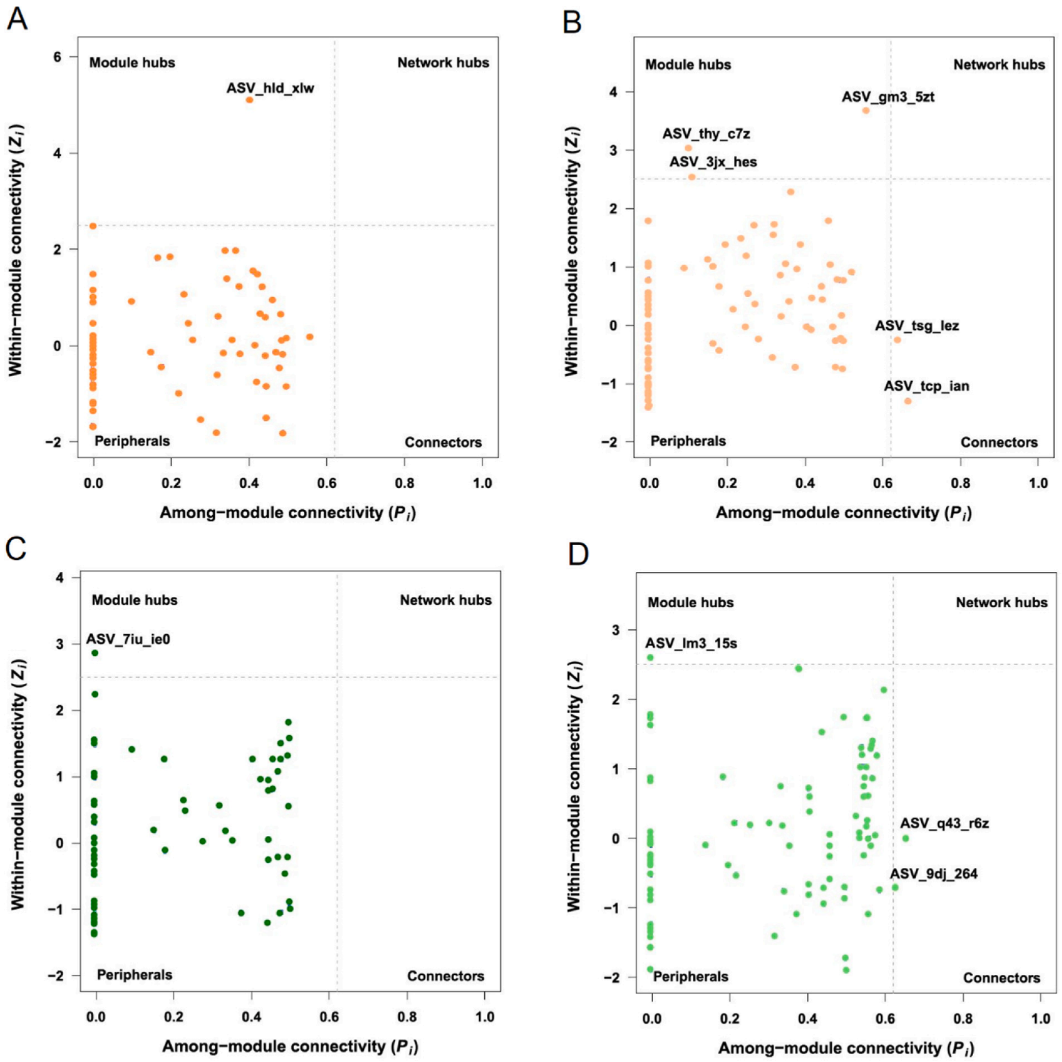

Fig. 8. Topological roles of keystone taxa in investigated lakes. (A) Crniševo; (B) Oćuša; (C) Kozjak; (D) Prošće. Module hubs are identified as Zi ≥ 2.5, Pi < 0.62, network hubs as Zi ≥ 2.5, Pi ≥ 0.62 and connectors as Zi < 2.5, Pi ≥ 0.62.

community structure (Knelman et al., 2014). The contrasting stability trends observed between FL and PA communities in Lake Kozjak may be attributed to seasonal changes that differentially affect these communities. During winter, lower temperatures and stratification can alter nutrient availability and particle dynamics, leading to increased stability in FL communities and decreased stability in PA communities (Yang et al., 2024b). As the lakes transition to summer, increased mixing and higher temperatures may homogenize environmental conditions, resulting in similar stability levels for both communities (Bižić-Ionescu et al., 2014). This seasonal variability underscores the importance of temporal factors in shaping microbial community dynamics. In Lake Prošće, the seasonal shift in stability aligns with patterns reported by Shilei et al. (2020), where seasonal stratification and mixing events significantly altered microbial community structure. The negative effects of DOM and nutrients on FL communities, alongside the positive influence of physico-chemical properties on PA communities, further emphasize the complex interplay of abiotic and biotic factors in shaping community stability, as noted in a similar study (Yan et al., 2024). In Lake Prošće, the stability values of FL and PA communities were more similar due to the more stable and balanced environmental conditions, lower DOM values, and consistent physico-chemical properties. This stability reduces environmental stress on PA communities, allowing them to behave more similarly to FL communities.

