Microbial communities in *Phragmites australis* biofilms from Masurian lakes (Poland) with varying trophic states

Valentina Smacchia[1], Michał Karlicki[1], Bartosz Kiersztyn[2], Anna Karnkowska[1]*

[1] University of Warsaw, Faculty of Biology, Institute of Evolutionary Biology, Żwirki i Wigury 101, Warsaw, Poland

[2] University of Warsaw, Faculty of Biology, Department of Hydrobiology, Żwirki i Wigury 101, Warsaw, Poland

* Corresponding author; email: a.karnkowska@uw.edu.pl

ABSTRACT

Biofilms, consisting of bacteria and protists, are key components of freshwater microbial communities and contribute to ecosystem functioning. Although biofilm and planktonic communities may share taxa, the extent of their overlap and the drivers of differentiation are not fully understood. We used 16S and 18S rRNA gene metabarcoding to examine bacterial and protist communities in *Phragmites australis* stem biofilms and planktonic fractions across lakes with differing trophic states in the Great Masurian Lakes (NE Poland). Biofilm communities on reed stems were compositionally distinct from planktonic ones, sharing only a limited core microbiome. The biofilm core was larger than that of planktonic communities and included taxa identified by machine learning as characteristic of biofilm assemblages. Planktonic communities varied among lakes, whereas *P. australis*-associated biofilms were comparatively similar, which may reflect host association and lifestyle-related buffering. Additionally, biofilm suspensions showed higher physiological activity per inoculum volume and broader substrate use under the assay conditions than free-living communities. These results highlight the distinct nature of freshwater biofilms and underscore the importance of jointly examining biofilm and planktonic fractions of prokaryotic and eukaryotic communities in studies of microbial diversity and function in lake ecosystems.

Keywords: epiphytic biofilm, bacteria, freshwater lake, metabarcoding, protist

Introduction

Microbial communities in freshwater ecosystems are complex and dynamic, with multiple interacting components that promote ecosystem stability, water quality, and resilience. Within these communities, two main life strategies can be identified: free-living plankton and community-aggregated assemblages known as biofilms. Although planktonic microbial communities have traditionally been the focus of research, biofilms are increasingly recognised as crucial ecological players (Battin et al., 2003, 2016), involved in maintaining a healthy aquatic environment by facilitating primary production, supporting nutrient cycling, and purifying water (Besemer, 2015; Bighiu and Goedkoop, 2021; Bonnineau et al., 2020). All members of biofilm microbial communities collectively drive biofilm physiology: heterotrophic bacteria and fungi mediate carbon utilisation and nutrient cycling, while protists exert top-down control by feeding on bacteria (Amado and Roland, 2017; Hoque et al., 2023; Perujo et al., 2025) and contribute to biofilm cohesion and architecture (Grossart et al., 2005; Flemming and Wingender, 2010; Makk et al., 2024). Among the various types of freshwater biofilms, epiphytic biofilms – those that develop on the stalks of aquatic plants – are particularly important. These biofilms exhibit greater species diversity and unique microbial compositions and functions compared to other periphytic communities (Levi et al., 2017; Wijewardene et al., 2022). Studies suggest that epiphytic biofilms display elevated metabolic rates, actively contributing to nitrification and denitrification (Levi et al., 2015). Interestingly, they appear to be less influenced by ambient environmental changes and more dependent on their interactions with macrophytes (Sentenac et al., 2022; Wijewardene et al., 2022). Moreover, submerged macrophytes such as *Phragmites australis* actively shape their surrounding microbiome through the release of root exudates – mixtures of sugars, amino acids, and secondary metabolites – which selectively recruit beneficial microorganisms (Bais et al., 2006; Berendsen et al., 2012; Vandenkoornhuyse et al., 2015). One of the most widespread freshwater biofilms develops on *P. australis*, a globally distributed aquatic plant with significant ecological and environmental impact (Meyerson et al., 2016; Milke et al., 2020). In lakes, biofilms on *P. australis* surfaces can significantly enhance nutrient and contaminant

removal, acting synergistically with the plant's natural filtration and assimilation capabilities (Hiraki et al., 2009; Zhao et al., 2012).

Biofilm development is closely linked to the surrounding planktonic, free-living microbial community, with each likely influencing the other. For example, in Lake Baikal, biofilms shared taxa with plankton, although their overall community structures differed (Sorokovikova et al., 2013). Similarly, unique taxa have been reported in both biofilm and planktonic communities in Lake Erie (Robinson et al., 2025). However, comparative studies of plankton and biofilm communities in lake ecosystems remain scarce and do not yet provide a clear picture of their reciprocal influences. Consequently, less is known about the protist components of these communities, particularly at the molecular level, and how bacteria and protists jointly structure and interact within biofilms compared to open-water environments (Arndt et al., 2003; Mukherjee et al., 2024; Alacid et al., 2025).

To investigate the influence between biofilms and the free-living fraction, we analysed bacterial and protist communities on *P. australis* stalks and compared them with planktonic communities in the lakes of the Great Masurian Lake District (GMLD), Northeastern Poland. Although the GMLD lakes share a geological and climatic background, they differ in trophic state (Siuda et al., 2020) and have been used to demonstrate strong eutrophication effects on bacterial communities (Kiersztyn et al., 2019; Grabowska-Grucza and Kiersztyn, 2023), making them a suitable system for studying microbial diversity.

