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DOCTORAL THESIS

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Prirodoslovno-matematički fakultet

Biološki odsjek

Ivana Stanić

Uloga prokariota u kruženju metana u Baćinskim i Plitvičkim jezerima

DOKTORSKI RAD

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This doctoral dissertation was carried out in Laboratory for precipitation processes, Division of materials chemistry at Ruđer Bošković Institute, under the supervision of Sandi Orlić, PhD, within the Postgraduate doctoral programme in Biology at the University of Zagreb, Faculty of Science, Department of Biology. The research has been supported by the Croatian Science Foundation under the project MALENA (IP-2020-02-9021).

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Dr. Orlić has participated in multiple research projects and has served as principal investigator on both national and international projects. He has published more than 60 scientific papers and holds an h-index of 29. He is actively involved in the organisation of different national and international conferences and is a member of International Society of Microbial Ecology (ISME) serving as the ISME ambassador for Croatia. Dr. Orlić is also the member of editorial boards of ISME Communications, Microbiology Spectrum and Annals of Microbiology, and a reviewer for different journals such as Nature communications, Nature and iScience.

University of Zagreb
Faculty of Science
Department of Biology

Doctoral thesis

THE ROLE OF PROKARYOTES IN METHANE CYCLE OF BAĆINA AND PLITVICE LAKES

IVANA STANIĆ
Ruđer Bošković Institute

Karst lakes play a significant role in global methane cycling, yet the microbial mechanisms underlying methane production and oxidation in these environments remain unexplored. This thesis investigates the diversity and function of methane-cycling prokaryotes in two lake systems in Croatia. Using 16S rRNA gene sequencing and qPCR targeting *mcrA* and *pmoA* genes, prokaryotic communities were analyzed across multiple seasons and environmental gradients. Laboratory enrichment experiments were conducted using a water sample collected from Lake Crniševo to assess the impact of increased salinity on methane-cycling prokaryotic communities. Results showed greater microbial diversity in Baćina Lakes, with oxygen and salinity as key structuring factors. Methanotrophs were more abundant compared to methanogens in both lake ecosystems, especially at oxic-anoxic interfaces. Elevated salinity reduced diversity and favored halotolerant taxa, with methanotrophs showing greater adaptability than methanogens. These findings underscore the importance of environmental gradients in shaping methane-cycling communities and are essential for predicting methane emissions under climate change.

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Neven Cukrov, PhD, scientific advisor

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ULOGA PROKARIOTA U KRUŽENJU METANA U BAĆINSKIM I PLITVIČKIM JEZERIMA

IVANA STANIĆ
Institut Ruđer Bošković

Krška jezera imaju značajnu ulogu u globalnom ciklusu metana, no mehanizmi koji stoje iza njegove produkcije i oksidacije u tim ekosustavima još su uvijek nedovoljno istraženi. Ovaj rad proučava raznolikost i funkciju prokariota uključenih u ciklus metana u dva jezerska sustava u Hrvatskoj. Korištenjem sekvenciranja 16S rRNA gena i qPCR analize ciljane na *mcrA* i *pmoA* gene, prokariotske zajednice analizirane su kroz više sezona u različitim okolišnim uvjetima. Kako bi se procijenio utjecaj povećanja saliniteta na prokariotske zajednice povezane s ciklusom metana, uzorci su kultivirani u obogaćenom mediju. Rezultati su pokazali veću mikrobnu raznolikost u Baćinskim jezerima, pri čemu su kisik i salinitet bili glavni čimbenici oblikovanja zajednica. Metanotrofi su dominirali nad metanogenima u oba jezerska ekosustava, osobito na granici između oksičnih i anoksičnih slojeva. Povišeni salinitet smanjio je raznolikost te doprinio rastu halotolerantnih vrsta, pri čemu su metanotrofi pokazali veću prilagodljivost od metanogena. Dobiveni rezultati ističu važnost okolišnih gradijenata pri oblikovanju zajednica uključenih u ciklus metana te su ključni za predviđanje emisija metana u kontekstu klimatskih promjena.

(99 stranice, 20 slika, 5 tablica, 174 literaturnih navoda, jezik izvornika: engleski)
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1. INTRODUCTION

1.1. Research context

Natural environments harbor complex and diverse microbial ecosystems, many of which remain poorly characterized. A significant proportion of microbial life, often referred to as "microbial dark matter," consists of uncultivated and phylogenetically novel taxa whose ecological roles are largely unknown (Hug et al., 2016; Rinke et al., 2013). Microorganisms are central to biogeochemical processes, including carbon cycling, nutrient transformation, and energy flow, making the study of microbial communities essential for understanding ecosystem function (Cavicchioli et al., 2019; Falkowski et al., 2008).

Advancements in molecular biology, particularly next-generation sequencing (NGS) technologies, have revolutionized microbial ecology. These methods allow high-throughput, parallel sequencing of environmental DNA, enabling comprehensive analysis of both taxonomic composition and functional potential of microbial communities (Caporaso et al., 2012; Quince et al., 2017). Culture-independent techniques have made it possible to identify taxa present at low abundance and to link microbial diversity with ecosystem processes (Bahram et al., 2018).

Freshwater lakes are particularly valuable for studying microbial ecology due to their sensitivity to environmental change and their role in global biogeochemical cycles, including methane production and oxidation (Bastviken et al., 2004; Tranvik et al., 2009). Methane in lakes is primarily produced through microbial methanogenesis in anoxic sediments and bottom waters, carried out by methanogenic archaea. Its removal occurs via aerobic and anaerobic methane oxidation, largely mediated by specialized methanotrophic bacteria and archaea (Knief, 2019; Oswald et al., 2016). The balance between these processes determines whether a lake is a net emitter or consumer of methane. Environmental gradients such as oxygen availability, redox potential, nutrient concentrations, and salinity have a profound effect on microbial community structure and, consequently, on the efficiency and direction of methane cycling (Mayr et al., 2020).

Furthermore, freshwater karst systems, such as those found in Croatia, represent unique and understudied habitats where microbial processes intersect with distinct hydrological and geochemical conditions. They are formed in soluble limestone or dolomite terrains, and are characterized by complex hydrology, stratified water columns, and strong geochemical gradients (O. Bonacci, 2001; Stevanović, 2015). Due to their geological and hydrological distinctiveness, these lakes often exhibit high sensitivity to environmental perturbations, including eutrophication, climate change, and salinization. Seasonal stratification can result in hypoxic or anoxic conditions in bottom layers, fostering redox-sensitive processes such as sulfate reduction and methanogenesis (Diao et al., 2017).

Climate change poses an additional challenge for karst lake ecosystems. Rising temperatures influence thermal stratification, ice cover duration, and evaporation rates, while increasing precipitation variability and anthropogenic pressure alter water balance and nutrient loading (Adrian et al., 2009). In coastal or low-lying karst areas, salinization due to sea intrusion or water extraction further complicates microbial processes by selecting for halotolerant or halophilic species and inhibiting processes like methanogenesis in favor of alternative electron acceptors such as sulfate (Lozupone & Knight, 2007). Salinization poses a growing threat to freshwater ecosystems, particularly in vulnerable karst environments where hydrological connectivity and limited buffering capacity make these systems highly sensitive to changes in salinity. Increases in salinity, whether from climate-induced drought, sea water intrusion, or anthropogenic activity, can disrupt microbial community structure, impair biogeochemical processes, and ultimately affect ecosystem stability and function. These environmental stressors are expected to reshape microbial community composition and function, with implications for biogeochemical cycling and greenhouse gas emissions.

1.2. Aims and scope

This doctoral dissertation focuses on two distinct lake systems in Croatia: an oligotrophic system with unique geomorphological, hydrological, and biological processes forming tufa barriers (Plitvice Lakes) and a mesotrophic ecosystem experiencing seasonal anoxia and salinization at the lake bottom (Baćina Lakes). These systems provide a unique opportunity to investigate how microbial communities respond to differing environmental pressures and contribute to methane cycling in freshwater environments.

The objectives of this research were to explore the diversity, distribution, and functional roles of prokaryotic communities in two karst lake systems with contrasting trophic and physicochemical characteristics. Specifically, the aims of the study were to (1) analyse the diversity of prokaryotic communities in the water column of Baćina and Plitvice Lakes, (2) describe the seasonal distribution of prokaryotic communities in the water column of Baćina and Plitvice Lakes, (3) analyse the impact of environmental factors on prokaryotic communities involved in the methane cycle, and (4) analyse the functional genes involved in the methane cycle. The proposed hypotheses were: (1) the diversity of prokaryotic communities varies according to the trophic status of the lake, (2) the seasonal distribution of prokaryotic communities is shaped by dynamic environmental conditions in each lake system, (3) the composition and abundance of methanogens and methanotrophs are primarily driven by oxygen concentration, and (4) the abundance of functional marker genes involved in the methane cycle reflects the oxygen concentration in the water column.

The expected scientific contribution of this research will be a detailed description of the biodiversity and distribution of prokaryotic communities in karst lakes across seasons, insights into the ecological functions of dominant microbial groups, particularly those involved in methane cycling, identification of prokaryotes and genes involved in the methane cycle and the environmental factors that influence them, and better understanding of how climate-related factors, such as warming and salinization, may influence microbial processes and greenhouse gas dynamics in freshwater ecosystems. By advancing our understanding of fundamental biological and ecological processes in lakes, this research will offer novel insights into the methane cycle in karst lakes and enhance scientific approaches to studying karst freshwater systems. By applying culture-independent methods, including 16S rRNA gene amplicon sequencing for microbial taxonomic profiling and quantitative PCR (qPCR) for the detection and quantification of functional genes involved in methane metabolism (*mcrA*, *pmoA*), this study employs a high-resolution, multi-target approach that integrates taxonomic and functional data to assess microbial community dynamics and metabolic potential in freshwater karst environments. In addition, a salinity enrichment experiment was conducted to simulate future environmental stress scenarios, enabling the evaluation of microbial community responses to salinization and the identification of functionally important and potentially resilient taxa and genes involved in methane cycling under altered conditions. Ultimately, the findings will contribute to the broader field of aquatic microbial ecology by offering new perspectives on microbial functions in karst environments and informing future conservation and management strategies for sensitive freshwater systems.

2. LITERATURE REVIEW

2.1. Molecular techniques for microbial ecology

The field of microbial ecology has undergone significant advancements over the past few decades, driven by the development and application of molecular techniques. Transition from traditional cultivation-based approaches to cutting-edge molecular techniques had a significant impact on our understanding of microbial communities and their functions in various ecosystems.

Historically, the study of microbial diversity relied heavily on cultivation-based methods that involve isolating and characterizing microorganisms using growth media (Kirk et al., 2004; Madigan et al., 2021). However, while microorganisms' rapid growth in controlled conditions makes them suitable for research, these approaches provided an incomplete representation of microbial communities, as the majority of microorganisms in many habitats remain unidentified and uncultured due to slow growth, dependence on other species, or environmental factors (Horner-Devine et al., 2004). The 1980s marked a pivotal shift in microbial ecology research with the introduction of molecular techniques. Woese et al. (1990) recognized the potential of ribosomal RNA for phylogenetic analysis, and Pace et al. (1986) proposed a method for analyzing rRNA genes by isolating and cloning DNA from environmental samples without the need for microbial cultivation.

Technical progress was achieved with the development of polymerase chain reaction (PCR) technology and probes for amplifying gene regions of interest (Giovannoni et al., 1990). PCR-based methods have revolutionized the field of microbial ecology by allowing researchers to study microorganisms that are difficult or impossible to culture using traditional techniques (Wintzingerode et al., 1997). These methods include fingerprinting techniques like denaturing/temperature gradient gel electrophoresis (DGGE/TGGE), terminal restriction fragment length polymorphism (T-RFLP) and automated ribosomal intergenic spacer analysis (ARISA), which enabled community profiling based on genetic differences (Su et al., 2012). While fingerprinting techniques remain useful for targeted studies or as complementary techniques, they are increasingly being replaced by more advanced technologies due to their superior resolution and ability to provide functional insights.

Simultaneously, microscopy-based molecular methods evolved, reviving interest in visualizing microbes in situ. A key development was fluorescence in situ hybridization (FISH), which enabled taxonomic identification of microorganisms directly within their environmental context (Bottari et al., 2007; Handelsman, 2005). More recently, techniques like bioorthogonal non-canonical amino acid tagging (BONCAT) have allowed for the detection of metabolically active cells by tagging newly synthesized proteins (Madigan et al., 2021), while Raman microspectroscopy and NanoSIMS provided insights into microbial physiology and elemental composition at the single-cell level (Gyngard & Steinhauser, 2018; Edward K. Hall et al., 2011).

Additional techniques developed to link ribosomal gene sequences with specific metabolic functions include DNA microarrays (Bodrossy & Sessitsch, 2004), quantitative PCR (qPCR) (Smith & Osborn, 2009), stable isotope probing (DNA-SIP) (Radajewski et al., 2000), single cell genomics (Ishoey et al., 2013), and next-generation sequencing (NGS) (Shokralla et al., 2012). Advancement of NGS technologies led to the rapid development of "omics" methods, enabling characterization of genetic and functional diversity of microorganisms as a whole through the analysis of DNA (metagenomics), RNA (metatranscriptomics), proteins (metaproteomics), or metabolites (metametabolomics) (Bouchez et al., 2016; Madigan et al., 2021). The development of environmental DNA (eDNA) sequencing techniques has allowed researchers to explore the genetic diversity within a wide range of ecosystems, providing functional insights beyond taxonomy (Ruppert et al., 2019). Contemporary microbial ecology databases are primarily constructed using PCR-amplified and sequenced DNA regions of interest. These databases facilitate the exploration of microbial diversity across novel ecosystems, environmental conditions, and comparative studies. Specific genomic regions serve as key markers for identifying different microbial groups: 16S rRNA (V3/V4 regions) for bacteria and archaea, 18S rRNA (V9 region) for eukaryotic microbes and ITS (internal transcribed spacer) region for fungi. These markers are central to taxonomic identification and are the foundation of global microbial ecology databases.

Modern metagenomics avoids traditional cloning, using shotgun sequencing to capture entire genomes from environmental samples. This enables reconstruction of metagenome-assembled genomes (MAGs), functional gene discovery, and metabolic pathway reconstruction (Simon & Daniel, 2011). It has proven especially valuable in exploring extreme environments (hydrothermal vents, hypersaline lakes), deep subsurface and sediment layers and engineered systems like wastewater treatment. Such studies reveal microbial roles in biogeochemical cycles, resilience to environmental stressors, and potential for biotechnological applications (Albertsen et al., 2013; Bahram et al., 2018).

Integration of multi-omics data is now at the forefront of microbial ecology enabling the linking of community structure with metabolic function and ecosystem processes, critical for modeling microbial responses to climate change, pollution, and resource availability (Louca et al., 2018). Ongoing developments in single-cell genomics, long-read sequencing, and bioinformatics pipelines will continue to improve resolution, accuracy, and interpretability of microbial ecology data. Future research will likely emphasize real-time monitoring, predictive modeling, and the integration of molecular data into global environmental management.

2.2. Freshwater ecosystems as indicators of environmental change

Freshwater ecosystems are vital natural resources that fulfill a wide range of human needs, including water supply, food production, habitat provision, hydropower generation, and recreational services. Ongoing population growth and agricultural expansion are causing an imbalance between water demand and supply, placing increasing stress on available freshwater sources. The quality of both groundwater and surface water is influenced by natural processes and human activities. Natural factors include mineral weathering, evapotranspiration, wind-blown sediment deposition, leaching of nutrients and organic matter from soil, and biological and hydrological processes that can alter the physical and chemical properties of water (Khatri & Tyagi, 2015). In contrast, anthropogenic pollution sources include agriculture, domestic wastewater, industrial discharge, mining, forestry, landfill runoff, and other human activities. Salinization of groundwater may result from point sources such as wastewater leakage, or from diffuse sources like agricultural runoff, seawater intrusion, and saline water infiltration from adjacent aquifers. In recent decades, declining precipitation, excessive groundwater extraction, rising air temperatures, and sea level rise have increasingly endangered water resources across the Mediterranean coastal zone.

Approximately 35% of Europe consists of karst terrain, and in some countries, as much as 50% of the public water supply comes from karst aquifers (Lewin & Woodward, 2009) Karst landscapes cover over 40% Croatia (Ilijanić et al., 2025). Due to the secondary porosity of carbonate rocks, karst water resources are highly susceptible to contamination and, in coastal areas, to salinization. Karst aquifers face multiple pressures, most commonly from climate change and intensive water extraction. Climate modeling study concluded that despite increased climatic extremes, the natural water level in the aquifer would have remained sufficient to sustain the spring over time, albeit with a slight downward trend (Hartmann et al., 2012). Therefore, in coastal karst regions, it is especially important to implement sustainable water management practices, including aquifer capacity monitoring and measures to prevent over-extraction, in order to protect these fragile and vital freshwater systems.

These ecosystems encompass a variety of habitats, particularly lotic (flowing) and lentic (still water) systems. Lentic systems are generally classified by size, volume, and trophic state, and include ponds, marshes, wetlands, and lakes. Lakes themselves are categorized typologically based on altitude, hydrographic location, mean depth, surface area, lithological substrate and origin, trophic status, thermal stratification, and dissolved oxygen distribution (Tranvik et al., 2009).

Lakes and other freshwater ecosystems are increasingly recognized as sensitive and integrative indicators of environmental change (Juutinen et al., 2009). These dynamic systems not only reflect the consequences of local disturbances but also respond to global-scale phenomena such as climate change, land-use shifts, and atmospheric deposition. Among them, lakes play a particularly vital role in the global carbon cycle, acting as both sources and sinks of greenhouse gases (DelSontro et al., 2018; Tranvik et al., 2009). Lakes actively participate in carbon transport, transformation, and sequestration, often retaining large amounts of allochthonous organic carbon from surrounding landscapes. They also act as significant emitters of methane (CH_4), especially under eutrophic and stratified conditions (Bastviken et al., 2004). Methane output from inland waters is now understood to contribute substantially to global greenhouse gas budgets, despite their relatively limited surface area (Holgerson & Raymond, 2016).

Due to their sensitivity to external stressors, lakes are effective early-warning systems for environmental degradation (Adrian et al., 2009; Juutinen et al., 2009). Changes in lake stratification, oxygen dynamics, and nutrient cycling often reflect broader climatic or anthropogenic pressures. For example, prolonged warming enhances thermal stratification, reducing vertical mixing and leading to oxygen depletion in deeper waters—a condition that favors methanogenesis and disrupts aquatic food webs (Woolway et al., 2021). These anoxic layers also promote the release of nutrients from sediments (internal loading), further accelerating eutrophication and altering lake productivity (Jenny et al., 2016). Eutrophication, whether driven by natural processes or anthropogenic nutrient enrichment, accelerates the aging and eventual terrestrialization of lakes (Carpenter, 1981; V. H. Smith, 2003). Under such conditions, microbial decomposition of organic matter in the absence of oxygen intensifies, leading to increased emissions of methane. Eutrophic and anoxic lakes emit significantly more methane than oligotrophic systems with high oxygen availability and low nutrient concentrations (Clayer et al., 2016; Juutinen et al., 2009). Importantly, freshwater ecosystems do not only reflect environmental change—they are also vulnerable to feedback mechanisms that can amplify global processes (Solomon et al., 2015).

2.3. Microorganisms in biogeochemical cycle

Microorganisms are a diverse group of organisms smaller than ~100 µm, encompassing representatives from all three domains of life: bacteria, archaea, and eukaryotes (Madigan et al., 2021). Their adaptability and genetic diversity allow them to colonize all habitats on Earth, playing crucial roles in regulating ecosystem processes (Bouchez et al., 2016). These microbes constantly interact with their environment and other organisms, with changes in their surroundings leading to alterations in microbial biomass, diversity, and activity. They typically exist in complex communities where their actions are regulated by mutual interactions (Madigan et al., 2021).

Microbial processes are fundamental drivers of global biogeochemical cycles. Microorganisms actively regulate the flux of organic carbon and facilitate nutrient recycling across ecosystems (Ed K. Hall et al., 2018). They play essential roles in the fixation and regeneration of key biogenic elements, such as carbon, nitrogen, sulfur, and phosphorus (Segovia et al., 2015; Shao et al., 2023). Microorganisms in the water column can be classified into free-living, particle-associated, and those that transition between both (Rösel & Grossart, 2012). Particles create microhabitats with altered environmental conditions such as suboxic zones or nutrient-rich hotspots (Hu et al., 2020; Lapoussière et al., 2011). Because only a small proportion of bacteria can thrive in both fractions, the community structure of particle- associated microbes is typically distinct from free-living ones and responds differently to abiotic factors (Hu et al., 2020; Rösel et al., 2012; Urvoy et al., 2022; Yang et al., 2024). Understanding microbial diversity and function is essential for microbial ecology, particularly in the context of environmental monitoring, climate change, bioremediation, and biotechnology (Rastogi & Sani, 2011).