4.3. *Community assembly mechanisms and microbial interactions in FL and PA bacteria*

The assembly of FL and PA bacterial communities across the studied lakes is influenced by both homogeneous selection and stochastic processes. Homogenous selection, the dominant mechanism, suggests that consistent environmental factors exert strong selective pressures, leading to a more uniform community composition. This process favors specific taxa adapted to stable conditions, resulting in a non-random distribution of microbial taxa that reflect prevailing environmental conditions. Consequently, communities exhibit more similar structures when influenced by homogenous abiotic and biotic conditions.

In contrast, stochastic processes played a significant role in Lake Crniševo, as indicated by βNTI exceeding 2, which suggests variable selection. This divergence occurs when communities experience different selective pressures due to temporal or spatial changes, such as seasonal anoxia and varying salinity levels in the lake's bottom waters. Dispersal limitation, more prominent in PA communities, suggests random variations in community composition. This is likely due to the restricted movement associated with particles, resulting in increased variability and random assembly of these communities. Network analysis of Lake Crniševo displays networks with most isolated and modular structure. These results suggest that the network might be less efficient in terms of information or resource flow across the network, implying lower resilience. Low resilience in a microbial network indicates a diminished capacity of the community to recover its original structure and function after disturbances. Such communities are more susceptible to environmental fluctuations, which can lead to alterations in microbial composition, potential loss of key functions, and reduced ecosystem stability (Philippot et al., 2021). Elevated salinity can significantly influence microbial community resilience. Studies have shown that increased salinity often reduces bacterial diversity and enhances community differentiation, making these ecosystems more vulnerable to environmental changes (Xin et al., 2024).

In contrast, Lake Oćuša's compact network with high clustering and shorter APL implies greater efficiency and stability (Zhang et al., 2021), supported by a Z-P plot showing several connectors. This indicates good within-module interactions but less interconnectedness, reflecting a distinct ecological strategy. Lake Kozjak displayed the lowest modularity and shortest path length, suggesting a simpler network with a more uniform interaction structure, which may indicate higher efficiency and stability despite less ecological complexity (Liu et al., 2022c). Lake Prošće, with moderately low modularity, balanced clustering, and an average path length, presents a well-organized modular structure with good inter-module connectivity. The Z-P plot highlights several nodes functioning as connectors and hubs, suggesting a complex yet resilient network. Overall, these findings suggest that the structural organization and ecological dynamics of microbial communities differ among lakes, influenced by varying environmental conditions.

5. Conclusion

This study provides key insights into the ecological dynamics of microbial communities in freshwater lakes, emphasizing the distinct roles of free-living (FL) and particle-associated (PA) bacterial groups and their responses to environmental gradients. Our findings demonstrate that seasonal stratification, mixing events, and local environmental factors drive community assembly and stability, with FL and PA communities showing differential responses to these dynamics.

Given the high number of samples and the detailed partitioning of microbial fractions, this study highlights the value of incorporating FL and PA differentiation in biomonitoring programs. We recommend that future biomonitoring efforts systematically include both fractions to better capture microbial responses to environmental stressors such as salinity fluctuations, anoxia, and seasonal mixing events. This partitioning strategy can improve the detection of ecological shifts and provide early warning signals of environmental change, particularly in sensitive ecosystems such as saline-influenced and stratified lakes. The impact of salinity on microbial community stability was particularly evident in Lake Crniševo, where seasonal salinity variations contributed to stronger selection pressures, shaping microbial assembly processes over time and influencing community stability. These findings suggest that increasing salinity can drive community divergence and potentially reduce microbial resilience, highlighting the need to monitor salinity-driven shifts in microbial composition as an indicator of ecosystem change. Furthermore, our results suggest that microbial co-occurrence networks can serve as a tool for assessing ecosystem resilience and functional redundancy. The observed differences in network complexity between Plitvice Lakes (more stable, functionally redundant networks) and Baćina Lakes (more dynamic, environmentally responsive networks) indicate that microbial interactions are key to ecosystem stability. Integrating network-based analyses in freshwater monitoring programs could enhance long-term assessments of microbial stability and ecosystem health, particularly in climate-sensitive and salinity-impacted lake systems.

Finally, understanding microbial community dynamics is critical for freshwater resource management. Our findings suggest that monitoring shifts in microbial assembly processes could help predict ecosystem responses to anthropogenic stressors, such as nutrient influx, salinity changes, and climate-driven alterations in stratification patterns. Future conservation and restoration strategies should consider microbial communities as bioindicators of ecosystem function and resilience, particularly in karstic and hydrologically dynamic freshwater systems.

CRediT authorship contribution statement

Ivana Stanić: Writing – original draft, Methodology, Formal analysis. **Katarina Kajan:** Formal analysis. **Lorena Selak:** Writing – review & editing, Formal analysis. **Sandi Orlić:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2025.113324.

Data availability

Data will be made available on request. Amplicon sequencing datasets presented in this article have been deposited in the NCBI repository under BioProject accession number PRJNA1167227.

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