In this study, we used 16S and 18S rDNA metabarcoding to compare bacterial and protist communities in biofilms and plankton across five GMLD lakes with different trophic states. Specifically, we aimed to: (1) identify biofilm and planktonic bacterial and protist communities across lakes with different trophic states; (2) determine shared and habitat-specific taxa in both communities; and (3) evaluate bacterial functional diversity and physiological activity to reveal habitat-specific traits.

Materials and Methods

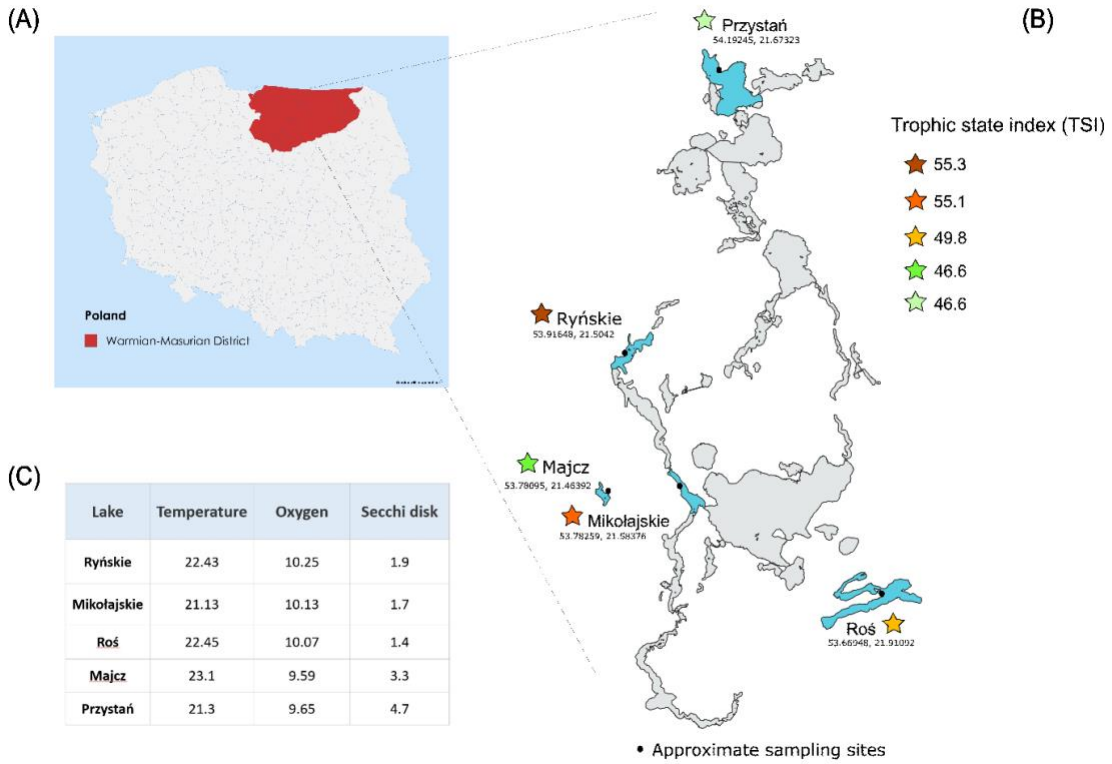
Sampling

Five lakes in the Great Masurian Lake District (GMLD, NE Poland) were selected as sampling sites (Fig. 1). Although they share a similar origin, geology, catchment characteristics, and climate, they differ in trophic status according to Carlson's classification (Table S1, Fig. 1). Four lakes – Ryńskie (RYN), Roś (ROS), Mikołajskie (MIK), and Przystań (PRZ)—are part of the interconnected Great Masurian Lake System (GMLS), whereas Majcz (MAJ) is isolated from this network and represents a mesotrophic system under low anthropogenic pressure.

From each lake, four subsample types were collected: biofilm from *Phragmites australis* stems (B), littoral water within (LP) and outside (L) the *P. australis* field, and pelagic water (P). L samples were collected at least 10 m from the nearest *P. australis* field, and P samples at least 200 m from the littoral zone. At each site, 5 L of surface water was collected. Biofilm samples from *P. australis* stems were collected following Tsuchiya et al. (2011) with modifications. Stems were cut into 30 cm sections, starting 5 cm below the water surface and extending to a depth of 35 cm; the upper 10 cm were discarded, and ten fragments per lake were retained and placed in a sterile plastic bottle filled with lake water from the corresponding *P. australis* field. Biofilms from 10 cm segments were removed using a sterile brush and suspended in 100 ml of 0.2 µm-filtered site water. A volume correction factor was applied to standardise results to water samples. This factor was calculated as the ratio of the suspension volume (100 ml) to the mean volume of a 10 cm stem segment (determined from ten segments), allowing biofilm-associated microbial activity to be expressed per equivalent water volume and compared with

littoral and pelagic samples. Enzyme activities were therefore expressed per sample volume and were not normalised to biomass or cell abundance.

For taxonomic composition analysis, each water and biofilm sample was filtered under low air pressure using Nucleopore filters (Whatman, Maidstone, UK) with a 0.2 µm pore size. The filters were stored at -80 °C until DNA extraction.