Microbial community composition and functional adaptations in lakes are strongly influenced by both trophic status and climatic conditions. In oligotrophic lakes, characterized by low nutrient availability, microbial communities tend to be dominated by oligotrophic taxa such as members of the Actinobacteria and Alphaproteobacteria, which exhibit small cell sizes and streamlined genomes—an adaptation to nutrient-poor conditions (Giovannoni et al., 2014; Neuenschwander et al., 2018). These microbes are often highly efficient at utilizing dissolved organic carbon and exhibit slower growth rates. In contrast, eutrophic lakes, rich in nutrients, host more diverse and metabolically flexible communities dominated by copiotrophic organisms such as Bacteroidetes and Gammaproteobacteria, which thrive on organic matter and can respond rapidly to phytoplankton blooms (Newton et al., 2011; Shade et al., 2007). Climatic conditions further shape microbial assemblages through temperature-driven shifts in metabolism and biogeochemical cycling. For example, lakes in cold, temperate regions typically harbor psychrotolerant microbes adapted to lower temperatures and slower decomposition rates, while warmer, tropical lakes show accelerated microbial turnover and higher microbial diversity due to increased metabolic activity and stratification (Lindström et al., 2006; Wilhelm et al., 2020). Additionally, climate-driven changes in stratification patterns and mixing regimes affect oxygen availability, leading to niche differentiation between aerobic and anaerobic taxa, particularly in hypolimnetic layers of stratified lakes (Peura et al., 2012).

The impact of environmental stress on microbial ecosystems can be assessed through resistance or the community's ability to remain unchanged, and resilience or its capacity to return to its original state after disturbance (Shade et al., 2012). Environmental changes can be short-term or long-term disturbances (Shade et al., 2012). Monitoring changes in microbial community structure in response to anthropogenic pressures helps predict ecosystem function shifts and informs water resource management (Santillan et al., 2019).

Salinity is recognized as a global-scale driver of microbial community composition and functional diversity in aquatic and terrestrial ecosystems (Lozupone & Knight, 2007). The physiological sensitivity of microbes to osmotic pressure arises from their cellular architecture: bacterial cells are enclosed by a semi-permeable cytoplasmic membrane and a porous peptidoglycan cell wall. When exposed to low external osmotic pressure, water influx may lead to cell swelling and lysis;

conversely, high osmotic pressure can cause cellular dehydration and loss of turgor. To survive under osmotic stress, microorganisms must tightly regulate intracellular water balance and solute concentration through a process known as osmoregulation (Wood, 2011). Osmoregulation typically involves the accumulation of osmolytes—small molecules that help maintain cytoplasmic stability without interfering with cellular processes. These include inorganic ions (K^+, Cl^-) and low-molecular-weight organic compounds (ectoine, glycine, betaine, glutamine, glutamate) (Sharma et al., 2019). Strategies for coping with high salinity environments are the salt-in strategy, in which cells accumulate high intracellular concentrations of inorganic ions, a method used by extreme halophiles (Vaidya et al., 2018), and the compatible solute strategy, which relies on the biosynthesis or uptake of organic osmolytes (Imhoff et al., 2021). The latter approach is more energetically expensive but less disruptive to enzyme function and is employed by a wide range of organisms, including bacteria, eukaryotes, and some methanogenic archaea (Gregory & Boyd, 2021).

Organisms unable to effectively manage osmotic stress may enter dormancy or succumb to cell damage (Mazhar et al., 2022). Consequently, increasing salinity in aquatic ecosystems, whether through natural processes or anthropogenic salinization, can lead to significant shifts in microbial community composition, favoring halophilic and halotolerant taxa while excluding more sensitive species. Beyond community structure, salinity exerts functional constraints as well. Elevated ion concentrations can denature extracellular enzymes and reduce their solubility, thereby inhibiting extracellular enzymatic activity essential for organic matter degradation (Salazar-Alekseyeva et al., 2024). Indirect effects include salinity-induced altered nutrient availability, which can suppress microbial activity and limit overall ecosystem functioning (Chaves et al., 2009). Furthermore, salinity can restructure microbial metabolic pathways by suppressing methanogenesis and promoting alternative anaerobic processes such as sulfate reduction, which outcompete methanogens for substrates in saline environments (Maltby et al., 2018).

2.4. Methane cycle in lake ecosystems

Methane, along with other greenhouse gasses, significantly contributes to global warming. Following carbon dioxide, methane is the second most important greenhouse gas linked to human activity. Since monitoring began, atmospheric methane concentrations have shown an upward trend with acceleration in the last decade, and although its atmospheric concentration (1940 ppb) is lower than that of carbon dioxide (425 ppm), methane has 28 times greater global warming potential than carbon dioxide on a 100-year timescale (Global Monitoring Laboratory, 2024). It accounts for about 16% of the warming effect and stays in the atmosphere for 12 years. Emissions from human activities such as agriculture, ruminants, fossil fuel exploitation, landfills and biomass burning, account for 60% of the methane in the atmosphere and drive the enhanced greenhouse effect. The remaining 40% of the methane in the atmosphere comes from natural sources such as wetlands and other freshwater systems (World Meteorological Organization, 2022).

The methane cycle in lakes involves a complex interplay of various microorganisms that mediate the production, consumption and flux of methane (Torres-Alvarado et al., 2005). These microorganisms include methanogens, methanotrophs and associated bacterial communities whose interactions profoundly influence the impact of methane on lacustrine environments. Methane production rates depend on temperature, availability of organic matter and absence of oxygen (Samad & Bertilsson, 2017). Majority of methane is produced in the anoxic lake sediments by methanogens and is diffused toward the upper more oxidized zone where it can be subsequently utilized by methanotrophs as their sole carbon and energy source in both oxic and anoxic conditions (Bastviken et al., 2004) thereby reducing methane emissions to the atmosphere. If not oxidized, methane can reach the atmosphere via ebullition (bubble release) or diffusion. Local environmental factors directly and indirectly influence the microbially mediated processes of methane production and consumption. Alterations in environmental conditions can consequently lead to shifts in local microbial communities and affect the pathway and rate of methane emission to the atmosphere (Dean et al., 2018).

2.4.1. Biogenic methanogenesis and methane paradox

Biogenic methanogenesis is the final stage of organic matter degradation carried out by methanogenic archaea using CO2, hydrogen, acetate or methylated compounds as substrates coupled to the generation of energy and methane production (Conrad, 2020). This process occurs strictly under anoxic conditions and is carried out predominantly by archaea from the phylum Euryarchaeota, which includes five main orders: Methanobacteriales, Methanomicrobiales, Methanococcales, Methanosarcinales, and Methanopyrales (Madigan et al., 2021). Notably, metagenomic studies have revealed additional methanogenic lineages that are phylogenetically distinct from Euryarchaeota. In natural environments, two dominant methanogenic pathways are observed: the biological breakdown of fatty acids by heterotrophs and the reduction of carbon dioxide by autotrophs. Based on substrate type and associated coenzymes, methanogenesis can be classified into hydrogenotrophic, methylotrophic, and acetoclastic pathways. Acetoclastic methanogens convert acetate into methane and carbon dioxide, process well-characterized in the order Methanosarcinales. Acetoclastic methanogenesis is predominant in freshwater sediments. However, some acetoclastic methanogens can also utilize methylated compounds such as methanol or methylamines, functioning as methylotrophic methanogens to generate ATP. In the absence of alternative terminal electron acceptors, hydrogenotrophic methanogens use hydrogen as an electron donor and carbon dioxide as the terminal electron acceptor, releasing methane as a byproduct. A key enzyme in the last step of all mentioned pathways is the methyl-coenzyme M reductase (MCR), whose gene subunits (*mcrA*) are widely used in metagenomic studies as biomarkers for methanogenic archaea (Guerrero-Cruz et al., 2021; Thauer, 1998). It is thought that methanogenesis is inhibited during exposure to oxygen due to high oxygen sensitivity of the key enzyme (Bižić et al., 2020) but methane production in oxic conditions has been reported for an increasing number of lakes (Bogard et al., 2014; Grossart et al., 2011; Günthel et al., 2019; Hartmann et al., 2020; Ordóñez et al., 2023; Tang et al., 2014). This contradiction was named the “methane paradox”. Several biotic and abiotic mechanisms have been proposed to explain the “methane paradox” including the formation of microenvironments suitable for classical anaerobic methanogenesis as well as novel pathways (Bižić et al., 2020).

The prevalence and type of anaerobic metabolism are influenced by a range of physicochemical parameters, including substrate composition, nutrient concentrations, pH, nitrogen content, fermentation temperature, salinity, toxic compound load, dissolved oxygen levels, and the profile of volatile fatty acids (Rajendran et al., 2014).

2.4.2. Aerobic and anaerobic methane oxidation

Methane can be oxidized aerobically by methane-oxidizing bacteria (MOB) belonging to classes Alphaproteobacteria (Type II methanotrophs) and Gammaproteobacteria (Type I methanotrophs) and the Verrucomicrobia phylum (Guerrero-Cruz et al., 2021). These organisms convert methane into bicarbonate ions, retaining it in the water column. Aerobic methane oxidation predominantly occurs at the oxic-anoxic interface. First step in methanotrophs' metabolism, oxidation of methane to methanol, is catalyzed by the one of the two enzymes: particulate methane monooxygenase (pMMO) or soluble methane monooxygenase (sMMO) (Khider et al., 2021). An exception is the methanotroph Methylocella, which lacks both enzymes. The gene *pmoA*, encoding the alpha subunit of pMMO, is widely used as a molecular marker in metagenomic studies. After the initial oxidation of methane to methanol, aerobic methanotrophs diverge in subsequent metabolic pathways (RuMP, Calvin–Benson–Bassham [CBB], or serine cycle) to incorporate carbon into biomass. Aerobic methanotrophs can also thrive under oxygen limitation and even in anoxic conditions (Guerrero-Cruz et al., 2021).

In addition to aerobic processes, anaerobic oxidation of methane (AOM) has been identified. AOM can be carried out by the Methylomirabilis genus of the NC10 phylum and members of the Euryarchaeota referred to as anaerobic methane-oxidizing archaea (ANME). Using alternative electron acceptors such as sulfate, nitrite, nitrate, or metal oxides, they oxidize methane via reverse methanogenesis and then transfer electrons to bacterial partners that reduce the terminal electron acceptors (Guerrero-Cruz et al., 2021). It has been reported that ANME uses the reverse methanogenesis pathway to convert methane to CO2 (Versantvoort et al., 2018). Consequently, the *mcrA* gene coding for the alpha subunit of MCR has been widely used as the functional biomarker gene for detecting both methanogens and ANME archaea (Speth & Orphan, 2018). A well-studied example is the syntrophic partnership between sulfate-reducing bacteria and ANME-1 archaea. In this system, electrons from methane oxidation are transferred to the sulfate reducer, which then reduces sulfate to sulfide. Although the energy yield is low, the process remains exergonic and, in energy-limited anoxic sediments such as marine environments, may account for over 90% of methane oxidation (Madigan et al., 2021). Some ANME members, such as ANME-2d, are capable of performing AOM independently by coupling it to nitrate reduction. This group also participates in AOM with metal oxides as electron acceptors (such as chromium oxide) which provides the highest energetic yield among known AOM pathways (Timmers et al., 2017).

2.4.3. Functional markers in methane metabolism

Understanding the microbial mediation of methane metabolism is critical for studying carbon cycling and predicting greenhouse gas fluxes from natural ecosystems. Molecular markers, specific gene sequences associated with key enzymatic functions, have become indispensable tools for identifying and quantifying methane-cycling microbial populations and their activities in diverse environments. These functional genes allow researchers to move beyond taxonomy, enabling insight into metabolic potential and ecological function.

The most widely used marker in methane production is *mcrA*, which encodes the alpha subunit of methyl coenzyme M reductase (MCR)—the enzyme responsible for the final step in methane biosynthesis. Due to its high conservation among methanogenic Archaea and its essential role in methane production, *mcrA* has become the standard functional marker for detecting methanogens in environmental samples (Steinberg & Regan, 2008). Variants of the *mcrA* gene are also found in anaerobic methanotrophic archaea (ANME), allowing researchers to detect anaerobic methane oxidation (AOM) through the same molecular framework (Evans et al., 2015).

For methane oxidation, the *pmoA* gene is the most commonly used functional marker for aerobic methanotrophs. It encodes a subunit of the particulate methane monooxygenase (pMMO), the primary enzyme responsible for methane oxidation under oxic conditions. Due to its ubiquity and diagnostic specificity, *pmoA* has been used extensively for phylogenetic analysis, diversity assessments, and quantification of methanotroph populations (Dumont et al., 2014; Kolb et al., 2003). The mmoX gene, encoding the soluble form of methane monooxygenase (sMMO), serves as a complementary marker in environments where pMMO is absent or repressed (McDonald et al., 2008).

In practical applications, these markers are used in molecular approaches such as qPCR for gene abundance estimation, amplicon sequencing for community profiling, and metagenomics and metatranscriptomics for functional and gene expression analysis. The development and refinement of functional markers have enabled targeted, high-resolution studies of methane metabolism across gradients of climate, trophic status, and environmental conditions. Their continued application is essential for integrating microbial ecology into broader models of carbon cycling and climate feedback mechanisms.

2.5. Study area

Sesasonal variations in microbial communities were analyzed across four lakes located in two distinct climatic regions in Croatia: the Plitivce Lakes in the Alpine biogeographical region and the Baćina Lakes in the Mediterranean biogeographical region (Figure 1). Both lake systems consist of multiple interconnected lakes, but differ in their hydrological, climatic, and trophic properties (Figure 2). The Plitvice Lakes system is hydrologically organized as a cascading chain of 16 karst lakes, descending from 636 m a.s.l. at Lake Prošće to 503 m a.s.l. at Lake Novakovića Brod (Radišić et al., 2021). Lake Prošće, the first lake in the system and the second largest (surface area ≈ 0.68 km², max depth ≈ 37 m), receives inflow from the Matica River (formed by the confluence of Bijela and Crna Rijeka) and drains over the Labudovac tufa barrier through a series of smaller lakes into Lake Kozjak, located at ~534 m a.s.l. (Klaić et al., 2020). Kozjak, the largest and deepest lake in the system (surface area ≈ 0.82 km², max depth ≈ 46 m), serves as the hydrological transition between the Upper and Lower Lakes, discharging toward the Korana River through a series of smaller downstream lakes. According to the Köppen climate classification, the Plitvice Lakes fall within a temperate continental climate with warm summers (Cfb). This climate type 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 & Filipčić, 2003). The Plitvice Lakes are oligotrophic and dimictic, with low concentration of nutrients and dissolved organic matter (DOM) (Kajan et al., 2023), and high levels of dissolved oxygen (Miliša & Ivković, 2023).

Figure 1. Sampling locations. Locations of sampled lakes (indicated by red dots) and their placement within major biogeographical regions in Croatia. The study sites include Lake Kozjak (KOZ) and Lake Prošće (PRO) located in the Alpine region (pink), and Lakes Crniševo (CRN) and Oćuša (OCU) located in the Mediterranean region (orange).

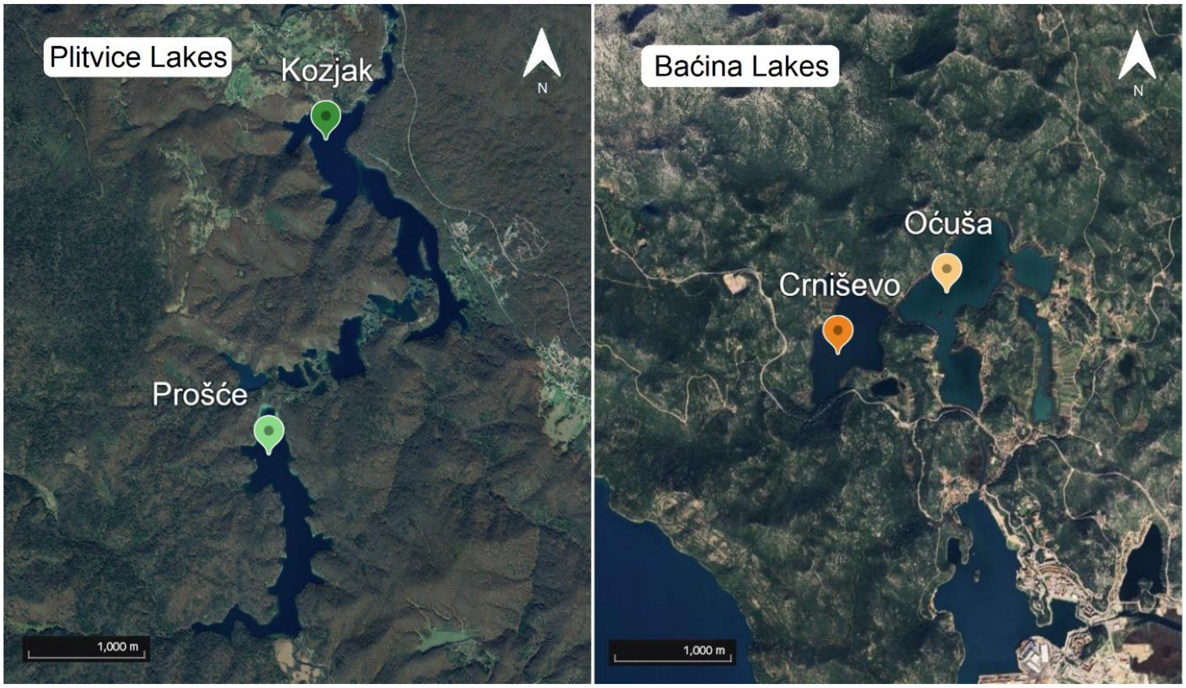

Figure 2. Large scale satellite image of investigated systems: Plitvice Lakes (left) and Baćina Lakes (right). Sampling locations (Kozjak, Prošće, Crniševo, and Oćuša) are labeled.

In contrast, 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 exceeds 22 °C, and the driest period occurs in the warm season, receiving less than 40 mm of precipitation (Miko & Ilijanić, 2015; Šegota & Filipčić, 2003). The Baćina Lakes are a connected system of seven karst cryptodepression lakes covering ~1,38 km², with Crniševo being the deepest (surface area ≈ 0,43 km², max depth ≈ 34 m) and Oćuša the largest by surface extent (surface area ≈ 0,55 km², max depth ≈ 19 m). These lakes are mesotrophic and monomictic, undergoing summer stratification and winter mixing (Hanžek et al., 2021; Miko & Ilijanić, 2015). Due to its cryptodepression nature and karst conduits connecting the aquifer to the Adriatic Sea, the Baćina Lakes system is susceptible to seawater intrusion (Miko & Ilijanić, 2015). The porosity of the limestone, combined with reduced summer precipitation, leads to seasonal declines in groundwater levels, facilitating the intrusion of denser seawater into the aquifer (Alfarrah & Walraevens, 2018; Srzić et al., 2020). Salinization primarily affects Lake Crniševo, where salt water enters through a bottom sinkhole and the underwater salty spring Mindel (Miko & Ilijanić, 2015). Despite its connection to Lake

Crniševo via a narrow channel, Lake Oćuša maintains significantly lower salinity, suggesting limited water exchange between the two lakes (Ognjen; Bonacci & Roje-Bonacci, 2020).

3. MATERIALS AND METHODS

3.1. Sampling and environmental parameters

Water samples were collected from the deepest parts of investigated lakes: Lake Kozjak (KOZ) and Lake Prošće (PRO) within the Plitvice Lakes system and Lake Crniševo (CRN) and Lake Oćuša (OCU) within the Baćina Lakes system. Samples were collected monthly from four lakes over a two-year period, from May 2021 to February 2023. Sampling depths were determined based on the presence of a thermocline or oxycline. When a thermocline was present, samples were taken from at least three depths: above, within and below the thermocline. In the absence of stratification, samples were collected from two depths: approximately 5 m below the surface and 5 m above the bottom. A total of 141 samples were obtained throughout 23 sampling campaigns, distributed as follows: 46 from Lake Crniševo, 35 from Lake Oćuša, and 30 each from Lake Kozjak and Lake Prošće.

Key environmental parameters, including dissolved oxygen (DO), temperature, pH, fluorescent dissolved organic matter (fDOM), turbidity, conductivity, and salinity, were measured *in situ* using an EXO3 Multiparameter Sonde (YSI, Yellow Springs, OH, USA). At each depth, two-liter water samples were collected using a UWITEC water sampler (GmbH, Mondsee, Austria), transported in sterile Nalgene PC bottles, and immediately filtered through polycarbonate membranes (Whatman Nuclepore Track-Etch, 47 mm diameter) with pore sizes of 3 µm for particle-associated (PA) and 0.2 µm for free-living (FL) microbial communities. Sequential filtration was performed using a peristaltic pump (Masterflex, Cole-Palmer, Vernon Hills, IL, USA), resulting in a total of 282 filters, which were stored at -80 °C until DNA extraction. The filtrate was used for ion chromatography (analyzing Ca^{2+}, Mg^{2+}, K^+, Na^+, NH_3^+, SO_4^{2-}, Cl^-, NO_3^-, and PO_4^{3-}) with a Dionex ICS-6000 DC ion chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) and for dissolved organic carbon (DOC) analysis using a QBD1200 analyzer (Hach Company, Loveland, CO, USA).

3.2. DNA extraction and sequencing

Genomic DNA was extracted using the DNeasy PowerWater Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. The hypervariable V4 region of the prokaryotic 16S rRNA gene was amplified via polymerase chain reaction (PCR) using the 515F (Parada et al., 2016) and 806R (Apprill et al., 2015) primer pair. Amplicons were 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 detailed in Pjevac et al., (2021). Demultiplexing was conducted using the demultiplex Python package (Laros, 2023), permitting one mismatch for barcodes and two for linkers and primers. Amplicon sequence variants (ASVs) were inferred using the DADA2 R package v1.42, following the standard workflow (Callahan et al., 2016). FASTQ reads 1 and 2 were trimmed at 220 nt and 150 nt, respectively, with an expected error threshold of 2. Taxonomic classification was performed using the SILVA database (Ref NR 99 release 138.1) via the SINA version 1.7.2 classifier (Pruesse et al., 2012) with a confidence threshold of 0.5. To reduce bias, singletons and ASVs classified as eukaryotes, mitochondria, chloroplasts, or unclassified organisms were excluded from downstream analyses. The amplicon sequencing datasets associated with this research have been deposited in the NCBI repository under BioProject accession number PRJNA1167227.

3.3. Data analyses

All statistical analyses were conducted in R (v.4.4.2, R Core Team, 2024) using functions from the phyloseq (McMurdie & Holmes, 2013), vegan (Oksanen et al., 2018), and ggplot2 (Wickham, 2016) packages.

Microbial community diversity was assessed using both alpha and beta diversity indices at the ASV level. The dataset was rarefied to an equal sequencing depth (>5000 reads per sample). Alpha diversity was quantified using observed, Chao1, and Shannon indices, with group comparisons performed via the Kruskal-Wallis test. Pairwise significance testing was applied to compare specific sample groups. Beta diversity was estimated using Bray-Curtis dissimilarity, and non-metric multidimensional scaling (NMDS) was used to visualize differences in microbial community composition. Statistical significance between groups was determined via permutational multivariate analysis of variance (PERMANOVA, 999 permutations). Additionally, site-specific analyses were conducted separately for each lake to assess the effects of fraction type, oxygen concentration, temperature, sampling month, and season on microbial beta diversity using PERMANOVA. Hierarchical clustering was performed to examine sample relationships based on ASV abundances. Hellinger transformation was applied for normalization, and Bray-Curtis dissimilarities were calculated. Clustering was performed using the complete linkage method, with results visualized as a dendrogram, annotated with sampling points and fraction types. To evaluate the influence of environmental factors on microbial community composition, Pearson's correlations were used with a Mantel test. The relationship between microbial communities and environmental parameters was further explored using Hellinger-transformed OTU tables, standardized (Z-score transformation), and visualized with network-style correlation plots. A co-occurrence network was constructed using the integrated network analysis pipeline (Feng et al., 2022). Because microbial community data were non-normally distributed, Spearman's rank correlation was applied to evaluate pairwise associations among taxa. Correlations with coefficients $|R| > 0.6$ and p-values < 0.01 were considered strong and statistically significant. To confirm that the constructed ecological network was not generated by random associations, 100 random networks with equivalent size and connectivity were generated for comparison using a z-test. The resulting networks were visualized in Gephi (v0.10.1), and key topological properties (average degree, clustering coefficient, average path length, and modularity) were calculated to characterize overall network structure.

3.4. Quantitative PCR

Functional gene abundances of the total microbial community, methanotrophs and methanogens were determined using qPCR (Table 1). The analysis was conducted with a CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA). Extracted genomic DNA from each sample and linearized plasmid standards were analysed in triplicate to ensure reproducibility. Specific primer pairs targeting 16S rRNA (515F and 806R), *pmoA* (A189F and mb661R), and *mcrA* (mlas and mcrA-rev) genes were used to assess microbial community composition and functional potential. Cycling conditions followed a three step protocol, including an initial denaturation, followed by amplification and melt curve analysis to verify product specificity. To confirm amplicon specificity, melt curve analysis was performed from 60°C to 96°C using Bio-Rad CFX Maestro software, with fluorescence data collected at 0.5°C increments. Negative controls (no template controls) were included in each run to monitor contamination. Standard curves were generated for each gene target to assess PCR efficiency and ensure accurate quantification. Data analysis included the calculation of gene copy numbers per nanogram of DNA, based on the standard curves and sample Ct values.

Table 1. Optimized quantitative PCR protocol specifications for targeting three functional genes: 16S rRNA, *pmoA* and *mcrA*. Listed are the forward and reverse primers with their sequences, number of PCR cycles, denaturation, annealing, and elongation conditions, final primer concentrations, expected amplicon lengths, and relevant literature references.

Target gene	**16S rRNA**	***pmoA***	***mcrA***
Primers	505F 806R	A189F Mb661R	mlas mcrA-rev
Sequence (5’-3’)	GTGYCAGCMGCGCGGTAA GGACTACNVGGGTWTCTAAT	GGNGACTGGGACTTCTGG CCGGMGCAACGTCYTTACC	GGTGGTGTMGGDTTCACMCARTA CGTTCATBGCGTAGTTVGGRTAGT
Cycles	39	42	40
Denaturation	95°C, 30 s	95°C, 10 s	95°C, 10 s
Hybridization	50°C, 1 min	62°C, 1 min	57°C, 20 s
Elongation	72°C, 45 s	87°C, 8 s	72°C, 30 s
Final primer concentration (µM)	0.5	0.5	0.5
Amplicon length (bp)	292	508	494
References	Parada et al., 2016 Apprill et al., 2015	Kolb et al., 2003	Steinberg and Regan, 2008

3.5. Enrichment experiment

To examine the effects of salinization on prokaryotic communities, an enrichment experiment was conducted using a water sample from Lake Crniševo collected in September 2022. Serum vials containing 45 mL of NMS medium were flushed with nitrogen (N_2), inoculated with 5 mL of sampled lake water from 22 m depth, and supplemented with 5 mL of saline solution. Methane was then added to create a 20% methane atmosphere. The experiment was set up in triplicate for four different salinity levels: 0, 2, 13, and 20. The serum vials were crimp-sealed and incubated at *in situ* temperature for two years. Methane concentrations were measured at three time points (September 2023, March 2024, and October 2024) using a 7890B GC System (Agilent Technologies, Santa Clara, CA, USA). In January 2025, samples were filtered through 0.2 µm pore-size polycarbonate membranes (Whatman Nuclepore Track-Etch, 47 mm diameter). DNA extraction, sequencing, and data analysis were performed following previously described protocols.

4. RESULTS

4.1. Composition and diversity of prokaryotic communities

Sequencing of 16S rRNA gene amplicons yielded 5716 unique ASVs, with sequencing depths ranging from 26 to 33463 reads per sample. Following quality filtering and rarefaction, 3945 ASVs were retained for further analysis. These ASVs were classified into 58 distinct phyla, with the most dominant being Proteobacteria (24.98%), Verrucomicrobiota (18.97%), Actinobacteriota (16.41%), Cyanobacteria (15.07%), and Bacteroidota (11.52%).

Hierarchical clustering of the samples revealed two primary clusters corresponding to the lake systems, with further subdivision based on the fraction (Figure 3). However, some exceptions were observed, where certain samples from Baćina Lakes clustered with those from Plitvice Lakes.

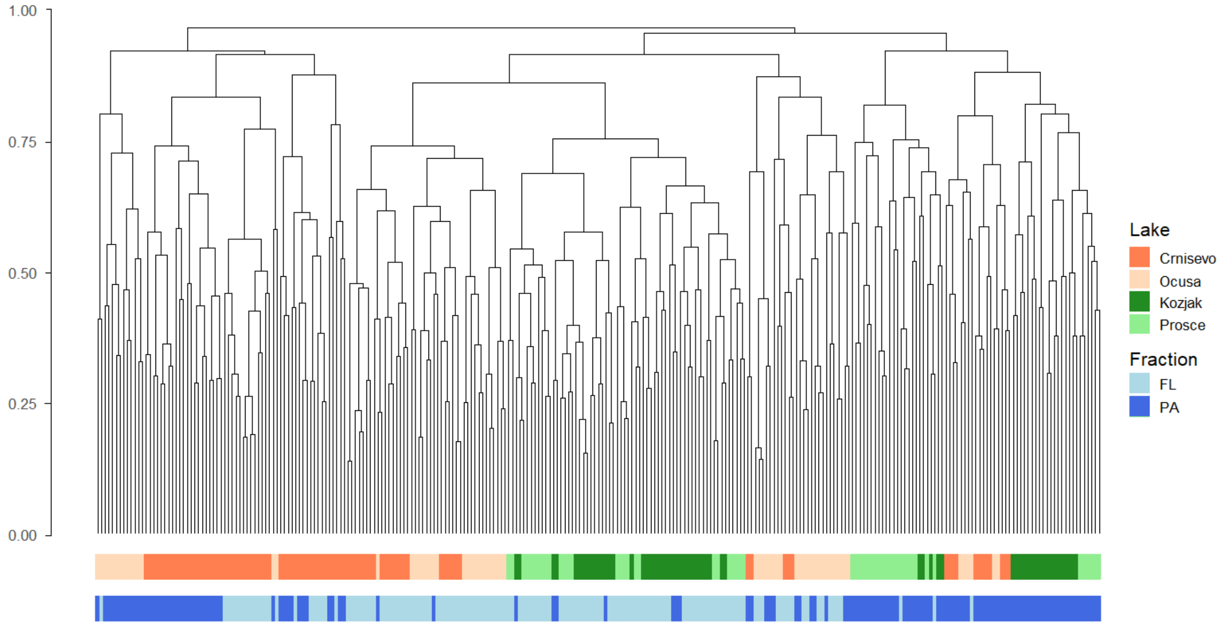

Figure 3. Hierarchical clustering dendrogram showing the similarity of prokaryotic community compositions based on 16S rRNA gene amplicon data across all lake samples. Each line represents a sample and colored bars showcase sample origin: the top bar denotes the lake (Crniševo, Oćusa, Kozjak, Prošće), and the bottom bar shows the microbial size fraction (FL, PA).

Microbial community richness (observed) and diversity (Shannon) were higher in Baćina Lakes compared to Plitvice Lakes (Figure 4). While observed richness measures were similar between FL and PA communities, Shannon diversity varied between fractions (Figure 4). Non-metric multidimensional scaling (NMDS) demonstrated clear grouping of microbial communities by lake ecosystem and fraction (Figure 5).

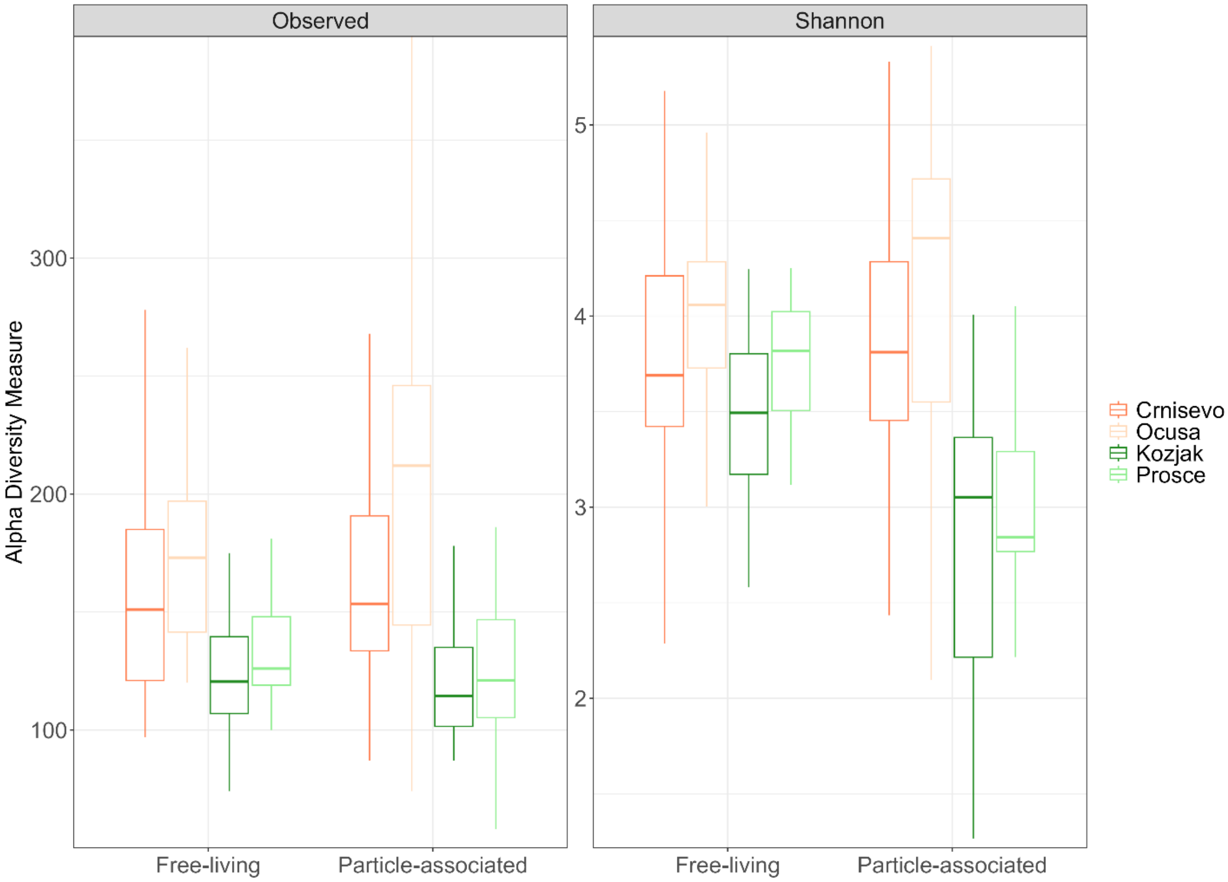

Figure 4. Alpha diversity of microbial communities across lakes and size fractions using two diversity metrics: observed ASVs reflecting richness and Shannon index reflecting richness and evenness. Samples are grouped by lake and fraction.

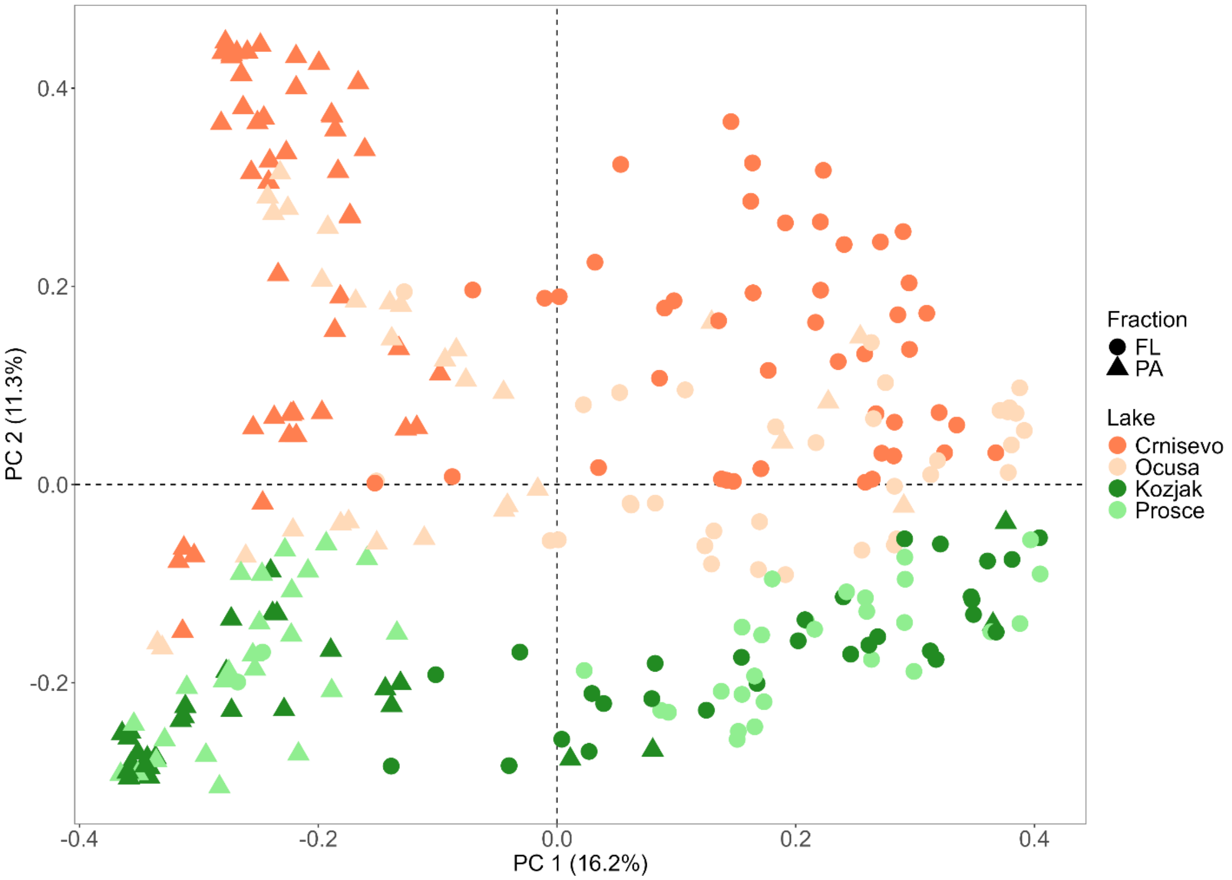

Figure 5. Principal Coordinates Analysis of prokaryotic community composition based on Bray-Curtis dissimilarity across four studied lakes. Samples are colored by lake (Crniševo and Oćusa in shades of orange, Kozjak and Prošće in shades of green) and shaped by fraction: free-living (circles) and particle-associated (triangles). The first two principal coordinate axes (PC1 and PC2) explain 16.2% and 11.3% of the total variation, respectively.

PERMANOVA analysis indicated that differences between lakes accounted for more variation (14.37%) than differences between fractions (10.98%) (Table 2). Even though differences between sampling months explain the same amount of variation as differences between lakes, their F-value is lower (4.76 compared to 14.8) indicating higher within group variations. Site-specific variations were primarily driven by sampling month, which explained the highest proportion of variation across all lakes (R^2 = 24–35%). Fraction was a key factor in shaping community composition in Lakes Crniševo, Kozjak, and Prošće (R^2 = 17–23%), whereas its influence was lower in Lake Oćuša (R^2 = 9%). Temperature had a consistent effect across all lakes (R^2 = ~5%–9%), while oxygen concentration was a significant factor only in Lake Crniševo (R^2 = 7.2%).

Table 2. Results of PERMANOVA analysis showing the effects of environmental and categorical variables on prokaryotic community composition across all samples (Whole community) and within individual lakes (Lake Crniševo, Lake Oćuša, Lake Kozjak, and Lake Prošće). The table includes the coefficient of determination (R²), F-values, and significance levels (*** $p \leq 0.001$) for each tested variable.