Lake	Temperature	Oxygen	Secchi disk
Ryńskie	22.43	10.25	1.9
Mikołajskie	21.13	10.13	1.7
Roś	22.45	10.07	1.4
Majcz	23.1	9.59	3.3
Przystań	21.3	9.65	4.7

Fig. 1. Geographic location of the studied lakes. *(A) The map shows the Great Masurian Lake District (GMLD) in northeastern Poland, where the sampled lakes are located (highlighted in red, upper left inset). (B) The five sampled lakes are shown with marked sampling sites. Lakes are classified according to their Trophic State Index (TSI).*

Physical and chemical metadata

In situ temperature and oxygen concentration were measured at each sampling point using a YSI ProODO probe (Yellow Springs, YSI Inc./Xylem Inc., USA). Photic zone depth was estimated using a LiCor LI-250A with LI-193R PAR sensor and a Secchi disk. The Trophic State Index (TSI) was calculated as the mean of values derived from total phosphorus measured over three consecutive summers (2021–2023; Table S1) using the Carlson formula (Carlson, 1977). Dissolved organic carbon (DOC), total phosphorus (TP), phosphate (PO_4^{3-}), and total nitrogen (TN) concentrations were determined as described in Kiersztyn et al. (2019).

Physiological fingerprint diversity and Leucine-aminopeptidase activity

The Biolog® EcoPlates™ (Biolog Inc., Hayward, CA, USA) method was used to determine community-level physiological profiles, with modifications as described by. Kiersztyn et al. (2019) Maximal potential leucine-aminopeptidase activity (Vmax_LAP) was measured fluorometrically following the methods of Chróst and Siuda, (2006) and Kiersztyn et al. (2012).

DNA extraction, amplification, and sequencing

DNA was isolated using the GeneMATRIX Soil DNA Purification Kit (EURx Sp. z o.o., Gdańsk, PL) from filters, following the manufacturer's protocol with minor modifications: after

the addition of 60 µL lysis buffer, samples were homogenised for 10 minutes with a vertical homogeniser, stored at -80°C for 1 hour, and homogenised again for 5 minutes at maximum speed. For the final elution, 30 µL DNase/RNase-free water was used.

Extracted DNA was quantified using a NanoDrop (Implen/Thermo Fisher Scientific, USA), and fragment size was verified on a 1% agarose gel. Samples were sequenced at the University of Warsaw (CeNT Genomics Core Facility, Poland) on a NovaSeq 6000 platform following amplification and library preparation.

The prokaryotic V4-V5 and eukaryotic V4 hypervariable regions of the 16S and 18S rRNA genes, respectively, were amplified following Romac, (2022a, 2022b) with minor modifications: primer concentrations were increased to 0.2 µM for prokaryotes and reduced to 0.4 µM for eukaryotes; for the prokaryotic primers, 20 cycles were used for amplification. Amplifications were performed in triplicate, pooled, and purified using a PCR clean-up kit (Syngen).

Sequence and statistical analysis

Sequence quality checks were performed on raw sequencing data using FastQC (Andrews, 2010). Reads were imported into the QIIME2 environment for demultiplexing, adapter trimming, and primer removal (Bolyen et al., 2019; Martin, 2011). Denoising was conducted using the DADA2 algorithm in QIIME2 (Callahan et al., 2016).

Taxonomic assignment of the V4-V5 16S rRNA and V4 18S rRNA sequences was performed using USEARCH global alignment implemented in VSEARCH (Rognes et al., 2016), with a minimum identity threshold of 70% and a minimum query coverage of 90%. The SILVA 138.1 database (Quast et al., 2012) was used for 16S rRNA classification, while the Protist Ribosomal Database PR2 5.0.0 was used for 18S rRNA classification (Guillou et al., 2012). All taxonomic and diversity analyses were conducted in RStudio IDE using the phyloseq package (Allaire, 2012; McMurdie and Holmes, 2013) after removal of ASVs classified as Eukaryota, chloroplasts, or mitochondria from the 16S dataset, and ASVs assigned to Metazoa, Embryophyta, Bacteria, or Archaea from the 18S dataset.

Statistical analyses, packages used, and the analytical workflow were based on the code published by Karlicki et al. (2024) with modifications (Zenodo DOI 10.5281/zenodo.17552625). Eukaryotic and prokaryotic prevalence analyses were performed using the microbiome and microViz packages (Barnett et al., 2021; Lahti and Shetty, 2017). Taxonomic analyses were conducted on non-normalised data, transformed to relative abundances of ASVs. For diversity and core microbiome analyses, samples were grouped as biofilm- or water-associated. To account for sequencing depth differences, data were normalised using Scaling with Ranked Subsampling (Beule and Karlovsky, 2020), and all subsequent analyses used normalised data. Alpha diversity was assessed using the Shannon index, and differences between groups were evaluated via pairwise Wilcoxon tests. To define the core microbiome, ASV tables were filtered based on sample association with either biofilm or water. Core ASVs were defined following Neu et al. (2021) as those with ≥0.001% relative abundance present in ≥90% of samples within each group. Given the unequal group sizes (5 biofilm and 15 water column samples), this threshold was approximated to the nearest integer: 4 out of 5 samples for biofilm (80%) and 14 out of 15 samples for the water column (93%).

To identify 16S rDNA and 18S rDNA ASVs whose abundance correlated with the matrix group (biofilm: n = 5; water: n = 15 per sequencing marker) across samples, we applied a supervised machine learning algorithm, Random Forest (RF) (Breiman, 2001), following the methodology of Karlicki et al. (2024). For each dataset, we selected the top 60 ASVs with the highest mean decrease in the Gini coefficient index (Fig. S1), representing those with the greatest impact on

sample classification. The classification achieved an out-of-bag (OOB) error rate of 0% for the 18S rDNA dataset and 5% for the 16S rDNA dataset.