Whole community	**Variable**	**R^2**	**F-value**	**Pr (>F)**
	Lake	0.14374	14.828	0.001 ***
	Month	0.14196	4.76	0.001 ***
	Fraction	0.10977	32.92	0.001 ***
	Sulfate	0.08216	23.90	0.001 ***
	Salinity	0.07119	19.93	0.001 ***
	DOM	0.0522	13.55	0.001 ***
	Oxygen	0.04188	11.36	0.001 ***
	Temperature	0.03642	10.09	0.001 ***
Crnisevo	**Variable**	**R^2**	**F-value**	**Pr (>F)**
	Month	0.24516	3.8509	0.001 ***
	Fraction	0.1781	19.286	0.001 ***
	Temperature	0.07734	7.4598	0.001 ***
	Oxygen	0.07264	3.4463	0.001 ***
Ocusa	**Variable**	**R^2**	**F-value**	**Pr (>F)**
	Month	0.34953	4.3755	0.001 ***
	Fraction	0.09646	6.7254	0.001 ***
	Temperature	0.08988	6.2219	0.001 ***
	Oxygen	0.05914	3.9598	0.001 ***
Kozjak	**Variable**	**R^2**	**F-value**	**Pr (>F)**
	Month	0.24686	2.8954	0.001 ***
	Fraction	0.20471	14.929	0.001 ***
	Temperature	0.05014	3.0619	0.001 ***
	Mixing	0.03852	2.3236	0.001 ***

Prosce	**Variable**	**R^2**	**F-value**	**Pr (>F)**
	Month	0.26729	2.7968	0.001 ***
	Fraction	0.20724	13.332	0.001 ***
	Temperature	0.08424	4.6916	0.001 ***
	Sulfate	0.06061	3.2903	0.001 ***

4.2. Seasonal and spatial distribution of prokaryotic communities

Based on prior analyses, including PERMANOVA and beta diversity, which revealed significant differences between free-living and particle-associated prokaryotic communities, we analysed the distribution of the two fractions separately to highlight the differences in taxonomic compositions. The five most abundant phyla exhibited shifts in dominance throughout the sampling period (Figure 6 and 7). Their vertical and temporal distribution varied across the four studied lakes and fractions. Notably, Cyanobacteria and Proteobacteria alternated as the dominant phyla in the Baćina Lakes in both fractions. Similar trends were reported in Lake Kozjak in the free-living fraction, while Proteobacteria and Verrucomicrobiota alternated in dominance in the particle-associated fraction as well as both fractions in Lake Prošće.

In the free-living fraction, Actinobacteriota were consistently abundant during summer and autumn months in all studied lakes. They occupied surface waters in summer months of the first sampling year in Plitvice Lakes, while appearing throughout the column in the second sampling year. In Baćina Lakes, Actinobacteriota occupied the whole column in summer months of the first sampling year and only bottom layers in the second sampling year. In contrast, Actinobacteriota were nearly absent in the particle-associated fraction across all lakes.

Proteobacteria were one of the most dominant phyla in both fractions, though their distribution varied with depth and time. In the free-living fraction, Proteobacteria were particularly abundant throughout the water column in Plitvice Lakes, while in Baćina Lakes they alternated in dominance with Cyanobacteria occupying the water column in summer and autumn months. In the particle-associated fraction, Proteobacteria peaked in summer months occupying depths around the thermocline in Baćina Lakes. In Lake Prošće, Proteobacteria were dominant in surface waters in summer months, while in Lake Kozjak they peaked in winter months when the water column was mixed.

Cyanobacteria showed a more dynamic pattern between fractions and depths. In the free-living fraction, Cyanobacteria peaked at thermocline depths during the summer and autumn months of the second sampling year in all of the studied lakes except Lake Prošće where they were in very low abundance. In the particle-associated fraction, Cyanobacteria were found throughout the water column in summer and autumn months. In Lake Oćuša they peaked in winter months of the second sapling year.

Bacteroidota were generally less dominant than other phyla occupying surface waters in winter months in the free-living fraction in all lakes. On the contrary, in the particle-associated fraction, they peaked in summer and autumn months.

Verrucomicrobiota were primarily associated with particle-attached communities and were higher in abundance in Plitvice Lakes where they dominated the whole water column, particularly during the spring and summer months. In contrast, they were minimally present in the free-living fraction, indicating a strong ecological preference for particle-rich environments.

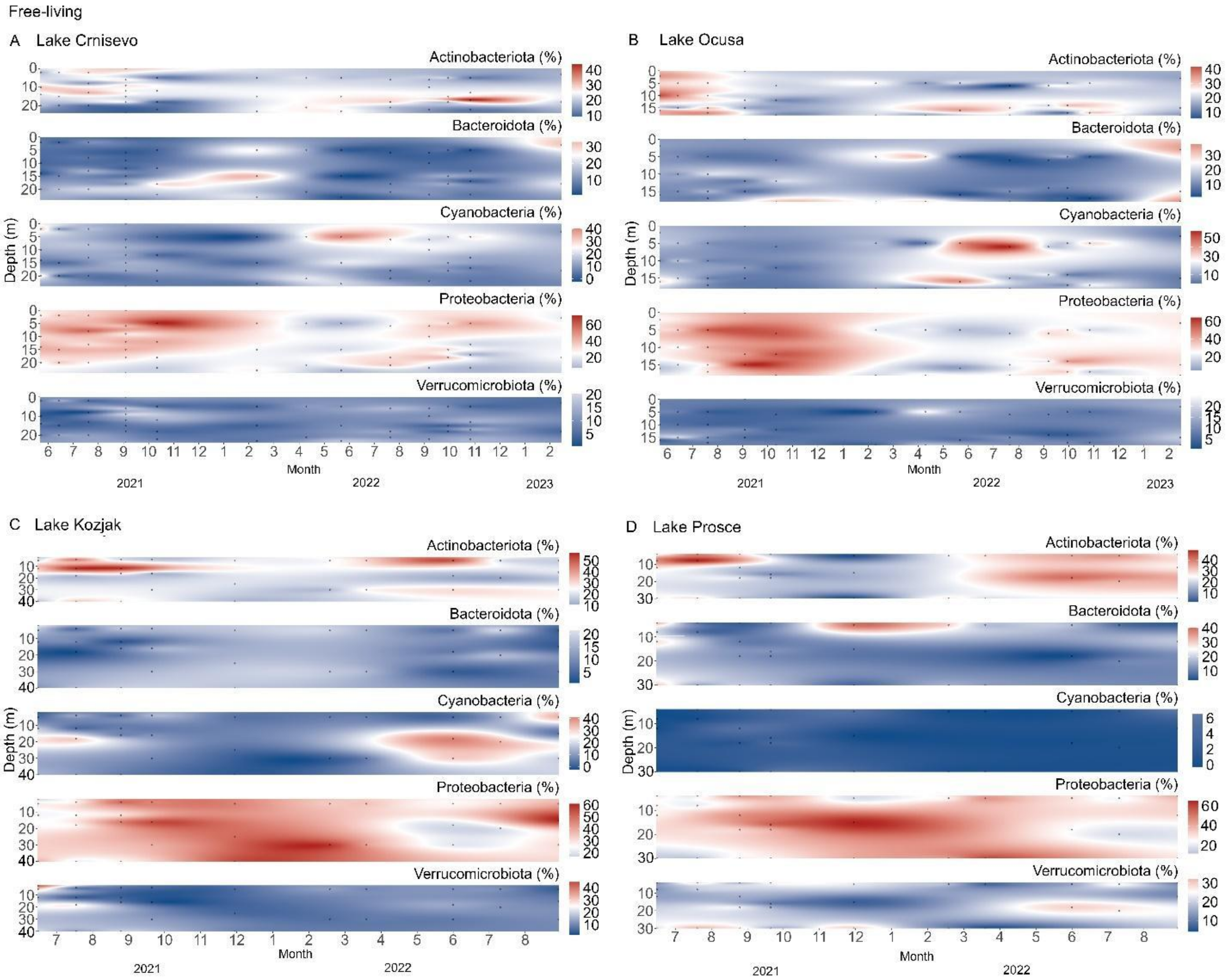

Figure 6. Relative abundance of major prokaryotic phyla in the free-living fraction across sampling months and depths in four studied lakes. Warmer colors indicate higher relative abundance.

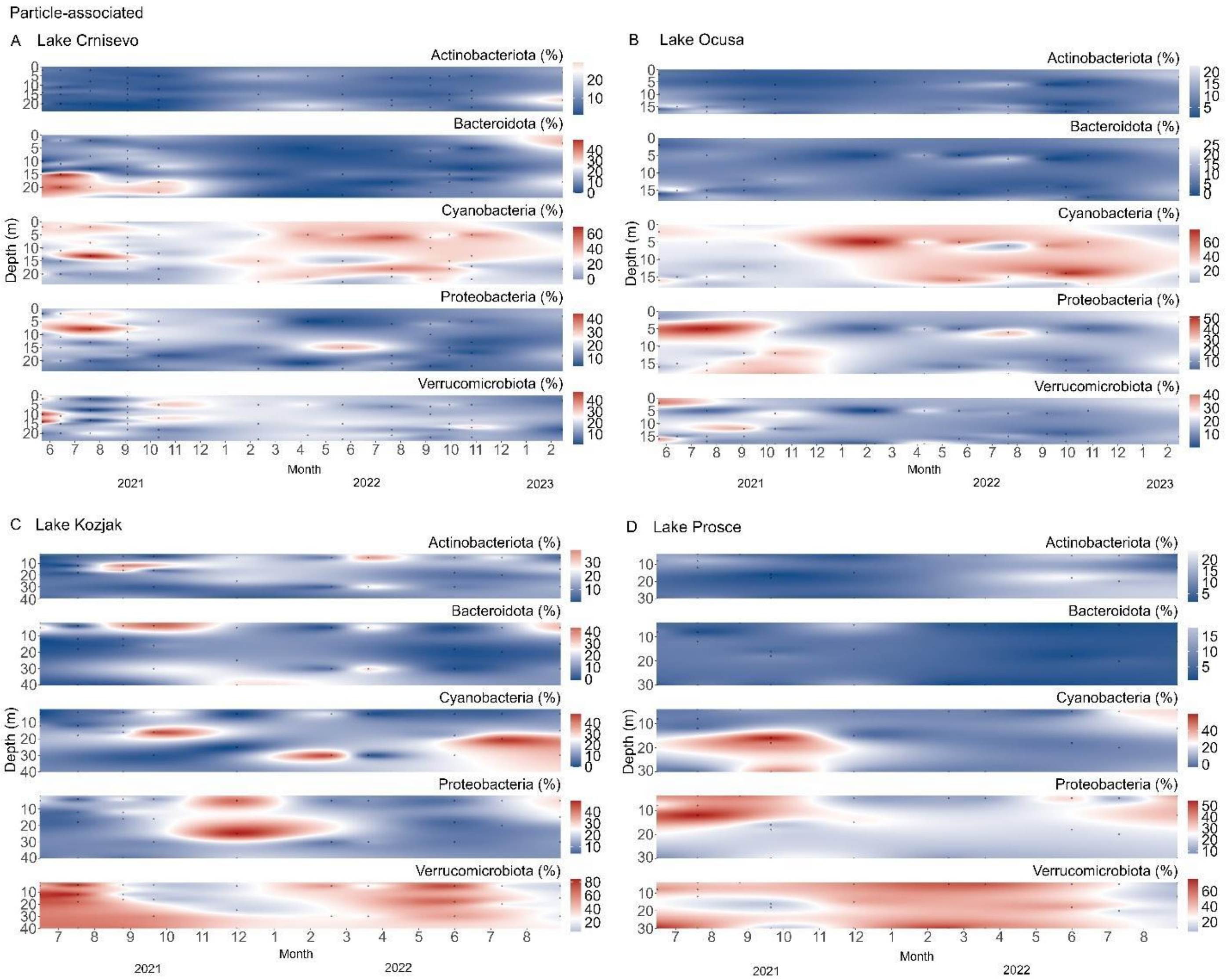

Figure 7. Relative abundance of major prokaryotic phyla in the particle-associated fraction across sampling months and depths in four studied lakes. Warmer colors indicate higher relative abundance.

In microbial co-occurrence networks, nodes represent individual ASVs, with larger nodes corresponding to highly connected taxa (hubs) that may hold important ecological functions. Edges denote significant co-occurrence relationships, indicating potential ecological associations among taxa. Modularity reflects the degree of network clustering, where higher modularity values suggest a more compartmentalized community structure, possibly shaped by environmental selection or functional specialization. Co-occurrence network analysis showed that positive associations dominated in all four lakes, forming cohesive green cores with fewer localized red edges (Figure 8). Lake Crniševo displayed the most heterogeneous structure, with multiple medium-to-large hubs distributed across several modules and numerous green bridges connecting compartments. Actinobacteriota, Proteobacteria, Bacteroidota, and Verrucomicrobiota occupied connector positions, while negative edges were concentrated at module margins. Lake Oćuša exhibited an elongated, less compact network organized around a dense central ribbon dominated by Actinobacteriota and Proteobacteria hubs and a sparsely connected periphery with short red edges extending toward the core. Lake Kozjak formed the most compact lattice, characterized by large Proteobacteria hubs embedded in a dense mesh of positive links and few peripheral negative edges. Lake Prošće showed a moderately compact core co-dominated by Proteobacteria and Actinobacteriota, with mid-sized hubs from Bacteroidota and Verrucomicrobiota and interspersed red edges within both core and periphery. Across all networks, Proteobacteria and Actinobacteriota consistently appeared as central hubs. Baćina networks were more heterogeneous and bridge-rich, whereas Plitvice networks, particularly Kozjak, were more compact and hub-centric.

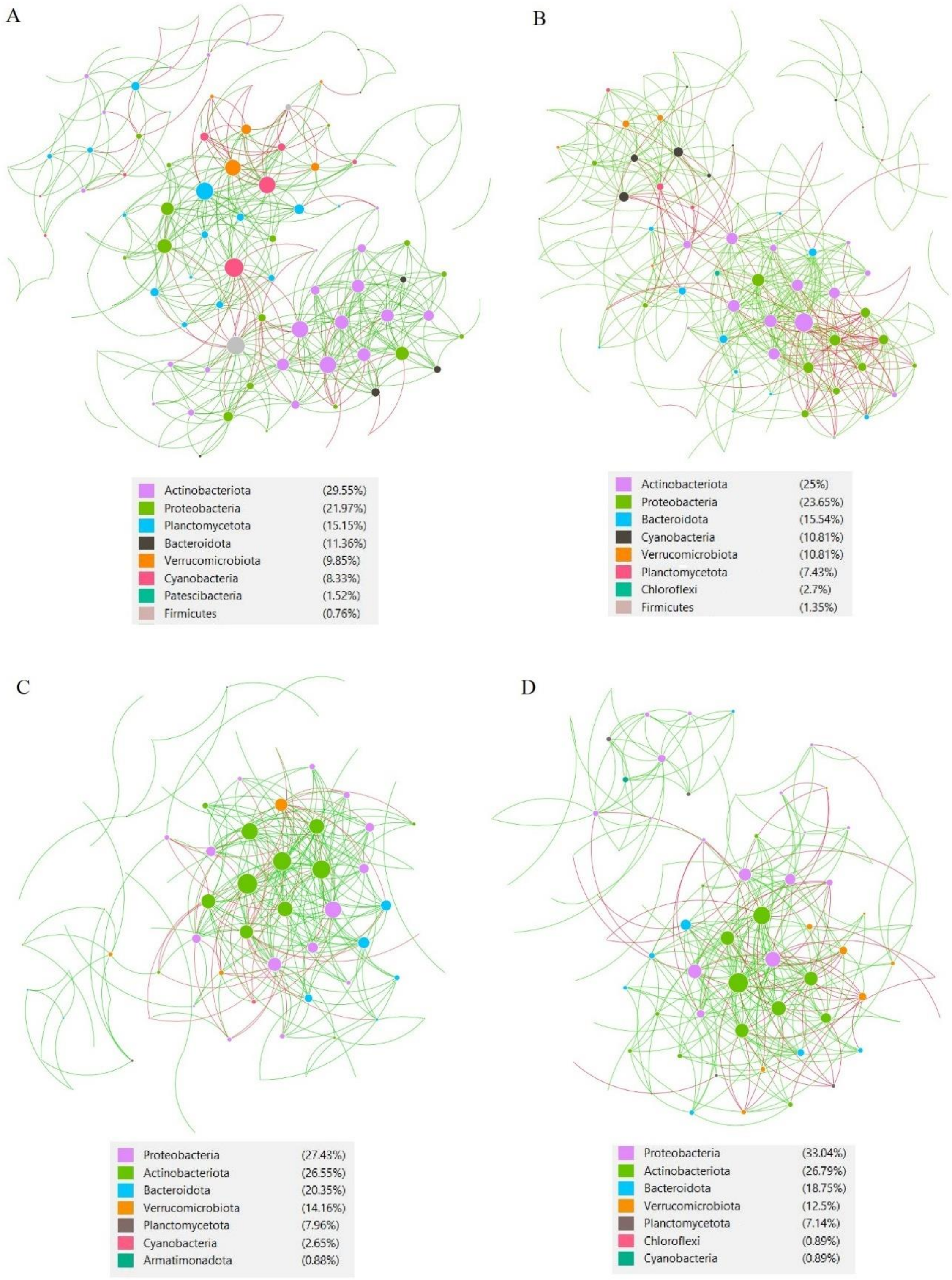

Figure 8. Co-occurrence network of bacterial communities. Visualization of microbial network patterns in four investigated lakes with the top 8 phyla represented by different colors. (A) Lake Crniševo, (B) Lake Oćuša, (C) Lake Kozjak, (D) Lake Prošće.

4.2.1. Methane-cycling microbial communities

The distribution patterns of methanotrophs and methanogens exhibited marked spatial and temporal variability across the four lakes (Figure 9). In Lake Crnisevo, methanotrophs were generally low in abundance, with only a transient peak in the upper 5 m during late summer 2021, while methanogens displayed a pronounced presence at 20 m depth during autumn 2021, suggesting potential anaerobic conditions or increased methane availability at that time and depth. Lake Ocusa showed a sharp peak in methanotrophs at 10 m depth during late autumn 2021, which coincided with the appearance of methanogens in the hypolimnion in early 2022, hinting at a potential spatial and temporal coupling between methane production and oxidation.

In contrast, Lakes Kozjak and Prosce revealed more consistent methanotrophic activity at deeper depths. In Kozjak, methanotrophs peaked at 20–25 m during autumn 2021 and early 2022, while methanogens remained nearly undetectable throughout the study period, suggesting limited methane production or strong oxidative filtering in this system. Similarly, in Lake Prosce, methanotrophs were elevated at 20 m in late 2021 and again in early 2022, while methanogens were only sporadically detected at low levels, primarily during winter 2022.

Overall, methanogens appeared more prominent in the shallower lakes (Crnisevo and Ocusa), where temporal anoxia may have supported their proliferation, whereas deeper and more stratified lakes (Kozjak and Prosce) favored methanotrophs at depth, with minimal methanogenic signatures. These trends suggest differing methane cycling dynamics between lake systems, likely driven by depth, oxygen availability, and thermal stratification.

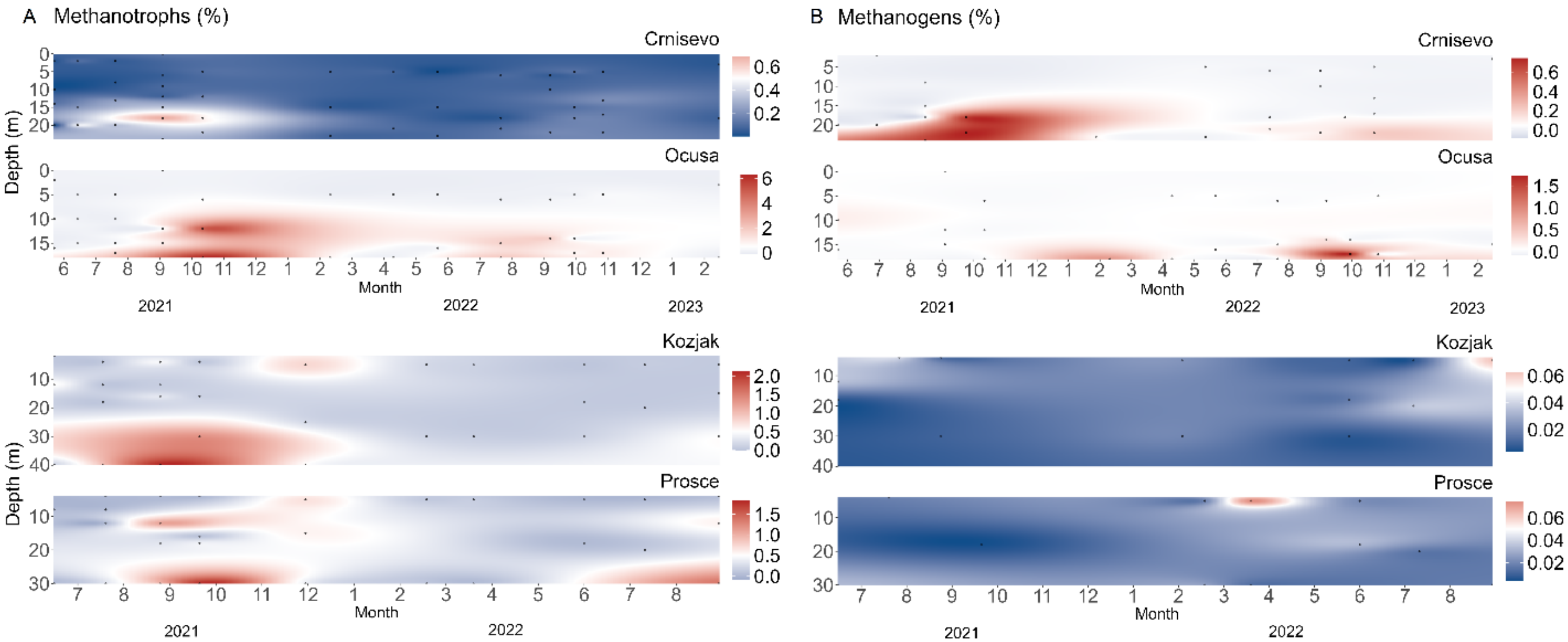

Figure 9. Relative abundance of methanotrophs and methanogens across sampling months and depths in four studied lakes. Warmer colors indicate higher relative abundance.

Functional group-specific PERMANOVA analysis showed distinct structuring patterns (Tables 3 and 4). Methanogens were primarily influenced by month ($R^2 = 11.1\%$), lake (7.2%), and oxygen, sulfate, and DOM, though these explained less variance individually (<6%). Notably, the F-values for methanogens were low ($F < 5$), indicating more diffuse group separation. In contrast, methanotrophs were strongly structured by lake ($R^2 = 14.8\%$, $F = 13.5$), followed by salinity, oxygen, and DOM (each ~4–6% R^2), with relatively high F-values (up to 16.5), suggesting sharper environmental filtering.

Table 3. Results of PERMANOVA analysis showing the effects of environmental and categorical variables on methanogenic community composition across all samples. The table includes the coefficient of determination (R^2), F-values, and significance levels (*** $p \leq 0.001$) for each tested variable.

Methanogens	**Variable**	**R^2**	**F-value**	**Pr (>F)**
	Month	0.11121	1.2651	0.001 ***
	Lake	0.07229	2.5193	0.001 ***
	Oxygen	0.05369	2.7516	0.001 ***
	Sulfate	0.04504	4.6696	0.001 ***
	DOM	0.0345	3.3593	0.001 ***
	Fraction	0.03069	3.1348	0.001 ***
	Salinity	0.02515	2.5284	0.001 ***
	Temperature	0.01871	1.8876	0.001 ***
	Mixing	0.01408	1.4135	0.001 ***
	Depth	0.01315	1.3188	0.001 ***

Table 4. Results of PERMANOVA analysis showing the effects of environmental and categorical variables on methanotrophic community composition across all samples. The table includes the coefficient of determination (R^2), F-values, and significance levels (*** $p \leq 0.001$) for each tested variable.

Methanotrophs	**Variable**	**R2**	**F**	**Pr(>F)**
	Lake	0.14839	13.5330	0.001 ***

	Month	0.0715	1.9424	0.001 ***
	Salinity	0.0673	16.5230	0.001 ***
	Oxygen	0.05053	11.8670	0.001 ***
	DOM	0.04909	11.2010	0.001 ***
	Sulfate	0.04592	11.3110	0.001 ***
	Temperature	0.04115	10.0850	0.001 ***
	Fraction	0.04009	9.2150	0.001 ***
	Depth	0.03098	7.5124	0.001 ***
	Year	0.02198	5.2824	0.01 *

4.3. Environmental factors

Vertical profiles of temperature, dissolved oxygen (DO), fluorescent dissolved organic matter (fDOM), and salinity revealed distinct stratification and mixing dynamics across the four lakes (Figure 10). Lake Crnisevo and Lake Ocusa exhibited similar seasonal stratification patterns, with mixing periods extending from December to April. During these months, thermal and oxygen gradients diminished across depth, indicating full water column mixing. In Lake Crnisevo, this was accompanied by a reduction in salinity gradients and a more homogeneous distribution of fDOM. In contrast, Lake Kozjak and Lake Prosce showed extended mixing periods, spanning from November to May. These lakes experienced more prolonged isothermal conditions, particularly in the upper 20–30 meters, with minimal stratification in temperature and DO during the mixing period. fDOM concentrations remained relatively low and stable throughout the water column in both lakes. Overall, the observed mixing periods coincide with winter and early spring, driven by decreased surface temperatures and increased wind-induced mixing, leading to vertical homogenization of physicochemical parameters.

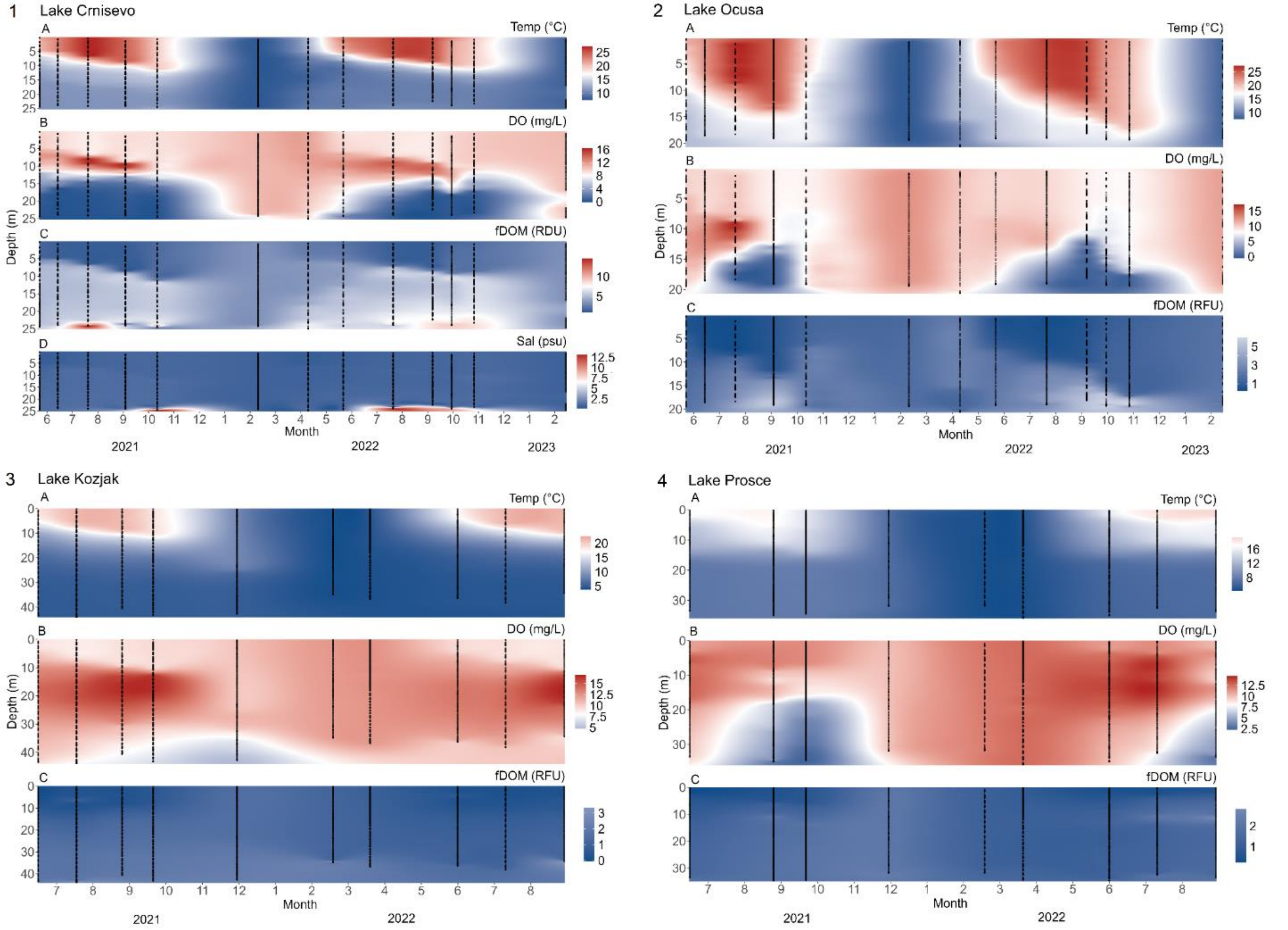

Figure 10. Key environmental parameters (temperature, dissolved oxygen, dissolved organic matter, and salinity) in four studied lakes across seasons and depths. Warmer colors indicate higher values.

Ion composition in the studied lakes varied with depth, time, and between lakes, reflecting seasonal patterns and stratification dynamics (Figure 11). Salinity indicators, chloride and sulfate, were notably higher in Baćina Lakes than in Plitvice Lakes. In particular, chloride concentrations in Lake Crniševo were thirty times higher than in Lake Oćuša, while sulfate levels remained similar in both. In Lake Crniševo, chloride concentrations increased with depth and were particularly elevated in the summer months, peaking in autumn 2021. Sulfate concentrations in Baćina Lakes were up to twenty times higher than in Plitvice Lakes, ranging from 13.5 mg/L to 141 mg/L, compared to 2.5 mg/L to 6.3 mg/L. Nitrate concentrations across all lakes reached a maximum of approximately 2.5 mg/L, though in most Baćina Lakes samples, nitrate levels were below the detection limit. A summary statistics of the measured physico-chemical parameters for each lake is shown in Table 5.

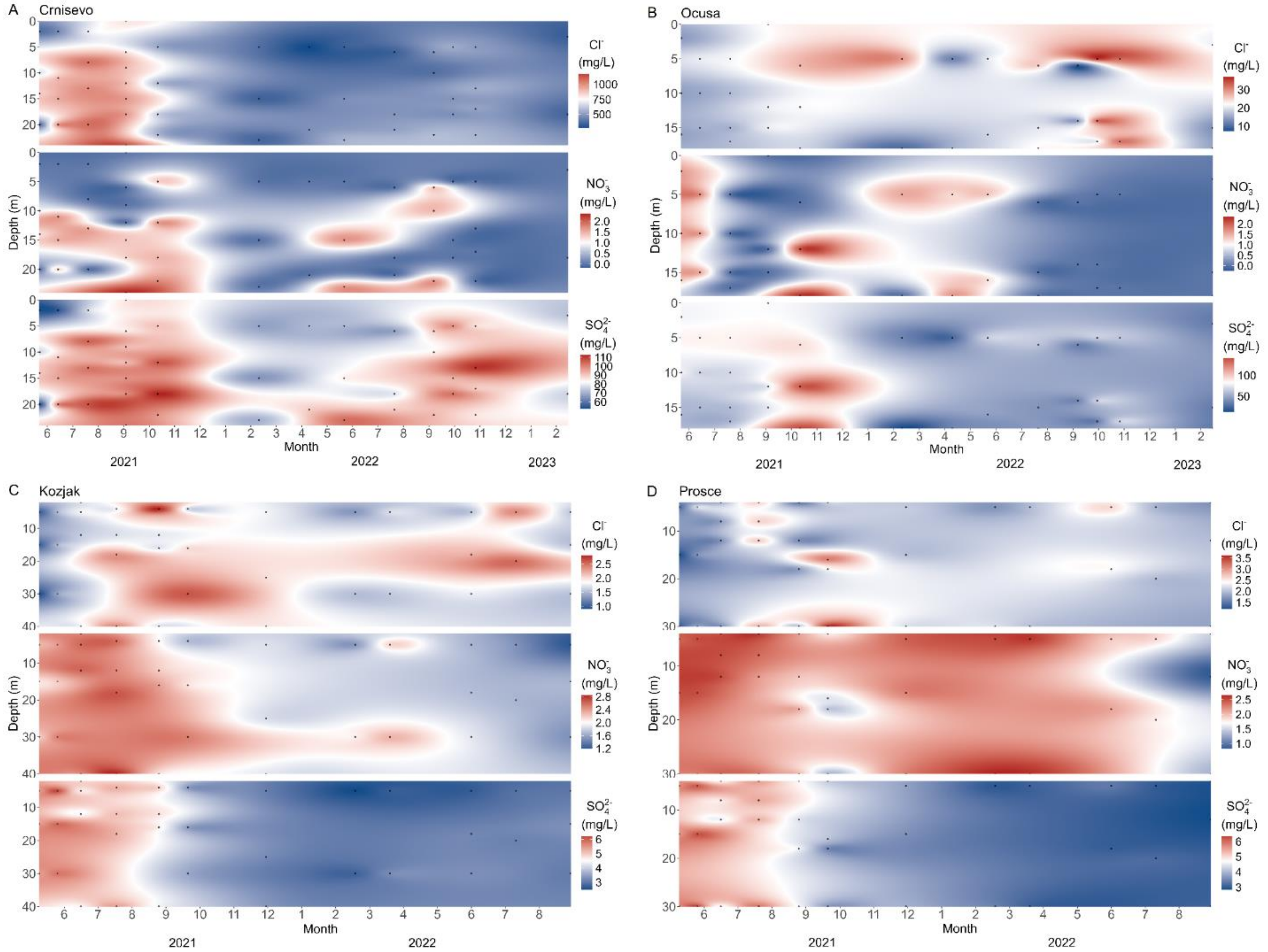

Figure 11. Distribution of chloride (Cl^-), nitrate (NO_3^-), and sulfate (SO_4^{2-}) concentrations in four studied lakes across seasons and depths. Warmer colors indicate higher values.

Table 5. Summary statistics table for key environmental parameters in four studied lakes showing mean, median, standard deviation (Std), minimum (Min), maximum (Max) and sample count.

Parameter	**Lake**	**Mean**	**Median**	**Std**	**Min**	**Max**	**Count**
Cl^- (mg/L)	Crniševo	575.78	542.39	241.67	276.51	1143.32	46
	Oćuša	18.93	20.21	6.36	8.54	35.54	35
	Kozjak	1.72	1.71	0.42	0.90	2.80	30
	Prošće	2.01	1.98	0.49	1.20	3.57	30
SO_4^{2-} (mg/L)	Crniševo	85.68	89.72	14.67	53.10	112.40	46
	Oćuša	55.16	61.00	27.04	13.45	141.22	35
	Kozjak	3.98	3.99	1.09	2.48	6.20	30
	Prošće	4.19	4.33	1.09	2.83	6.30	30
fDOM (RFU)	Crniševo	3.86	5.01	1.84	1.17	7.84	46
	Oćuša	1.59	1.85	1.09	0.30	5.26	35
	Kozjak	1.05	0.99	0.60	0.04	2.19	30
	Prošće	1.26	1.24	0.52	0.57	2.48	30
ODO (mg/L)	Crniševo	3.79	8.86	4.43	0.12	15.64	46
	Oćuša	6.62	9.16	3.82	0.50	16.19	35
	Kozjak	11.42	11.81	2.46	5.96	16.56	30
	Prošće	9.85	11.23	2.67	3.01	13.48	30
Salinity (psu)	Crniševo	1.00	1.06	0.22	0.57	1.85	46
	Oćuša	0.23	0.22	0.02	0.20	0.28	35
	Kozjak	0.18	0.18	0.01	0.17	0.20	30

	Prošće	0.20	0.20	0.01	0.18	0.21	30
Temperature (°C)	Crniševo	13.41	12.05	5.63	7.59	26.91	46
	Oćuša	16.45	16.83	5.39	7.58	26.92	35
	Kozjak	8.50	7.81	5.84	3.93	21.45	30
	Prošće	9.32	8.46	4.22	4.40	20.32	30

In Lake Crniševo, both PA and FL microbial communities exhibited a positive correlation with calcium ion concentrations, while the PA community also correlated positively with chloride (Figure 12A). Salinity showed a positive correlation with magnesium, chloride, sulfate, and nitrate, while negatively correlating with oxygen. Additionally, calcium concentration was negatively correlated with temperature and chloride. In Lake Oćuša, fDOM displayed a negative correlation with oxygen (Figure 12B). Sulfate and nitrate were positively correlated with the PA community, whereas chloride was positively associated with the FL community. In Lake Kozjak, sulfate and nitrate showed a negative correlation with calcium and magnesium concentrations (Figure 12C). Both PA and FL communities were positively correlated with calcium, magnesium, nitrate, and sulfate. In Lake Prošće, the PA community was positively correlated with calcium, magnesium, and sulfate, while the FL community correlated only with sulfate (Figure 12D). Regarding environmental parameters, calcium concentrations were negatively correlated with sulfate and temperature but positively correlated with magnesium.

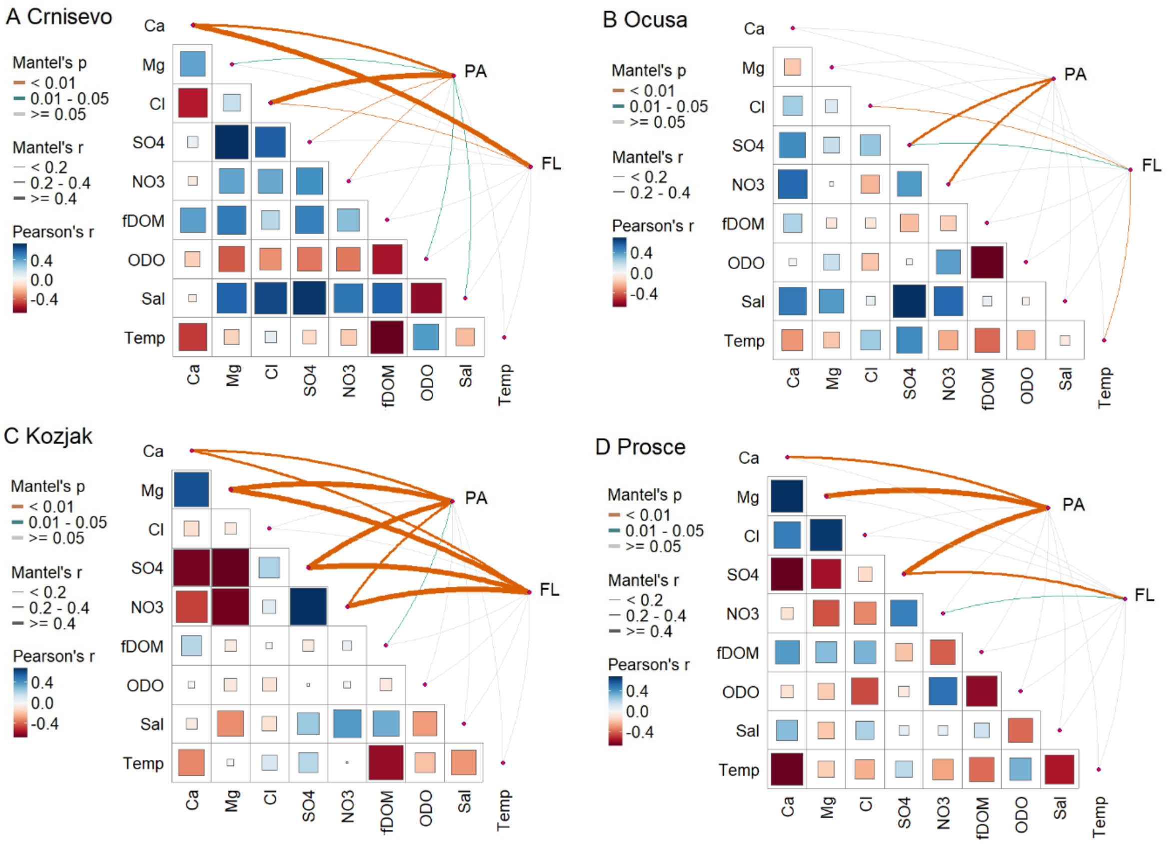

Figure 12. Pairwise comparison between environmental factors with a color gradient representing Pearson’s correlation coefficients for each lake. Partial Mantel test was performed for FL and PA communities and each environmental factor, respectively. Line width indicates the partial Mantel’s r statistic for corresponding correlations, with the larger values indicating stronger correlations and line color indicating significance.

Canonical correspondence analysis (CCA) showed clear separation in microbial community structure among the four lakes, with environmental gradients driving this variation (Figure 13). Plitvice Lakes associated with higher nitrate (NO_3^-) concentrations and elevated dissolved oxygen (ODO). In contrast, Baćina Lakes aligned more closely with higher concentrations of sulfate (SO_4^{2-}), calcium (Ca), magnesium (Mg), salinity (Sal), fDOM, and temperature.