Results and Discussion

Community structure of biofilm and planktonic assemblages

Metabarcoding sequencing generated 2.96 million 16S rDNA and 3.35 million 18S rDNA reads, of which 43.84% and 62.15% passed quality filtering, yielding 5,999 bacterial and 4,509 eukaryotic ASVs, respectively (Table S2, Fig. S2). The most prevalent bacterial phyla in both biofilm and water samples were Proteobacteria (21–67%), Actinobacteriota (0.2–42.8%) and Bacteroidetes (15.2–41.4%) (Fig. 2a, upper panel). The protist community mostly consists of Dinoflagellata (3.4–89.4%), Gyrista (1.5–38%), Perkinsea (0.7–28%), Ciliophora (2.5–24%), Chlorophyta (0.9–25.7%), Fungi (0.6–39.2%) and Cercozoa (0.16–30.9%) (Fig. 2a, lower panel). While the major phyla were similar among samples (Fig. 2), differences emerged at lower taxonomic levels, with biofilm communities containing more taxa per sample than planktonic ones (Fig. S3).

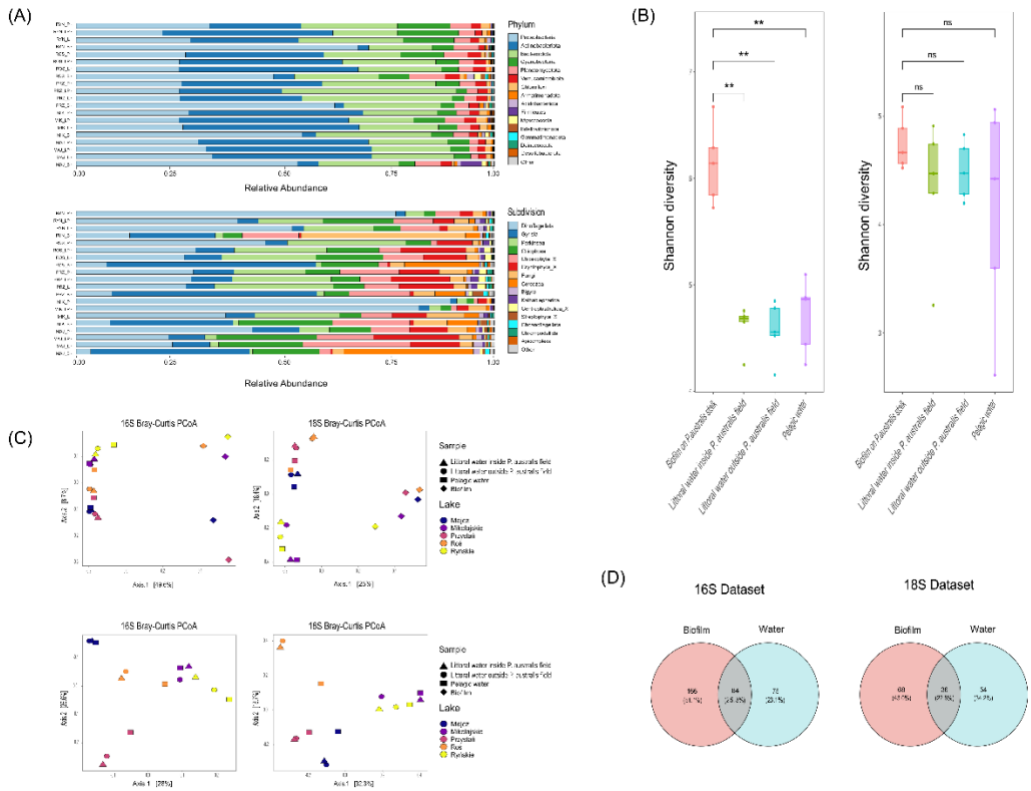


Fig. 2. Microbial community composition and diversity in biofilm and planktonic samples across five lakes. (A) *Relative abundance of bacterial (upper panel) and protist (lower panel) communities at phylum and subdivision level, respectively, across all biofilm and water samples. Samples are grouped by lake (Majcz, Mikołajskie, Przystań, Roś, Ryńskie) and sample type (biofilm, littoral water inside and outside P. australis bed, pelagic water).* ***(B)*** *Shannon diversity index for bacterial (left) and protist (right) communities across sample types. Boxes represent the interquartile range, with the median shown as a horizontal line; whiskers extend to 1.5× IQR; dots indicate outliers.* ***(C)*** *Principal Coordinate Analysis (PCoA) of Bray-Curtis dissimilarity for bacterial (16S, left) and protist (18S, right) communities. Upper panels show all sample types distinguished by shape, with lakes colour-coded; lower panels show only water samples to highlight between-lake variation.* ***(D)*** *Venn diagrams showing the number of shared and unique ASVs between biofilm and water samples for the 16S (left) and 18S (right) datasets.*

Prokaryotic biofilms appear to exhibit more complex community structures compared to planktonic communities across all parts of the lake (Fig. 2b), as supported by the Wilcoxon test ($p < 0.005$) (Supplementary Data 1) and the Shannon index, which ranges from approximately 5.7 to 6.7 for biofilms and 4.2 to 5.1 for water samples (Table S3). In contrast, no clear difference in richness or evenness was observed for protist communities between biofilm and plankton (Fig. 2b), consistent with the lack of significant differences detected by the Wilcoxon test for 18S rDNA data (Supplementary Data 2).