Moreover, free-living (FL) and particle-associated (PA) microbial fractions (circles and triangles, respectively) showed partial overlap, but with some separation along the CCA axes, suggesting niche differentiation by lifestyle type across lakes and environmental gradients.

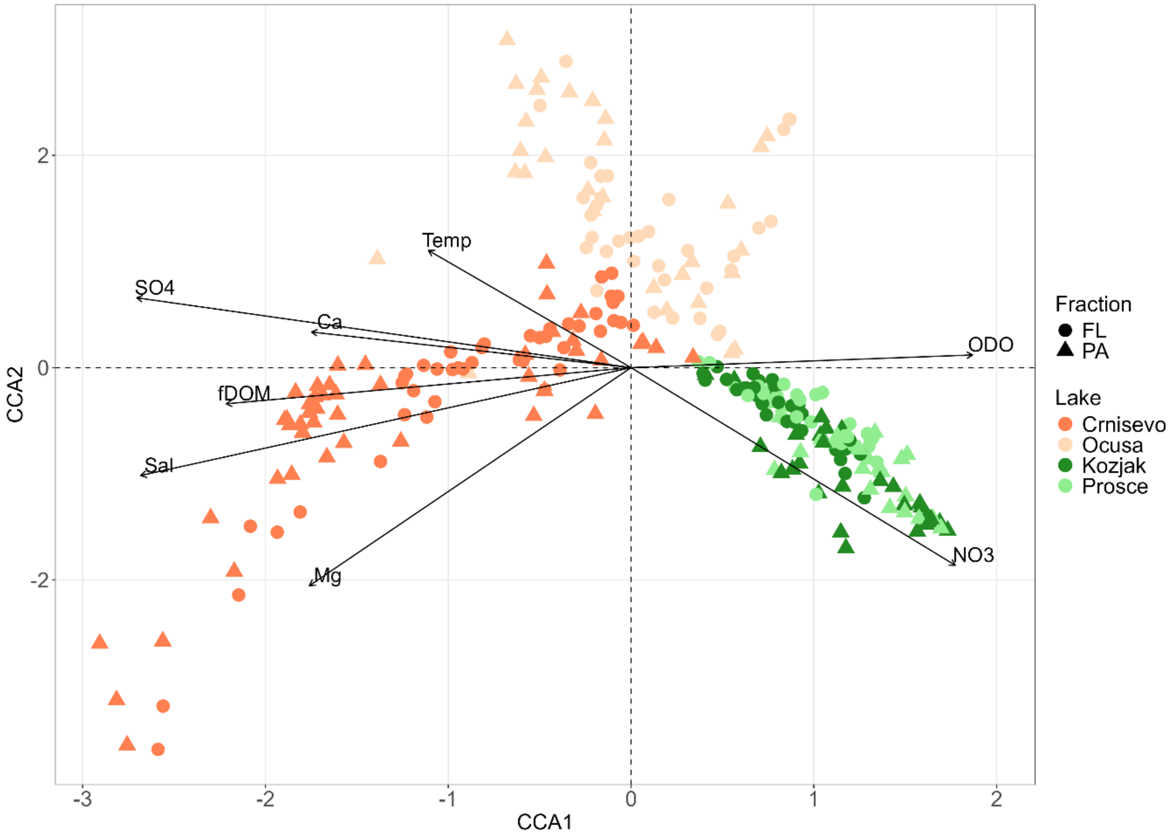

Figure 13. Canonical correspondence analysis ordination of prokaryotic community composition in four studied lakes based on environmental gradients. Samples are colored by lake (Crniševo and Oćusa in orange shades, Kozjak and Prošće in green shades) and shaped by fraction (circles: free-living, triangles: particle-associated). Arrows indicate the direction and strength of key environmental variables influencing community structure.

4.4. Functional gene analysis

The relative abundance of *pmoA* and *mcrA* genes showed distinct spatial and temporal patterns across the four studied lakes (Figure 14). The *pmoA* gene was generally more abundant than *mcrA*. In Lake Crnisevo, *pmoA* exhibited two main peaks - one during summer 2021 and another in autumn 2022 - concentrated primarily in the upper 5–15 m of the water column. In Lake Ocusa, *pmoA* abundance increased markedly from autumn 2021 through summer 2022, with the highest values observed at 10–15 m depth. Lake Kozjak showed moderate *pmoA* signals throughout the study period, with localized increases in the 10–20 m layer during autumn 2021. In contrast, Lake Prošće displayed relatively stable *pmoA* levels, with slightly higher values near the surface and at 10–15 m during summer 2021.

Patterns of *mcrA* abundance differed from those of *pmoA*. In Lake Crnisevo and Lake Prošće, *mcrA* remained consistently low throughout the sampling period, with values generally below 0.02%. Ocusa and Kozjak Lakes, however, showed transient *mcrA* increases. In Lake Ocusa, a pronounced *mcrA* peak occurred in autumn 2022 around 10 m depth, while in Kozjak, the highest *mcrA* abundance was detected between 10–20 m in spring 2022.

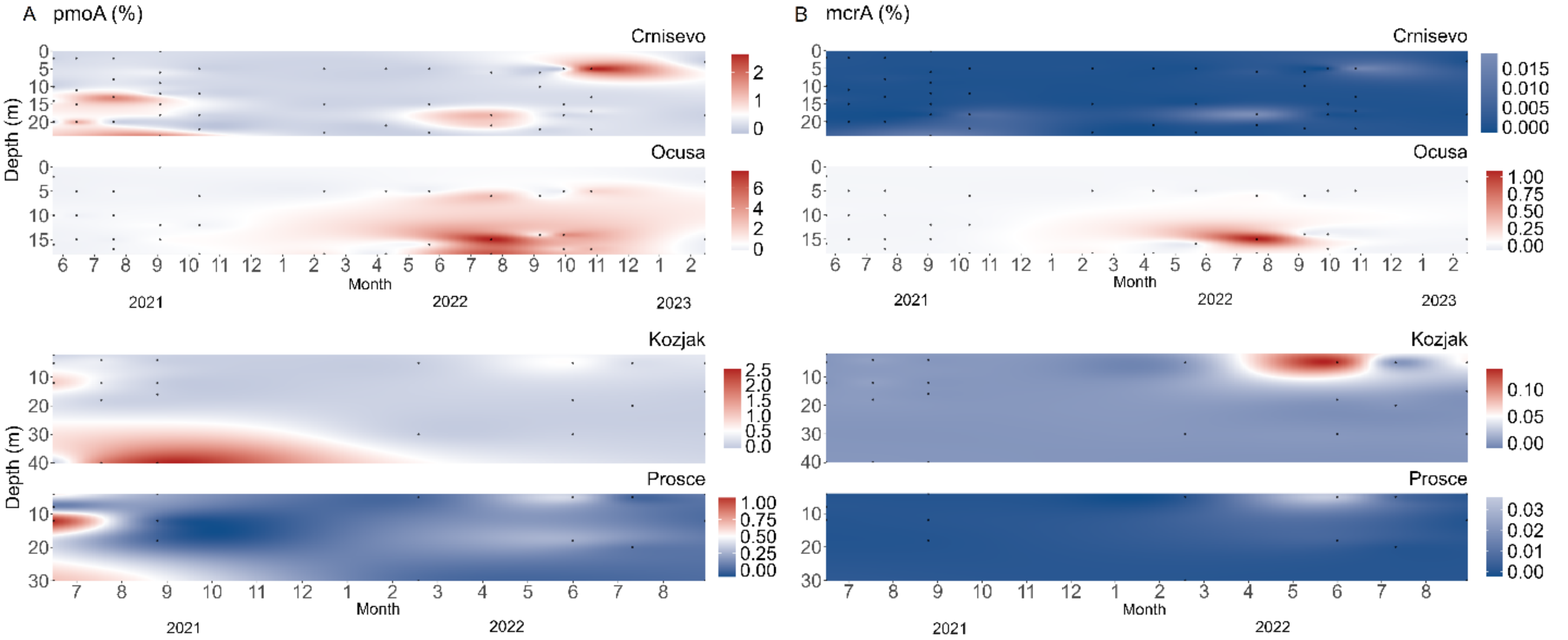

Figure 14. Relative abundance of *pmoA* and *mcrA* genes in four studied lakes across sampling depths and months. Warmer colors indicate higher relative abundance.

4.5. Enrichment experiment

Methane concentrations decreased over time in all treatments, indicating active methane consumption throughout the incubation period (Figure 15). The initial 20% methane concentration declined to values between 13.7% and 15.4% by October 2024. The rate of methane loss was comparable across treatments, with slightly higher residual methane observed at moderate salinity levels (S2 and S13) compared to the highest salinity (S20). By March 2024, all treatments showed a reduction of approximately 25–30% relative to the initial concentration, and the decline continued gradually until the final sampling.

Figure 15. Methane concentration (%) measured at different salinity treatments (S0, S2, S13, S20) over a two-year incubation period. Initial methane concentration was adjusted to 20% (v/v) in November 2022. Measurements were taken in September 2023, March 2024, and October 2024.

Sequencing of enrichment samples resulted in 2316 unique ASVs, with sequencing depths ranging from 11389 to 19271 reads per sample. Following quality filtering and rarefaction to equal sequencing depth, 1662 ASVs were retained for further analysis.

Microbial community richness (observed ASVs) and diversity (Shannon index) were much higher in the original sample compared to enrichments, with low salinity enrichments (S0 and S2) having slightly higher alpha diversity indices compared to high salinity enrichments (S13 and S20 (Figure 16). This pattern suggests that salinity enrichment led to a selective pressure favoring specific microbial groups while reducing overall community diversity. Among the enrichment treatments, low salinity conditions (S0 and S2) maintained moderately higher alpha diversity indices compared to high salinity enrichments (S13 and S20). The S2 treatment showed the highest diversity among enrichments, while the most extreme salinity condition (S20) exhibited the lowest diversity metrics. Principal coordinates analysis (PCoA) showed differences between microbial communities from different salinity treatments and the original sample (Figure 17). The analysis demonstrated separation between high salinity enrichments, low salinity enrichments, and the original sample along the first principal coordinate axis. PERMANOVA analysis confirmed that salinity was the dominant environmental factor structuring these communities, accounting for 93.3% of the variation between microbial assemblages.

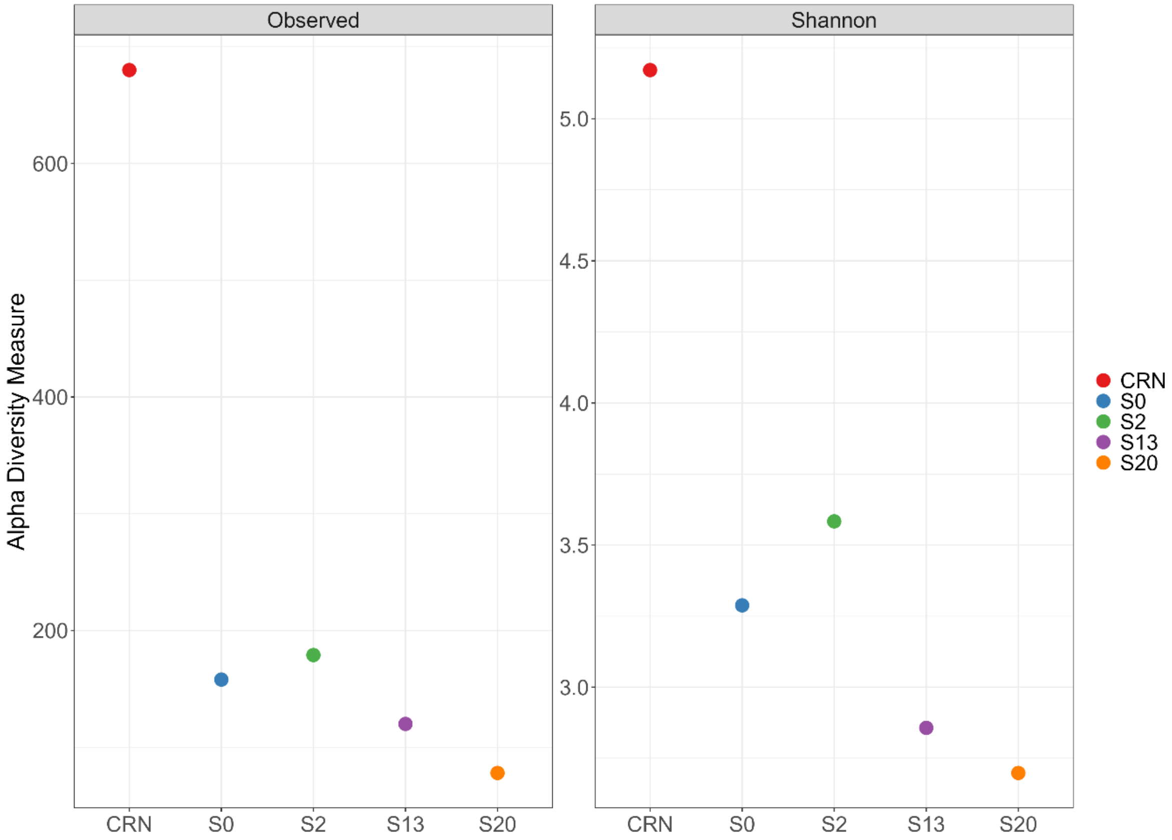

Figure 16. Alpha diversity of prokaryotic communities under varying salinity treatments (CRN, S0, S2, S13, S20), measured by observed species richness (left) and Shannon diversity index (right).

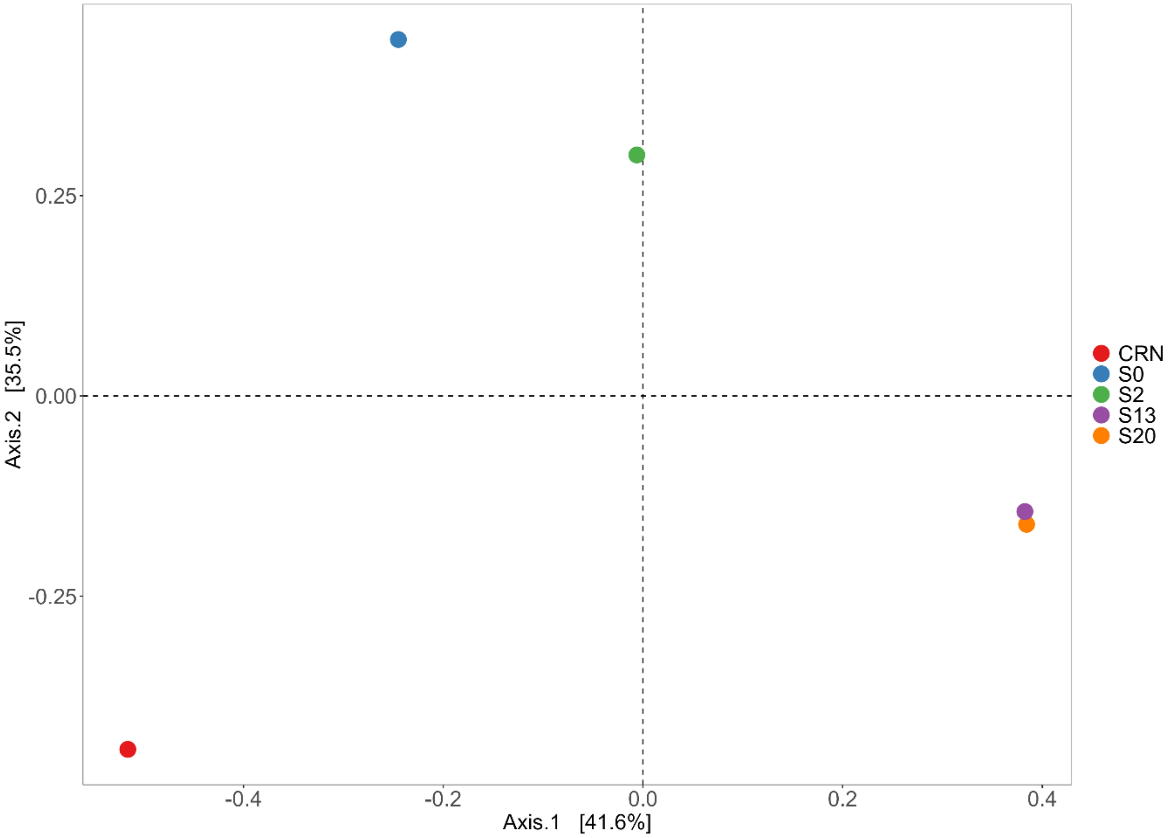

Figure 17. Principal Coordinates Analysis plot of microbial community composition under different salinity treatments (CRN, S0, S2, S13, S20). The first two axes explain 41.6% and 35.5% of the total variation, respectively.

Similar to other sampled lakes, the most abundant phyla from the original sample from Lake Crniševo were Actinobacteria (Actinomycetota), Bacteroidota, Cyanobacteria, Proteobacteria (Pseudomonadota) and Verrucomicrobiota (Figure 18). Compared to the original sample, the enriched samples were dominated by Proteobacteria and Bacteroidota with Campylobacterota relative abundance increasing as the salinity increased. The most common genera from Campylobacterota in the enriched samples were Arcobacter and Sulfurimonas with Sulfurovum appearing only in the S20 enriched sample.

The original Lake Crniševo sample exhibited a diverse taxonomic profile typical of freshwater lake ecosystems, with dominant phyla including Actinobacteria (Actinomycetota), Bacteroidota, Cyanobacteria, Proteobacteria (Pseudomonadota), and Verrucomicrobiota. This taxonomic diversity aligned with patterns observed in other sampled lakes from the study region. Salinity enrichment resulted in taxonomic restructuring, with enriched samples becoming strongly dominated by Proteobacteria and Bacteroidota. A notable feature of the enrichment communities was the progressive increase in Campylobacterota relative abundance corresponding to increasing salinity levels. Within this phylum, the genus Arcobacter emerged as a dominant component across most enrichment conditions, while Sulfurimonas showed consistent presence throughout the salinity gradient (Figure 19). Remarkably, Sulfurovum appeared exclusively in the highest salinity treatment (S20), suggesting this genus may represent a specialist adapted to extreme halophilic conditions.

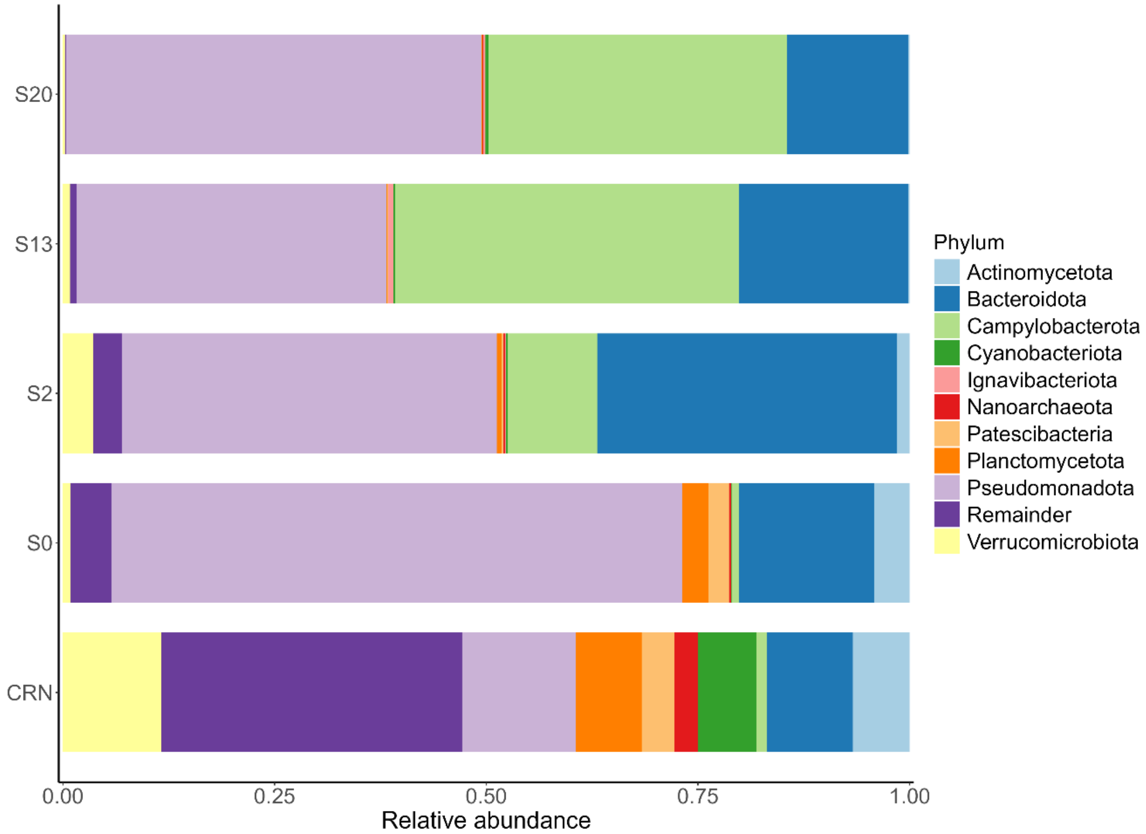

Figure 18. Relative abundance of dominant prokaryotic phyla under different salinity treatments (CRN, S0, S2, S13, S20).

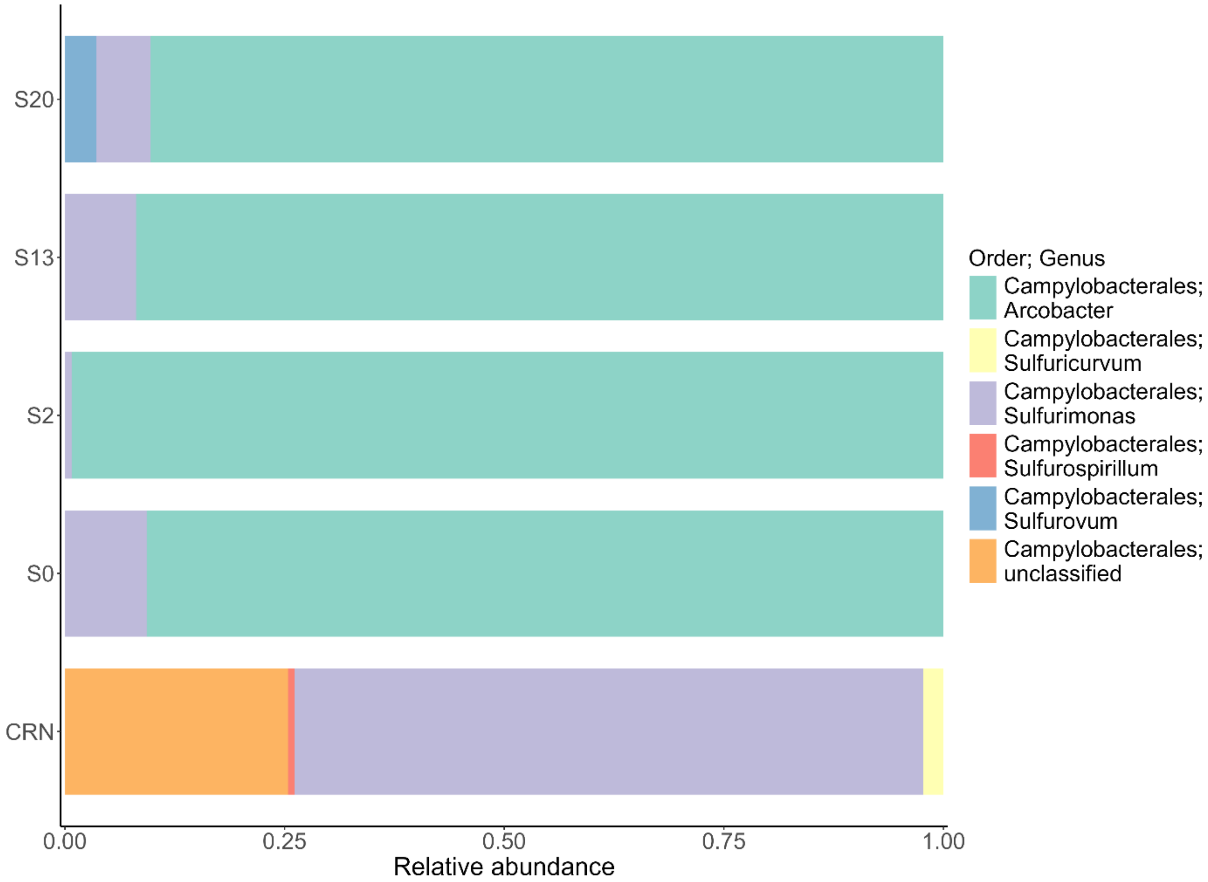

Figure 19. Relative abundance of dominant Campylobacterota genera across salinity treatments (CRN, S0, S2, S13, S20).

Regarding methane cycling prokaryotes, methanogens were not present in the enriched samples while methanotrophs differed based on salinity levels. The low salinity enrichments (S0 and S2) were dominated by Methylobacter and Methylocystis, while the high salinity enrichments (S13 and S20) had a higher abundance of Methylomonas and Crenothrix (Figure 20). The original sample consisted of Methyloparacoccus, Methylicorpusculum and Methylacidiphilales.

The enrichment experiment revealed significant shifts in methane-cycling microbial communities, with complete absence of methanogenic archaea in all enriched samples. This finding contrasts sharply with the original sample and suggests that the enrichment conditions were not conducive to methanogen survival or growth.

Methanotrophic bacteria exhibited salinity-dependent community structuring. Low salinity enrichments (S0 and S2) were predominantly colonized by Methylobacter and Methylocystis, representing typical Type I and Type II methanotrophs, respectively. In contrast, high salinity enrichments (S13 and S20) showed elevated abundance of Methylomonas and Crenothrix, indicating a shift toward halotolerant methanotrophic taxa. The original sample harbored a distinct methanotrophic assemblage comprising Methyloparacoccus, Methylicorpusculum, and members of the Methylacidiphilales order.

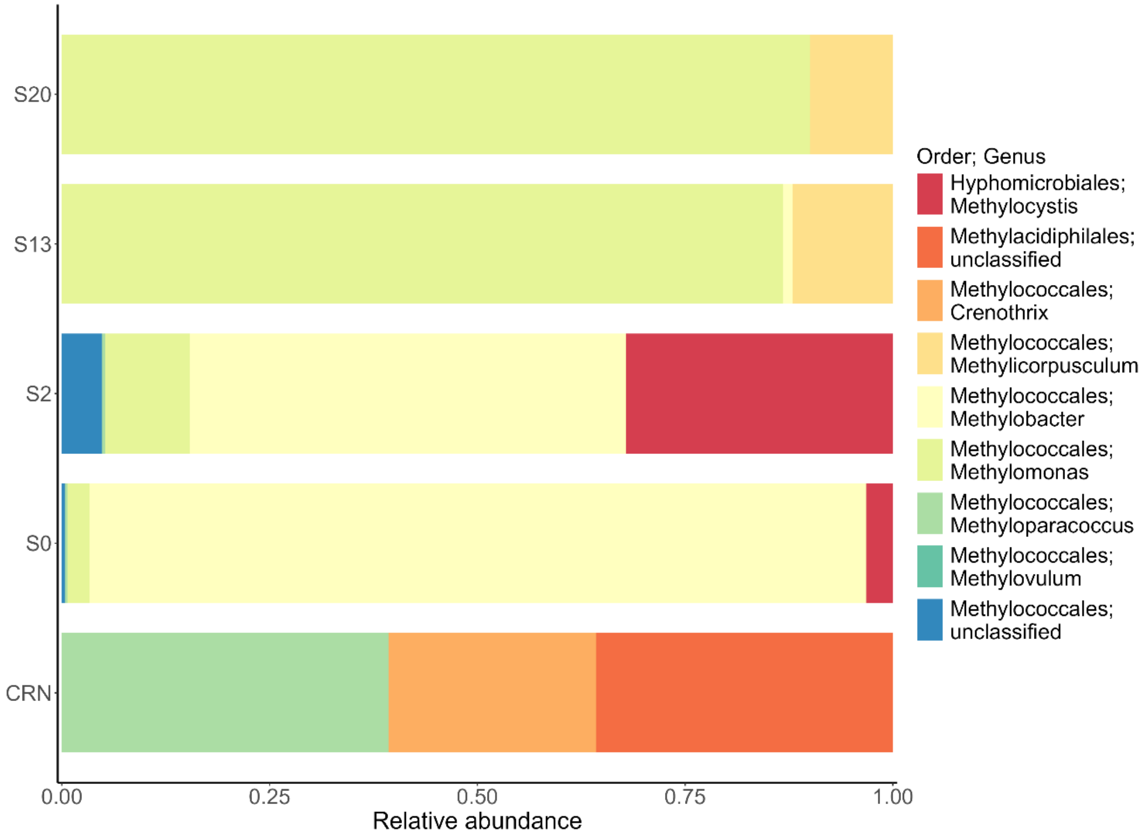

Figure 20. Relative abundance of dominant methanotrophic genera across salinity treatments (CRN, S0, S2, S13, S20).

5. DISCUSSION

5.1. Microbial community diversity and trophic status comparison

The strong association between microbial diversity and lake trophic status observed in this study aligns with broader patterns documented across freshwater ecosystems worldwide (Shen et al., 2019; Zwirglmaier et al., 2015). The higher prokaryotic richness and functional potential in the mesotrophic Baćina Lakes compared to oligotrophic Plitvice Lakes confirms established ecological principles regarding nutrient availability and microbial niche diversification (Shen et al., 2019; Wang et al., 2022). This pattern reflects fundamental differences in resource availability and environmental heterogeneity between contrasting trophic systems.

The dominance of specialized taxa in oligotrophic Plitvice Lakes, particularly Verrucomicrobiota and Actinobacteriota, demonstrates the selective pressure exerted by low-nutrient conditions. Verrucomicrobiota's preference for particle-associated lifestyles and their higher abundance during spring and summer in Plitvice systems reflects their well-documented ability to degrade complex polysaccharides and form biofilms in nutrient-poor environments (Chiang et al., 2018; Orellana et al., 2022). This phylum's adaptation to oligotrophic conditions and particle-rich environments represents a specialized ecological strategy that has been consistently observed across low-nutrient freshwater systems globally (Shen et al., 2019). Similarly, the consistent abundance of Actinobacteriota in surface waters during summer months, particularly in oligotrophic systems, reflects their competitive advantage under nutrient-limited conditions. These bacteria exhibit streamlined genomes and efficient nutrient utilization strategies that allow them to thrive in low-resource environments (Allgaier & Grossart, 2006; Newton et al., 2011). The seasonal dynamics observed, with peak abundances during summer and autumn months, correspond to previously documented patterns in temperate freshwater systems where Actinobacteriota respond to thermal stratification and reduced mixing (Diao et al., 2017).

In contrast, the mesotrophic Baćina Lakes supported copiotrophic groups including Bacteroidetes and Gammaproteobacteria, reflecting enhanced nutrient availability and more diverse metabolic opportunities. Bacteroidetes' prominent role in organic matter degradation, particularly their winter surface peaks in free-living communities and summer-autumn dominance in particle-associated fractions, indicates active participation in phytoplankton bloom senescence and organic matter processing (Meyer et al., 2022; Xie et al., 2024). This pattern of seasonal succession mirrors observations from other nutrient-rich freshwater systems where Bacteroidetes populations track organic matter availability and decomposition cycles (Woodhouse et al., 2016).

The hierarchical clustering results, showing occasional overlap between Baćina and Plitvice samples despite their contrasting trophic status, suggests the existence of shared core taxa performing essential metabolic functions across karst environments. This finding parallels observations from other comparative studies where core microbial communities maintain fundamental biogeochemical processes despite system-specific environmental pressures (Ruth et al., 2024). The presence of common taxa like Proteobacteria across both systems indicates universal metabolic pathways for carbon cycling in carbonate-rich karst environments, similar to patterns observed in subterranean cave microbiomes (Wang et al., 2022).

5.2. Seasonal dynamics and environmental drivers

The pronounced temporal variation explaining 24-35% of microbial community composition validates the critical role of seasonal dynamics in structuring freshwater microbial communities. This level of temporal control is consistent with studies from other temperate lake systems where seasonal stratification and mixing cycles create predictable environmental gradients that drive microbial succession (Diao et al., 2017; Morrison et al., 2017). The observation that monthly variation explained similar amounts of variance as lake identity, but with lower F-values indicating higher within-group variation, reflects the complex interplay between local environmental conditions and broader seasonal patterns.

The seasonal dominance patterns observed — Cyanobacteria and Proteobacteria alternating in Baćina Lakes during summer stratification, and Verrucomicrobiota prevailing in Plitvice during mixing periods — demonstrate predictable responses to stratification-mixing cycles. These patterns align with established models of microbial succession in stratified lakes, where thermal and chemical gradients create distinct ecological niches (Shade et al., 2007; Diao et al., 2017). The unique finding of recurrent Actinobacteriota dominance in Plitvice surface layers during stratification represents a novel adaptation to oligotrophic, low-oxygen conditions that has not been extensively documented in previous karst lake studies.

The dynamic vertical distribution of major phyla reveals sophisticated niche partitioning strategies. Cyanobacteria's presence at thermocline depths during summer and autumn, combined with their broader water column distribution in particle-associated fractions, suggests both active growth responses to optimal light-nutrient conditions and passive sedimentation dynamics (Paerl & Otten, 2013). This pattern of depth-specific distribution followed by redistribution through mixing has been observed in other stratified systems and reflects the complex interplay between active microbial ecology and physical transport processes.

Proteobacteria's consistent dominance across seasons and depths, but with varying abundances and phylogenetic composition, demonstrates the ecological versatility of this phylum in freshwater systems. Their alternating dominance with Cyanobacteria in Baćina Lakes reflects rapid responses to changing resource availability and redox conditions, consistent with their diverse metabolic capabilities and involvement in multiple biogeochemical processes (Jiang et al., 2022; Kim et al., 2021). The preferential association of Proteobacteria with thermocline regions during summer months suggests active responses to optimal temperature-oxygen-nutrient combinations.

The near absence of Actinobacteriota in particle-associated fractions across all lakes provides strong evidence for their specialized planktonic lifestyle. This pattern reflects their small cell size and metabolic adaptations to oligotrophic conditions, where particle association would be less advantageous than maintaining high surface area to volume ratios for efficient nutrient uptake (Newton et al., 2007). This lifestyle specialization represents a fundamental ecological strategy that distinguishes Actinobacteriota from more generalist taxa capable of exploiting both planktonic and particle-associated niches.

5.3. Environmental gradients and community assembly

The canonical correspondence analysis (CCA) results demonstrate clear environmental structuring of microbial communities along multiple gradients, with the first two axes explaining substantial variance in community composition. The separation of Baćina and Plitvice systems along primary environmental gradients reflects fundamental differences in geochemical regimes that extend beyond simple trophic classification. Salinity, sulfate, and dissolved organic matter (DOM) emerged as primary drivers distinguishing Baćina Lakes, while nitrate and oxygen characterized Plitvice systems, creating distinct selective environments for microbial taxa.

The elevated salinity and sulfate concentrations in Baćina Lakes, ranging from 13.5 to 141 mg/L for sulfate compared to 2.5-6.3 mg/L in Plitvice systems, reflect marine influence that create unique selective pressure on microbial communities, favoring halotolerant and sulfur-cycling taxa, as demonstrated by the enrichment of Campylobacterota and sulfur-oxidizing bacteria in these systems. This gradient effect has been documented in other coastal and inland saline systems where ionic strength fundamentally alters microbial community structure and metabolic potential (Lozupone & Knight, 2007).

The differential responses of free-living and particle-associated communities to environmental variables reveal sophisticated niche partitioning strategies. In Lake Crniševo, the particle-associated community's correlation with calcium and chloride reflects aggregation processes and mineral associations that create distinct microenvironments for attached bacteria (Grossart, 2010). Conversely, free-living communities' stronger associations with dissolved organic carbon and nitrate in various lakes indicate dependence on planktonic resource gradients and dissolved nutrient availability. PERMANOVA results showing that lake identity explained more variance (14.37%) than lifestyle fraction (10.98%) emphasizes the overriding importance of system-specific environmental conditions in community assembly. However, the consistent significance of fraction effects across individual lakes ($R^2 = 17\text{-}23\%$ in most systems) demonstrates that lifestyle partitioning represents a fundamental organizational principle that operates within broader environmental constraints. This hierarchical structuring — where environmental filtering establishes system-level patterns while lifestyle preferences create fine-scale niche differentiation — reflects established community assembly theory in microbial ecology.

The temperature and oxygen effects observed across all systems, explaining 5-9% of variance, represent universal environmental drivers that operate consistently across different karst lake types. Temperature's role as a metabolic control mechanism affects all microbial processes, while oxygen availability fundamentally determines available metabolic pathways and redox chemistry. The stronger oxygen effects in Lake Crniševo ($R^2 = 7.2\%$) likely reflect its greater propensity for seasonal anoxia, creating more dramatic redox gradients that strongly influence community structure.

5.4. Co-occurrence networks and microbial interactions

The co-occurrence network analysis reveals sophisticated patterns of microbial interaction that vary systematically across lake systems, providing insights into community stability and ecological functioning. The predominance of positive associations across all networks, forming cohesive cores with localized negative edges, aligns with theoretical frameworks suggesting that environmental filtering and metabolic complementarity drive community structure more than direct competitive exclusion (Eiler et al., 2012). This pattern indicates that cooperative or neutral interactions dominate over antagonistic relationships in these karst systems.

Lake-specific network architectures reflect underlying environmental and ecological differences. Lake Crniševo's heterogeneous structure with multiple medium-to-large hubs distributed across several modules indicates high environmental heterogeneity and functional redundancy. This architecture suggests a more resilient community capable of maintaining function through multiple metabolic pathways and taxon interactions. The presence of numerous positive edges connecting compartments reflects cooperative relationships that may facilitate resource sharing and metabolic coupling across different functional groups.

Lake Oćuša's elongated, less compact network organized around a dense central ribbon suggests a more streamlined community structure where key taxa (Actinobacteria and Proteobacteria hubs) coordinate community function. This architecture may reflect more stable environmental conditions that allow the establishment of core interaction partnerships without requiring extensive functional redundancy. The sparsely connected periphery with short negative edges indicates limited antagonistic interactions restricted to network margins.

Lake Kozjak's compact lattice structure characterized by large Proteobacteria hubs embedded in dense positive link networks represents the most integrated community architecture observed. This pattern suggests tight metabolic coupling and efficient resource utilization, potentially reflecting the lake's oligotrophic status where efficient nutrient cycling becomes critical for community maintenance. The few peripheral negative edges indicate that competitive interactions are minimized through tight niche differentiation and resource sharing.

Lake Prošće's moderately compact core with distributed hub taxa (Proteobacteria, Actinobacteria, Bacteroidetes, and Verrucomicrobiota) and interspersed negative edges represents an intermediate network structure. This architecture suggests balanced competitive and cooperative interactions, with multiple taxa sharing hub functions. The presence of negative edges within both core and periphery indicates ongoing niche overlap and resource competition that may drive continued community evolution.

The consistent emergence of Proteobacteria and Actinobacteria as central network hubs across all systems reflects their ecological versatility and fundamental roles in freshwater carbon and nutrient cycling. These taxa serve as "keystone" organisms that facilitate community stability through their extensive interaction networks and metabolic capabilities. Their hub status indicates that community disruption would likely propagate through these taxa, making them potential indicators of ecosystem health and stability (Zhang et al., 2022).

5.5. Free-living and particle-associated lifestyle niche specialization strategies

The consistent differentiation between particle-associated and free-living bacterial communities across all environmental gradients demonstrates fundamental lifestyle-based niche partitioning that operates as a universal organizing principle in these karst systems. The PERMANOVA results showing fraction effects explaining 10-23% of variance across lakes indicates that lifestyle choice represents a major ecological strategy that transcends system-specific environmental conditions.

Verrucomicrobiota's strong preference for particle-associated habitats across all systems provides compelling evidence for phylogenetically conserved lifestyle specialization. Their higher abundance in Plitvice Lakes, particularly during spring and summer when particle loads from primary production are elevated, reflects their documented ability to degrade complex polysaccharides and organic polymers associated with phytoplankton and detrital particles (Chiang et al., 2018). This specialization represents an evolutionary adaptation that allows exploitation of particle-associated resources while avoiding direct competition with planktonic specialists.

The near absence of Actinobacteriota in particle-associated fractions represents the opposite extreme of lifestyle specialization, reflecting adaptations to planktonic resource acquisition. Their small cell size, streamlined metabolism, and preference for dissolved organic carbon sources make particle association energetically unfavorable compared to maintaining high surface-area-to-volume ratios for efficient nutrient uptake (Newton et al., 2007). This lifestyle specialization allows Actinobacteriota to dominate in oligotrophic conditions where dissolved resources are limited but particle formation may be reduced.