The Bray-Curtis dissimilarity metrics, visualised using Principal Coordinate Analysis, also showed that biofilm and planktonic communities were separated across all three water zones in the five lakes, for both prokaryotes and protists (Axis 1, Fig. 2c, upper panel). Furthermore, biofilm samples clustered together across lakes, although single biofilm replicates per lake limit within-lake inference (Fig. 2c, upper panel). The compositional difference was supported by PERMANOVA (adonis) analysis ($p < 0.001$) and beta-dispersion analysis ($p > 0.05$) for both datasets (Supplementary Data 1, 2), further supported by PERMANOVA, which showed compositional differences between biofilm and water communities across all zones using balanced pairwise comparisons. Notably, bacterial communities within biofilms seem to differ between the less eutrophic and more eutrophic lakes (Axis 2 of Fig. 2c, upper panel). Beta-dispersion was also assessed among samples across lake zones (excluding biofilm samples) and revealed differences in community composition between lakes across all zones in both datasets (Fig. 2c, lower panel), suggesting relatively homogeneous planktonic communities within lakes.

Our metabarcoding analyses of 16S and 18S rDNA datasets indicate that microbial communities forming biofilms on *P. australis* stalks differ from those in the surrounding plankton, for both bacteria and protists. Biofilm communities exhibited higher overall diversity, particularly among bacteria, and the separation between biofilm-associated and planktonic assemblages was consistently observed across all lakes. Notably, within individual lakes, planktonic communities remained relatively stable across spatial zones regardless of their proximity to *P. australis* biofilms. These results align with previous studies suggesting that large-scale habitat characteristics can influence planktonic community structure more strongly than localised effects of adjacent biofilms (Iniesto et al., 2022; Yan et al., 2019).

Core and characteristic taxa of biofilm and planktonic communities

Among bacterial ASVs forming the core of both communities, 166 (51.1%) are specific to the biofilm and 75 (23.1%) to the water column (Fig. 2d). Similarly, for protists, 68 (43.0%) ASVs are exclusive to the biofilm core and 54 (34.2%) to the water column core (Fig. 2d), indicating a larger core in biofilm communities than in planktonic ones. As core size can be influenced by group size and prevalence thresholds (water samples, $n = 15$; biofilm, $n = 5$), we also calculated cores within each lake zone where sample numbers are balanced. The results confirm that biofilm communities consistently harbour a larger core than the corresponding planktonic communities (Fig. S4). The high number of unique taxa in lake biofilms compared to planktonic ones suggests that biofilms support a more complex and functionally diverse microbial assemblage (Besemer et al., 2012; Robinson et al., 2025).

The bacterial ASVs specific to the biofilm core were primarily affiliated with Proteobacteria (*Aeromonas, Acinetobacter, Rhizobium* s.l.) and Bacteroidota (*Fluviicola, Flavobacterium*). The core of protistan biofilm taxa consisted mostly of Chlorophyta, Bacillariophyta, Fungi, and Ciliophora. Most bacterial ASVs specific to the water column were affiliated with Actinobacteriota, Cyanobacteria, and Bacteroidota, and among the protists, Dinoflagellates, Chlorophyta, and Ciliophora (Table S4).