Proteobacteria's presence in both fractions but with varying phylogenetic composition suggests ecological versatility within this diverse phylum. Different proteobacterial lineages have evolved distinct strategies for exploiting particle-associated versus planktonic niches, allowing the phylum as a whole to occupy diverse ecological roles. This metabolic diversity explains their consistent network hub positions and their ability to maintain dominance across varying environmental conditions.

The seasonal dynamics of lifestyle partitioning, particularly Bacteroidetes' winter surface peaks in free-living communities versus summer-autumn dominance in particle-associated fractions, reveals sophisticated temporal niche specialization. This pattern suggests that Bacteroidetes can shift between exploitation of dissolved winter resources (possibly from mixing-driven nutrient release) and particle-associated summer resources (from phytoplankton production and senescence). Such temporal flexibility represents an advanced ecological strategy that maximizes resource acquisition across seasonal cycles.

5.6. Functional gene abundance and methane cycling potential

The role of lifestyle also extended to methane-cycling organisms. Methanogens were largely restricted to the hypolimnion of the mesotrophic Baćina Lakes, likely reflecting local anoxia and higher organic matter availability — conditions conducive to methanogenesis (Lueders et al., 2001). In contrast, Plitvice Lakes exhibited a near absence of methanogens, and a more consistent presence of methanotrophs, particularly in intermediate depths, pointing to efficient methane oxidation at oxic-anoxic boundaries. This pattern is consistent with oxidative barriers observed in stratified systems like Lake Zug, where methane rarely escapes into oxic waters (Oswald et al., 2015). The strong environmental structuring of methanotrophs, shown by both qPCR and PERMANOVA results, supports the notion that these organisms are more tightly controlled by physicochemical gradients such as oxygen, DOM, and salinity. Methanogens, showing weaker environmental separation, may rely more on microscale habitat heterogeneity, such as anaerobic microniches within aggregates (Rösel et al., 2012; Xiao et al., 2024). The observed differences in environmental sensitivity indicates that methanotrophs function as environmental sentinels, while methanogens tend to persist in hidden, often particle-associated niches, reflecting their distinct ecological strategies (Finn et al., 2020).

Salinity emerged as a potent determinant of microbial community structure. Enrichment experiments demonstrated a sharp decrease in diversity with increasing salinity, accompanied by a taxonomic shift toward halotolerant taxa such as Arcobacter and Sulfurimonas, mirroring patterns observed in saline lakes and estuaries (Ventosa, 2006). This simplification of community structure aligns with global biogeographic studies showing salinity as a primary environmental filter in aquatic microbiomes (Lozupone & Knight, 2007). The exclusion of methanogens from saline enrichments despite their presence in initial samples suggests high osmotic sensitivity, consistent with previous findings (Oren, 2024). Conversely, methanotrophs exhibited compositional shifts in response to salinity stress, transitioning from freshwater taxa (Methylocystis) to halotolerant types (Crenothrix, Methylomonas) as salinity increased — an adaptive flexibility also seen in estuarine and saline wetland studies (Biderre-Petit et al., 2011). These findings not only confirm known salinity constraints on methane cycling but also highlight specific microbial lineages capable of maintaining function under stress. In Lake Crniševo, the low overall abundance of methanotrophs, except for a short-lived peak in the upper 5 m during late summer 2021, contrasts with a pronounced methanogen presence at 20 m depth in autumn, suggesting that deeper, likely anoxic conditions supported methane production at that time (Grossart et al., 2011). In Lake Oćuša, methanotrophs peaked at 10 m in late autumn 2021, coinciding with methanogen emergence in the hypolimnion in early 2022, indicating a possible coupling between methane production and oxidation in time and space (Martinez-Cruz et al., 2017). Conversely, the deeper Lakes Kozjak and Prošće showed more consistent methanotrophic activity at depth, which suggests effective methane oxidation in these lakes and possibly limited anaerobic zones for methanogen proliferation (Zimmermann et al., 2021). In Prošće, methanotrophs were consistently elevated at depth, whereas methanogens were detected only sporadically and at low abundance, primarily in winter. These observations point to contrasting methane cycling regimes: shallow, temporally stratified lakes favored methanogenesis, while deeper, more stably stratified lakes facilitated methane oxidation in deeper oxic-anoxic transition zones (Johnson et al., 2022). PERMANOVA analysis further supported these trends, showing that methanogen community structure was primarily influenced by month ($R^2 = 11.1\%$), lake (7.2%), and chemical factors such as oxygen, sulfate, and DOM, though each of these explained only a small portion of the variance and resulted in diffuse groupings ($F < 5$). This suggests that methanogen dynamics are sensitive to short-term environmental variability and redox fluctuations

(Conrad, 2007). In contrast, methanotrophs showed clearer ecological structuring, being strongly influenced by lake identity ($R^2 = 14.8\%$, $F = 13.5$) and to a lesser extent by salinity, oxygen, and DOM (~4–6% R^2), reflecting sharper environmental filtering and niche specialization in methane oxidation (Kip et al., 2010).

Gene abundance data from qPCR provided further support for these functional patterns. The *pmoA* gene, indicative of aerobic methanotrophy, was consistently more abundant than *mcrA*, a marker for methanogenesis, by at least an order of magnitude in all lakes (Knief, 2015). In Lake Crniševo, *pmoA* abundance peaked in the free-living (FL) fraction below 10 m during summer 2021, while the particle-associated (PA) fraction showed peaks in upper layers during late 2022. In contrast, *mcrA* followed similar trends but remained at lower absolute levels, suggesting less widespread or less active methanogenesis under oxic or mildly hypoxic conditions (Bogard et al., 2014). The 10-fold higher *pmoA* vs. *mcrA* gene abundances across all lakes indicate methane oxidation dominates over production, consistent with oxic water column conditions favoring Type I/II methanotrophs (Li et al., 2021). However, the detection of *mcrA* in oxic layers supports the "methane paradox," potentially via microoxic microniches or novel aerobic methanogenesis pathways (Bižić et al., 2020). The PA fraction's higher methanotrophic capacity aligns with particle-associated hotspot dynamics observed in coastal sediments (Katsman et al., 2013). Lake Oćuša displayed the highest gene copy numbers overall in the PA fraction during summer 2022, with *pmoA* and *mcrA* peaking at 11% and 2%, respectively, in bottom waters, indicative of active methane cycling in deeper, likely low-oxygen layers (Yvon-Durocher et al., 2014). Seasonal differences were evident in the FL fraction, where *pmoA* dominated in summer and *mcrA* peaked in autumn and winter. In both lakes, *pmoA* was more abundant, especially in deeper layers, and reflected patterns consistent with active methanotrophy near or below the thermocline. For instance, *pmoA* in Lake Prošće reached up to 12% in the PA fraction during spring 2022, demonstrating a strong microbial response to methane gradients likely formed during stratification (Beck et al., 2013). These patterns suggest a general dominance of methanotrophy over methanogenesis across all lakes, with spatial variability reflecting redox gradients, stratification strength, and organic substrate availability. Moreover, the higher resolution offered by functional gene profiling helped disentangle niche differentiation and microbial adaptation to methane cycling processes across different lake environments.

5.7. Impacts of salinization on microbial community structure and function

The enrichment experiment revealed salinity-driven community shifts: low salinity (S0 and S2) favored Methylobacter (Type I) and Methylocystis (Type II), while high salinity (S13 and S20) selected for halotolerant Methylomonas and sulfur-cycling Sulfurovum. This mirrors estuarine gradients where salinity restructures methanotroph guilds and suppresses methanogenesis. The absence of methanogens in high-salinity treatments (20) supports sulfate-reducing bacteria outcompeting acetoclastic methanogens under elevated ionic strength, a critical consideration for coastal karst systems facing seawater intrusion (Sun et al., 2023). Among the enrichments, low salinity treatments (S0 and S2) showed moderately higher diversity than high salinity treatments (S13 and S20), with the S2 treatment exhibiting the highest richness and the S20 treatment the lowest, indicating a clear decline in microbial diversity with increasing salinity, a trend that reflects selective pressure favoring halotolerant or halophilic taxa under saline conditions (Lozupone & Knight, 2007). PCoA revealed distinct separation between microbial communities from different salinity treatments and the original sample, with PERMANOVA confirming salinity as the primary structuring factor, explaining the majority of the variation in community composition (Fierer & Jackson, 2006).

Methane concentration measurements over the two-year incubation further supported these microbial patterns. Initial methane concentrations (20%) declined steadily across all salinity treatments, reaching approximately 14–15% after 24 months. The magnitude of decline was similar among treatments, but the highest salinity (S20) showed slightly lower methane consumption compared with low and intermediate salinity levels (S0–S13). This consistent decrease indicates sustained, though moderate, methanotrophic activity under all tested conditions. The absence of detectable methanogens or anaerobic methane-oxidizing archaea (ANME) in the enriched samples suggests that methanogenesis was either inhibited or not re-established under the nitrogen-flushed conditions. Elevated salinity likely imposed osmotic stress that disrupted methanogenic metabolism, while the lack of suitable redox gradients and syntrophic partners further limited their proliferation. In contrast, residual methane oxidation may have been maintained by halotolerant methanotrophs capable of persisting under microaerophilic conditions, possibly using alternative electron acceptors such as nitrate or metal oxides (Oswald et al., 2016). The measured persistence of methane oxidation despite two years of incubation thus reflects the metabolic resilience of methanotrophs and their ability to adapt to suboptimal redox and saline environments.

Taxonomic analysis of the original Lake Crniševo sample revealed a diverse and typical freshwater community dominated by Actinobacteria (Actinomycetota), Bacteroidota, Cyanobacteria, Proteobacteria (Pseudomonadota), and Verrucomicrobiota, consistent with patterns observed in similar freshwater ecosystems (Newton et al., 2011). In contrast, the enriched samples were largely dominated by Proteobacteria and Bacteroidota, with a noticeable increase in Campylobacterota as salinity increased. Within this phylum, the genus Arcobacter was prevalent across all enrichment levels, while Sulfurimonas was consistently present and Sulfurovum appeared exclusively in the highest salinity treatment (S20), suggesting an adaptation to extreme halophilic conditions (Waite et al., 2017). Regarding methane-cycling microorganisms, methanogens were entirely absent from all enriched samples, suggesting the saline enrichment conditions were unsuitable for their survival or activity, likely due to their strict anaerobic and often low-salinity preferences (Conrad, 2007). High-salinity enrichments (S13 and S20) favored Methylomonas and Crenothrix, both associated with greater salt tolerance and potentially mixotrophic capabilities (Oswald et al., 2016). The methanotrophic community in the original sample consisted of Methyloparacoccus, Methylicorpusculum, and members of Methylacidiphilales, indicating a more diverse and possibly environmentally sensitive assemblage adapted to freshwater conditions (Kip et al., 2010).

Altogether, these results highlight salinity as a potent environmental filter that drives both community simplification and taxonomic restructuring, particularly within functionally relevant groups such as methane-cycling prokaryotes. The increasing dominance of specialized halotolerant and halophilic taxa with rising salinity suggests a strong adaptive capacity within Lake Crniševo's microbial community to respond to salinity changes, whether natural or anthropogenically induced.

5.8. Ecological implications and environmental management

The findings of this study have significant implications for understanding how karst lake ecosystems may respond to climate-driven environmental changes. The demonstrated sensitivity of microbial communities to salinity, temperature, and redox conditions suggests that these systems will experience substantial biological reorganization under projected climate scenarios.

The complete elimination of methanogenic capacity under saline conditions, combined with persistent methanotrophic activity, indicates that increasing salinization would fundamentally alter greenhouse gas dynamics in these ecosystems. The absence of methanogens and anaerobic methane-oxidizing archaea (ANME) in the enrichment experiments likely reflects a combination of physiological and environmental constraints. Both groups are highly sensitive to oxygen and salinity, requiring low-ionic-strength conditions and complex syntrophic partnerships to sustain activity (Oren, 2011). In the incubations, the nitrogen-flushed atmosphere and lack of stable redox gradients may have prevented the establishment of anaerobic niches, while prolonged exposure to elevated salinity created osmotic stress that inhibited methanogenic metabolism and cell integrity. Additionally, the absence of suitable substrates and potential competitors such as sulfate-reducing bacteria further limited methanogen survival (Conrad, 2007; Boetius & Wenzhöfer, 2013). Together, these factors suggest that under increasing salinization and redox instability, methane production and anaerobic oxidation become ecologically untenable, shifting ecosystem methane dynamics toward dominance by halotolerant aerobic or microaerophilic methanotrophs.

The strong environmental filtering effects observed suggest that climate-driven changes in precipitation patterns, temperature regimes, and hydrological connectivity will predictably alter microbial community structure and function. The differential responses of particle-associated and free-living communities to environmental gradients indicate that climate impacts will affect different components of the microbial community unequally, potentially disrupting established interaction networks and biogeochemical cycling patterns.

The network analysis results suggest that some systems (like Lake Kozjak with its highly integrated structure) may be more vulnerable to climate disruption than others (like Lake Crniševo with its modular, redundant architecture). Systems with tighter network integration may experience cascading effects from environmental changes that propagate through hub taxa, while more modular systems may maintain function through alternative pathways. This variation in network resilience should inform management strategies and monitoring priorities. The identification of core taxa and keystone hub species provides targets for monitoring ecosystem health and predicting community responses to environmental change. Taxa like Proteobacteria and Actinobacteria, which consistently serve as network hubs, represent early warning indicators whose abundance or activity changes may signal broader ecosystem disruption. Monitoring these hub taxa could provide efficient approaches for assessing ecosystem health and predicting climate change impacts.

This research contributes to broader understanding of microbial community assembly and functioning in freshwater ecosystems by demonstrating how multiple environmental filters interact to structure complex biological communities. The hierarchical effects observed — where system-level environmental conditions establish broad community patterns while lifestyle preferences and species interactions create fine-scale organization — illustrate fundamental principles of community ecology that extend beyond microbial systems (Leibold & Chase, 2017). The demonstration of predictable seasonal succession patterns, combined with system-specific network architectures, suggests that microbial communities in these karst systems exhibit both deterministic responses to environmental conditions and system-specific effects that create unique community properties. This combination of predictable and contingent factors represents a fundamental challenge for ecosystem management and climate change prediction.

Future research should focus on functional validation of the interaction networks identified through co-occurrence analysis. While correlation-based networks provide insights into potential interactions, experimental approaches are needed to confirm mechanistic relationships and quantify interaction strengths. Additionally, long-term monitoring programs should be established to validate the predictive frameworks developed from this cross-sectional study. The integration of environmental DNA approaches with traditional biogeochemical measurements demonstrated in this study provides a powerful framework for ecosystem assessment that could be applied to other freshwater systems. The combination of taxonomic profiling, functional gene quantification, and network analysis offers comprehensive insights into ecosystem structure and function that individual approaches cannot provide.

6. CONCLUSION

This doctoral research provides a comprehensive comparative analysis of microbial community dynamics, methane cycling, and physicochemical stratification across two contrasting karst lake systems: the oligotrophic Plitvice Lakes and the mesotrophic, seasonally anoxic, and partially saline Baćina Lakes. By integrating vertical profiling, 16S rRNA gene amplicon sequencing, functional gene quantification, and salinity enrichment experiments, this study establishes clear ecological links between microbial diversity, environmental gradients, and biogeochemical functioning in freshwater karst systems.

1. *The diversity of prokaryotic communities varies according to the trophic status of the lake (Hypothesis 1).*

   Microbial diversity was strongly associated with the trophic state of the lakes, confirming Hypothesis 1. The mesotrophic Baćina Lakes exhibited higher prokaryotic richness and functional potential than the oligotrophic Plitvice Lakes. Oligotrophic conditions favored specialized and streamlined taxa such as Verrucomicrobiota, adapted to low-nutrient and carbonate-rich environments, whereas copiotrophic groups (Bacteroidetes, Gammaproteobacteria) dominated Baćina, reflecting enhanced nutrient availability. These findings illustrate how trophic gradients act as ecological filters shaping microbial niche specialization and metabolic potential in karst lakes.

2. *The seasonal distribution of prokaryotic communities is shaped by dynamic environmental conditions in each lake system (Hypothesis 2).*

   Temporal variation explained 24–35% of the total variance in microbial community composition (PERMANOVA), validating Hypothesis 2. Seasonal stratification and mixing cycles produced predictable microbial shifts, with Cyanobacteria and Proteobacteria dominating Baćina during summer stratification, and Verrucomicrobiota prevailing in Plitvice during winter mixing. A novel finding was the recurrent dominance of

Actinobacteriota in Plitvice surface layers during stratification, indicating unique adaptations to oligotrophic, low-oxygen environments. These patterns reinforce the importance of redox-driven and seasonal processes in shaping microbial community structure and succession.

3. *The composition and abundance of methanogens and methanotrophs are primarily driven by oxygen concentration (Hypothesis 3).*

   Oxygen availability emerged as the primary driver of methanogenic (*mcrA*) and methanotrophic (*pmoA*) distributions, confirming Hypothesis 3. Methanogens were restricted to shallow, seasonally anoxic layers of Baćina Lakes, particularly in sulfate-rich zones favoring anaerobic metabolism, whereas methanotrophs were more broadly distributed and abundant at oxyclines, emphasizing oxic–anoxic coupling in methane turnover. The salinity enrichment experiments further extended Hypothesis 3 by revealing how ionic stress acts as an additional environmental filter. Increasing salinity (S0–S20) caused sharp declines in microbial diversity, suppressed methanogenic activity entirely, and selected for halotolerant and sulfur-oxidizing taxa such as Campylobacterota, Arcobacter, Sulfurovum, and Crenothrix. These findings demonstrate that both oxygen and salinity gradients jointly structure methane-cycling communities, with salinity posing an additional constraint on methanogenesis under changing environmental conditions.

4. *The abundance of functional marker genes involved in the methane cycle reflects the oxygen concentration in the water column (Hypothesis 4).*

   Quantitative functional gene analysis confirmed Hypothesis 4: *pmoA* gene abundance consistently exceeded *mcrA* by one to two orders of magnitude across all lakes, indicating that methane oxidation dominates over production. Methanotrophic activity was highest at oxyclines and in deeper stratified layers, especially during late summer and autumn in Baćina and in the bottom waters of Lake Prošće in Plitvice. The enrichment experiments reinforced this result, showing that under increased salinity, methanotrophs persisted while methanogens were fully inhibited. This supports the conclusion that salinity not only modulates community composition but also alters functional balance within the methane cycle, favoring oxidation pathways over production. Together, these findings confirm that oxygen availability and ionic composition regulate the abundance and functional activity of methane-cycling microorganisms in karst lake systems.

This study demonstrates that trophic status, seasonal stratification, redox gradients, and salinity jointly regulate microbial community composition, metabolic potential, and methane-cycling processes. The study highlights the ecological partitioning between particle-associated and free-living microorganisms, demonstrating how lifestyle and environmental factors co-structure microbial communities. Importantly, the identification of salinity-tolerant methanotrophs but salinity-sensitive methanogens indicates potential future imbalances in methane cycling under changing climate and hydrology. By experimentally integrating salinity perturbations with in situ microbial and functional analyses, this work provides novel insights into the resilience and vulnerability of methane-cycling microorganisms under environmental stress. The findings highlight the potential for salinity-driven suppression of methanogenesis and the persistence of methanotrophic activity under variable redox and ionic conditions.

Overall, this research advances the scientific understanding of microbial ecology in karst systems and contributes a predictive framework for assessing how climate-driven changes, altered hydrology, and freshwater salinization may affect greenhouse gas dynamics and biogeochemical feedbacks in sensitive freshwater environments.

7. LITERATURE

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8. CURRICULUM VITAE

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Education

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Work experience

2025 - present	Professional Specialist Institute of Immunology, Viral Vaccines Department, Zagreb
2021 - 2025	Assistant Ruđer Bošković Institute, Department for Materials Chemistry, Laboratory for Precipitation Processes, Zagreb
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Scientific publications

1. Stanić, Ivana; Čačković, Andrea; Orlić, Sandi
   Salinity Negatively Impacts Protistan and Fungal Community Stability and Shapes Assembly Processes in Freshwater Ecosystems // Environmental microbiology reports, 17 (2025), 5; e70209, 14. doi: 10.1111/1758-2229.70209
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1. Vojtkuf, Iva; Čačković, Andrea; Stanić, Ivana; Orlić, Sandi

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6. Stanić, Ivana; Ivanić, Maja; Fiket, Željka; Furdek Turk, Martina; Mešić, Armin
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