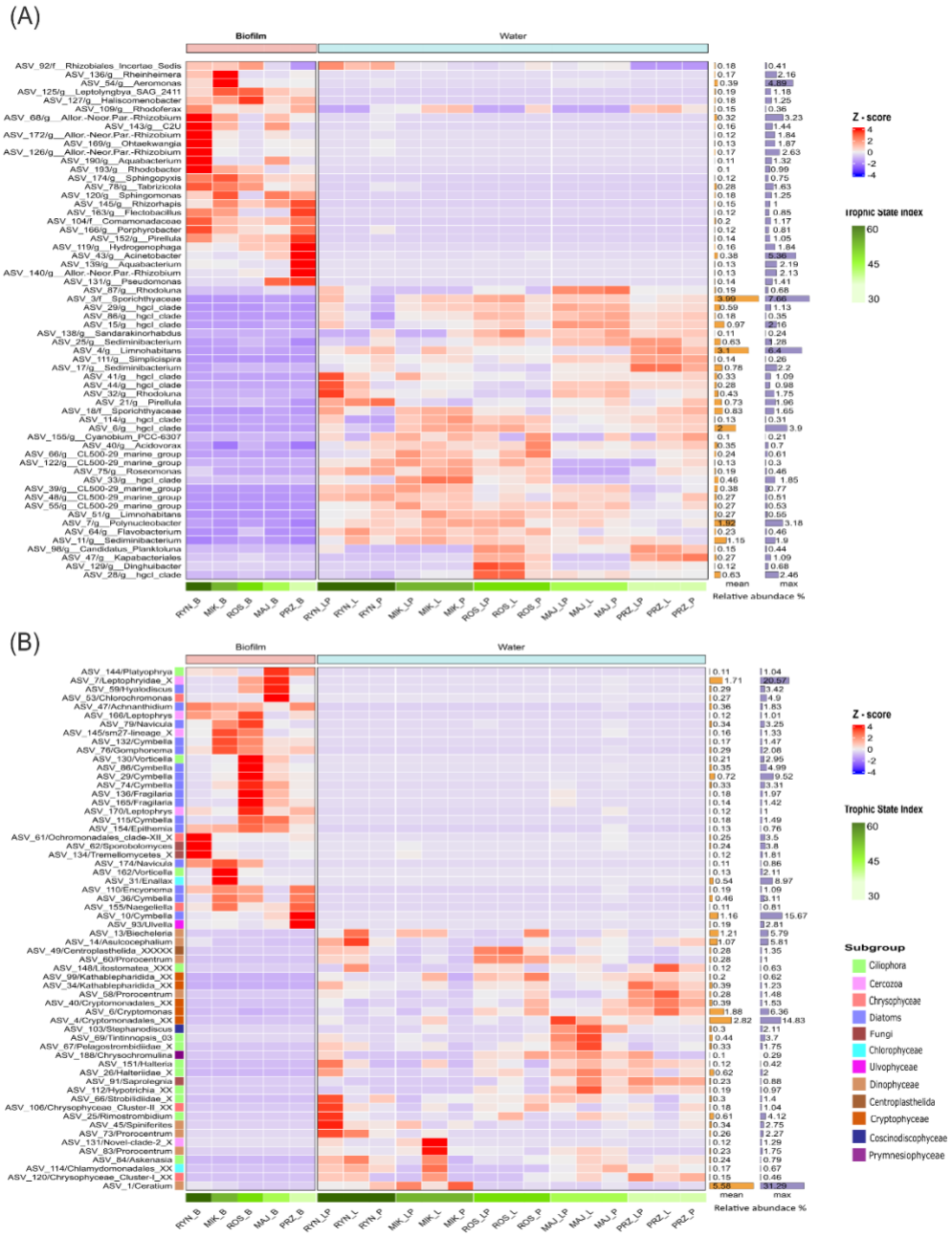


Fig. 3. *ASVs characteristic of biofilm and water communities identified by machine learning. Characteristic ASVs for each sample investigated were identified using a random forest algorithm. (A) Heatmap showing the abundance of the 60 ASVs selected by the random forest model across the 20 prokaryote samples. (B) Heatmap showing the abundance of the 60 ASVs selected by the model for protists. The first five samples represent biofilm communities, followed by water samples grouped by lake. Within each group, samples are arranged from higher to lower trophic level (left to right). The bar plots to the right of each heatmap display the overall relative abundance of each ASV across the entire dataset.*

Of the 60 selected ASVs in the 16S rDNA dataset, 26 were characteristic of the biofilm group, while 44 were characteristic of the water column (Fig. 3a). ASVs from the families *Rhizobiaceae, Rhodobacteraceae, Spirosomaceae, Saprospiraceae, Aeromonadaceae, Alteromonadaceae, Moraxellaceae, Pseudomonadaceae, Microscillaceae, Leptolyngbyaceae, and Sphingomonadaceae* define the characteristic prokaryotic biofilm community (Table S5).

In the 18S rDNA dataset, the 60 most characteristic ASVs are evenly distributed between biofilm and water column (30 ASVs each), with only a few biofilm-associated ASVs also present in the water column (Fig. 3b). The RF model for the 18S dataset identified 16 ASVs from nine genera of benthic diatoms as the most characteristic for protist biofilms (Fig. 3b). Other characteristic protist groups in biofilm samples include Chlorophyta, Ochrophyta, Cercozoa, Fungi and Ciliophora. A few genera are characteristic across all five biofilm samples, notably *Leptophrys*, *Achnanthidium* and *Epithemia*.

RF analysis of both 16S and 18S rDNA datasets further supported the separation of biofilm and planktonic clusters and highlighted the taxa most strongly driving this differentiation. Most ASVs identified overlapped with the biofilm core (Table S4), emphasising the central role of these taxa in sustaining biofilm structure and function. The bacterial ASVs identified by RF analysis as characteristic of biofilms included persistent freshwater colonisers. Genera such as *Aeromonas*, *Acinetobacter*, and *Flavobacterium* are associated with organic matter degradation and biofilm formation under variable conditions, while *Sphingomonas* and members of the Comamonadaceae contribute to biofilm structure and carbon cycling (Czieborowski et al., 2020; Grilo et al., 2021; Pandolfo et al., 2023). The presence of the *Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium* complex suggests associations with macrophytes that may enhance nitrogen availability and system stability. Some taxa, including *Sphingomonas*, are also recognised as plant growth-promoting bacteria (PGPB) (Flemming and Wingender, 2010). Additionally, several core genera (e.g., *Sphingomonas*, *Hydrogenophaga*, *Pseudomonas*, *Rhodoferax*, *Flavobacterium*) are commonly linked to diatom-associated biofilms, which were abundant in the samples (Bruckner et al., 2008; Grossart et al., 2005). The characteristic protistan biofilm community comprised phototrophic taxa, including Chlorophyta and benthic diatoms (e.g. *Cymbellales*, *Gomphonema*, *Navicula*), as well as fungal taxa likely acting as decomposers (Grossart et al., 2016; Klawonn et al., 2023) and potential bacterial grazers such as ciliates (e.g. *Rimostrombidium*).

Environmental drivers of community composition

The envfit function applied to the NMDS ordination enabled us to identify potential environmental variables influencing community composition. Six environmental variables appeared to correlate with free-living bacterial community composition, and temperature correlated with protists (Fig. S5). However, no clear associations were observed for biofilm communities (Table S6), although limited within-lake replication prevents firm conclusions.

The consistent biofilm composition and high number of unique ASVs (Fig. 2a, b, d) suggest that factors other than surrounding planktonic communities or lake-specific environmental conditions may contribute to biofilm structure (Rusznyák et al., 2007; Tsuchiya et al., 2011). Biofilm communities showed no significant associations with the measured bulk-water variables, in contrast to the planktonic communities (Table S6). However, this interpretation is limited by the small number of biofilm samples (n = 5) and possible scale mismatches. Given the limited sampling (one biofilm sample per lake, n = 5) and single timepoint, envfit results for biofilms have low power, and bulk-water variables may not reflect reed-surface microenvironments. Biofilm structure may instead reflect internal community dynamics, potentially associated with a stable core of bacterial and protist taxa.

While biofilms may appear similar when considering only the most abundant taxa (Fig. 2a), a more detailed examination of characteristic ASVs (Fig. 3a) revealed distinct taxonomic signatures often linked to lake trophic status (TSI). In more eutrophic lakes (Ryńskie, Mikołajskie, Roś), biofilm-associated ASVs included *Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium*, C2U/*Pseudoxanthobacter*, *Ohtaekwangia*, *Aquabacterium*, and members of *Rhodobacteraceae* and *Sphingomonadaceae*, with *Aeromonas* and *Rheinheimera*

characteristic of Mikołajskie. In less eutrophic lakes (Majcz, Przystań), biofilms were characterised by *Pseudomonas*, *Acinetobacter*, *Hydrogenophaga*, and *Aquabacterium*. Some ASVs (e.g. *Rhizobiales*, *Rhodoferax*) occurred in both biofilm and water. Protistan communities showed similar patterns: phototrophic taxa (*Chlorochromonas*, *Naegeliella*, *Ulvella*) characterised less eutrophic lakes, whereas fungal taxa (*Sporobolomyces*, *Tremellomycetes*) and heterotrophs (*Ochromonadales*, *Vorticella*) were more prominent in eutrophic systems.

Observed patterns correspond with known associations of taxa with trophic conditions, including nutrient-associated groups such as *Rhizobiales*, *Pseudoxanthobacter*, and *Ohtaekwangia*, as well as metabolically flexible taxa typical of meso- to oligotrophic environments (Wang et al., 2017; Czieborowski et al., 2020; Grilo et al., 2021; Pandolfo et al., 2023). The presence of *Aeromonas* and *Rheinheimera* in eutrophic lakes aligns with their association with nutrient-enriched or anthropogenically impacted waters (Janda and Abbott, 2010; Zhao et al., 2023; Lennert et al., 2024). Shared ASVs between biofilm and plankton may indicate exchange between habitats, potentially via dispersal or detachment processes (Grossart et al., 2005; Bruckner et al., 2008). Meanwhile, lake-specific patterns– such as the occurrence of photoheterotrophic taxa under reduced light conditions or taxa associated with high water clarity– suggest that local environmental factors influence biofilm composition (Kopejtka et al., 2020; Villena-Alemany et al., 2024). For protists, the distribution of phototrophic taxa in less eutrophic lakes and heterotrophic or decomposer taxa in more eutrophic systems is consistent with shifts in resource availability and trophic structure reported in freshwater ecosystems (Zhu et al., 2015; Nicholls and Wujek, 2015; Grossart et al., 2016; Nunes et al., 2022; Klawonn et al., 2023).

Functional differentiation of biofilm and planktonic communities

We assessed the physiological potential of bacterial communities by measuring leucine-aminopeptidase (LAP) activity and analysing community-level physiological profiles with Biolog EcoPlates. Maximal LAP activity, measured per sample volume, was 10–100 times higher in biofilm suspensions than in water column communities (Table S7). Likewise, biofilm-associated microorganisms consistently exhibited higher substrate utilisation rates than planktonic communities (Fig. 4a). Respiration profiles across major organic compound groups (Fig. 4b, Table S8) showed that carbohydrates and complex carbon sources were generally preferred in both community types. However, amine utilisation varied substantially between lakes and habitats. In Lakes Mikołajskie and Ryńskie, amine utilisation accounted for only 2–8% of total respiration in littoral and pelagic water samples, but increased to approximately 20% in biofilm communities associated with *P. australis*. In Lake Przystań, littoral plankton inside the *P. australis* zone also exhibited elevated amine utilisation (~21.9%), whereas the biofilm-associated community displayed a distinct substrate utilisation pattern. In contrast, biofilm communities in Lake Majcz utilised all carbon sources at relatively consistent rates (11–18%), indicating a more balanced metabolic profile.

Bray–Curtis dissimilarity and NMDS analyses based on the utilisation of 31 carbon substrates revealed clear clustering of biofilm samples, indicating a consistent physiological fingerprint (Fig. 4c, d). This separation was supported by PERMANOVA ($p < 0.001$) and beta-dispersion analysis ($p > 0.05$) (Supplementary Data 3). While planktonic communities from the same lake showed some similarity in substrate utilisation, biofilm communities remained distinct across lakes. Notably, the biofilm from Lake Mikołajskie deviated from the main cluster, indicating a unique metabolic profile in carbon utilisation (Fig. 4c, d).

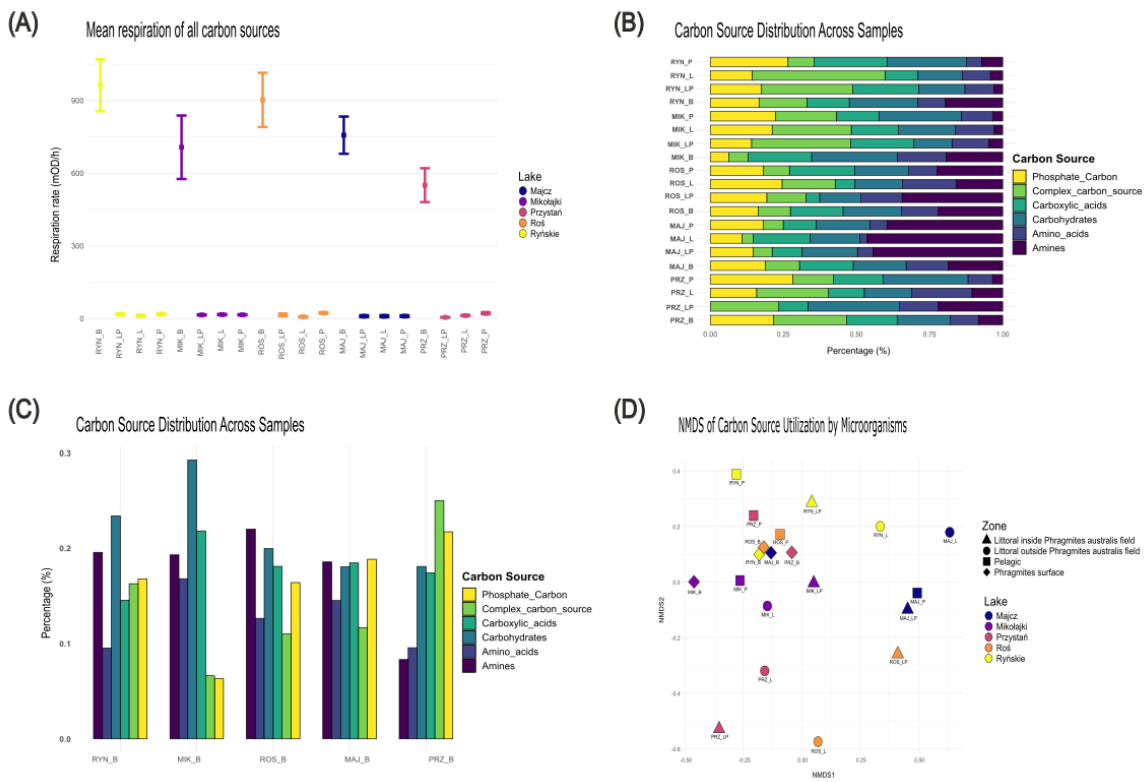


Fig. 4 *Visualization of Biolog EcoPlate Results. (A) Boxplot illustrates the average respiration rate of 31 carbon sources per sample. (B) Bar plot displays the average relative utilization of carbon sources, categorized by compound type for each sample. (C) A focused comparison of carbon sources used by biofilm samples is shown. (D) NMDS plot representing the Bray-Curtis dissimilarity analysis, highlighting variations in the utilization of 31 different carbon sources across samples.*

The higher LAP activity and substrate utilisation rates in biofilms are consistent with their role as metabolically active hotspots, where attached microbial communities efficiently process organic matter. The increased use of amines in biofilms and selected planktonic samples may reflect localised nitrogen availability and substrate specialisation, while the general preference for carbohydrates and complex carbon corresponds with the dominance of plant-derived and recalcitrant DOM in these systems. The distinct clustering of biofilm metabolic profiles suggests a degree of functional consistency across lakes, whereas the greater variability among planktonic communities likely reflects sensitivity to local environmental conditions such as nutrient availability, DOM composition, and microbial interactions (Zwart et al., 2002; Sala et al., 2020; Hu et al., 2022). Differences between littoral and pelagic zones further indicate spatial structuring, potentially linked to plant-derived inputs in littoral habitats (Lambert and Perga, 2019; Stets and Cotner, 2008).

Lake-specific patterns support this interpretation. In Lake Roś, broad substrate utilisation suggests diverse DOM sources and active microbial turnover (Kajan et al., 2023), whereas in Lake Majcz, elevated amine utilisation may indicate nitrogen-rich, phosphorus-limited conditions. In contrast, Lake Przystań communities showed a stronger preference for carbohydrates and complex carbon, consistent with degradation of plant-derived material under moderate nutrient conditions.

Overall, these results highlight that biofilm communities maintain distinct and relatively consistent metabolic profiles, while planktonic communities exhibit greater functional variability shaped by local environmental conditions.

Conclusions

Phragmites australis stem biofilms contain diverse and compositionally distinct microbial communities compared to surrounding plankton, with a consistent core assemblage and higher metabolic activity. These differences were supported by both taxonomic (16S, 18S) and functional analyses. Biofilm communities were relatively consistent across lakes, whereas planktonic communities exhibited greater variability, suggesting a stronger influence of local environmental conditions on free-living assemblages. Both bacterial and protistan groups contributed to these patterns, highlighting their roles in biofilm structure and function. By including protists alongside bacteria, this study extends previous molecular research, which has largely focused on prokaryotes (Souffreau et al., 2018; Fedorov et al., 2022), and reinforces the distinction between biofilm-associated and planktonic communities in freshwater ecosystems (Brablcová et al., 2013; Mou et al., 2013; Borsodi et al., 2020).

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Author contribution statement

Conceptualisation: AK, BK, VS. Developing methods: AK, BK, VS. Conducting the research: VS, MK, BK, AK. Data analysis: VS, BK. Data interpretation: VS, BK, AK. Preparation of figures and tables: VS. Writing original draft: VS. Writing review and editing: VS, AK, MK, BK.

Data Availability Statement

The sequencing data have been deposited in the EMBL-EBI European Nucleotide Archive (ENA) under the Project ID PRJEB90292. All the rest of the Supplementary materials can be found in the Zenodo repository under DOI 10.5281/zenodo.17552625.

Conflicts of Interest

The authors have declared that no competing interests exist.

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