Divergent biofilm and free-living microbial communities on *Phragmites australis* and in plankton across an eutrophication gradient in the Great Masurian Lakes (Poland)

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ABSTRACT

Biofilms are key components of freshwater microbial communities, contributing critically to ecosystem functioning. Composed of both bacteria and protists, biofilm communities are shaped by environmental conditions and the lifestyle traits of their constituent organisms. Although biofilm and planktonic communities may share some taxa, the extent of the overlap and the factors driving community differentiation remain poorly understood. Here, we used high-throughput metabarcoding of 16S and 18S rRNA genes to investigate bacterial and protist communities associated with *Phragmites australis* stem biofilms and planktonic fractions across five lakes spanning an eutrophication gradient in the Masurian Lake District, northeastern Poland. The biofilm communities on reed stems were highly distinct from planktonic ones, sharing only a small core microbiome. The biofilm core not only exceeded that of planktonic communities but also contained characteristic taxa, identified via machine learning, that were distinct from planktonic assemblages, including several with potential roles in bioremediation and as bioindicators. Planktonic communities were strongly influenced by environmental factors, whereas *Phragmites australis*-associated epiphytic biofilm communities appeared more uniform across lakes, consistent with host-associated selection and potentially with lifestyle-related buffering. Moreover, biofilm suspensions exhibited higher apparent physiological activity per inoculum volume and broader substrate use under our assay conditions than the free-living communities. Altogether, our findings demonstrate the ecological significance of biofilms in freshwater systems and underscore the need to integrate both biofilm and planktonic fractions of prokaryotic and eukaryotic organisms to fully capture microbial diversity, function, and ecosystem resilience.

Keywords: protist, bacteria, biofilm, free-living microbial community, freshwater lake, eutrophication

1. Introduction

Microbial communities in freshwater ecosystems are complex and dynamic, with multiple interacting components promoting ecosystem stability, water quality, and resilience (Hellawell 2012; Sagova-Mareckova et al. 2021). In those communities, we can identify two main life strategies: free-living plankton and community-aggregated assemblages known as biofilms. While planktonic microbial communities have traditionally been the focus of research, biofilms are increasingly recognized as crucial ecological players (Battin et al. 2003a; Battin et al. 2016) involved in maintaining a healthy aquatic environment (Besemer 2015; Bonnineau et al. 2020; Sentenac et al. 2022). Biofilms exist in various forms such as epilithic (on rocks), epiphytic (on plants) and benthic (on sediments), and support ecosystem stability by facilitating primary production, cycling nutrients, and purifying water (Besemer 2015; Bighiu and Goedkoop 2021). Their microbial communities also contribute to the degradation of pollutants - including heavy metals, herbicides and organic contaminants - and significantly improve the efficiency of activated sludge systems, particularly by promoting nitrogen removal through integrated fixed-film-activated sludge processes (Gebara 1999; Bighiu and Goedkoop 2021). Though purely bacterial communities are often the primary focus in biofilm studies, the role of protists and their interactions with bacteria remain understudied. This is a shame as they can act as important grazers on biofilm bacteria, affecting population dynamics (Gleason et al. 2008; Wey et al. 2012; Hoque et al. 2023). Diatoms, for instance, substantially contribute to biofilm cohesion and architecture, creating microhabitats and providing surfaces for algal–bacterial interactions (Battin et al. 2003b; Grossart et al. 2005; Flemming and Wingender 2010a; Makk et al. 2024). On the other hand, some might be hosts for pathogenic bacteria (Lau and Ashbolt 2009) and facilitate the spread of toxins and antibiotic-resistant genes (Greub and Raoult 2004). All members of biofilm microbial communities collectively drive biofilm physiology: heterotrophic bacteria and fungi mediate carbon utilisation and nutrient cycling, and protists exert top-down control by feeding on bacteria (Amado and Roland 2017; Sentenac et al. 2022; Hoque et al. 2023; Perujo et al. 2025).

Among the various types of freshwater biofilms, epiphytic biofilms - those that develop on the stalks of aquatic plants - are particularly important. These biofilms exhibit higher 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, playing an active role in 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 (Wijewardene et al. 2022; Sentenac et al. 2022), a stability in part attributable to the functional redundancy of microbial communities which ensures the maintenance of key ecosystem processes despite environmental disturbances (Yachi and Loreau 1999; Díaz and Cabido 2001; Biggs et al. 2020). Moreover, submerged macrophytes, like *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 the lakes, biofilms on *P. australis* surfaces can significantly improve nutrient and contaminant removal, acting synergistically with the plant's natural filtration and assimilation capabilities (Hiraki et al. 2009; Zhao et al. 2012). These same

properties are used in constructed wetlands where *P. australis* fosters microbial communities that aid in phytoremediation and environmental restoration by breaking down pollutants and improving wastewater treatment (Scholz and Xu, 2002; Scholz, 2003; Stottmeister et al., 2003; Kaseva, 2004).

Epiphytic biofilm microbial communities can serve as sensitive indicators of water quality. While organisms such as macroinvertebrates, fish, and diatoms are well-established bioindicators due to their high sensitivity to environmental change (Blackman et al. 2024), their morphology-based assessments are labour-intensive and often yield incomplete data. The integration of standardized eDNA sequencing with physicochemical measurements enables more accurate and spatially comprehensive monitoring. Diatoms, which frequently occur in sediments and biofilms due to their non-motile nature, are particularly informative; their detection within epiphytic biofilms can provide valuable insights for ecological assessment (Poulíčková et al. 2004; Arumugham et al. 2023; Blanco 2024).

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 provide yet a clear picture of their reciprocal influences. Even less is known about the protist components of these communities 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 from water collected from three lake zones (located at increasing distance from the reed stands) across five lakes in the Great Masurian Lake District (GMLD), Northeastern Poland. The GMLD lakes are an interconnected glacial lake system that holds about 20% of the country's freshwater. Despite their shared geological and climatic background, the lakes vary in trophic state (Siuda et al. 2020a), offering a natural gradient to explore biofilm and free-living plankton composition, ecological roles, and environmental interactions. Several studies investigated the diversity of free-living bacteria in the GMLD and demonstrated the strong impact of eutrophication on microbial communities. Eutrophication has been shown to profoundly affect microbial organic matter cycling in lakes and to reshape bacterial communities, leading to shifts in taxonomic composition, favouring potentially pathogenic bacteria, and promoting antibiotic resistance (Kiersztyn et al. 2019a; Grabowska-Grucza and Kiersztyn 2023). Protistan communities, however, have been comparatively understudied, particularly when it comes to using molecular approaches. Recent investigations in Lake Roś (a part of the GMLD system) have begun to address this gap, revealing strong seasonal dynamics and season-specific taxa within bacterial and protistan assemblages (Karlicki et al. 2024).

In this study, we used high-throughput sequencing of 16S and 18S rRNA genes to compare bacterial and protist communities in biofilms and plankton along an eutrophication gradient in selected GMLD lakes. Specifically, we aimed to: (1) identify shared and habitat-specific taxa in biofilm communities; (2) determine the impact of environmental conditions on biofilm

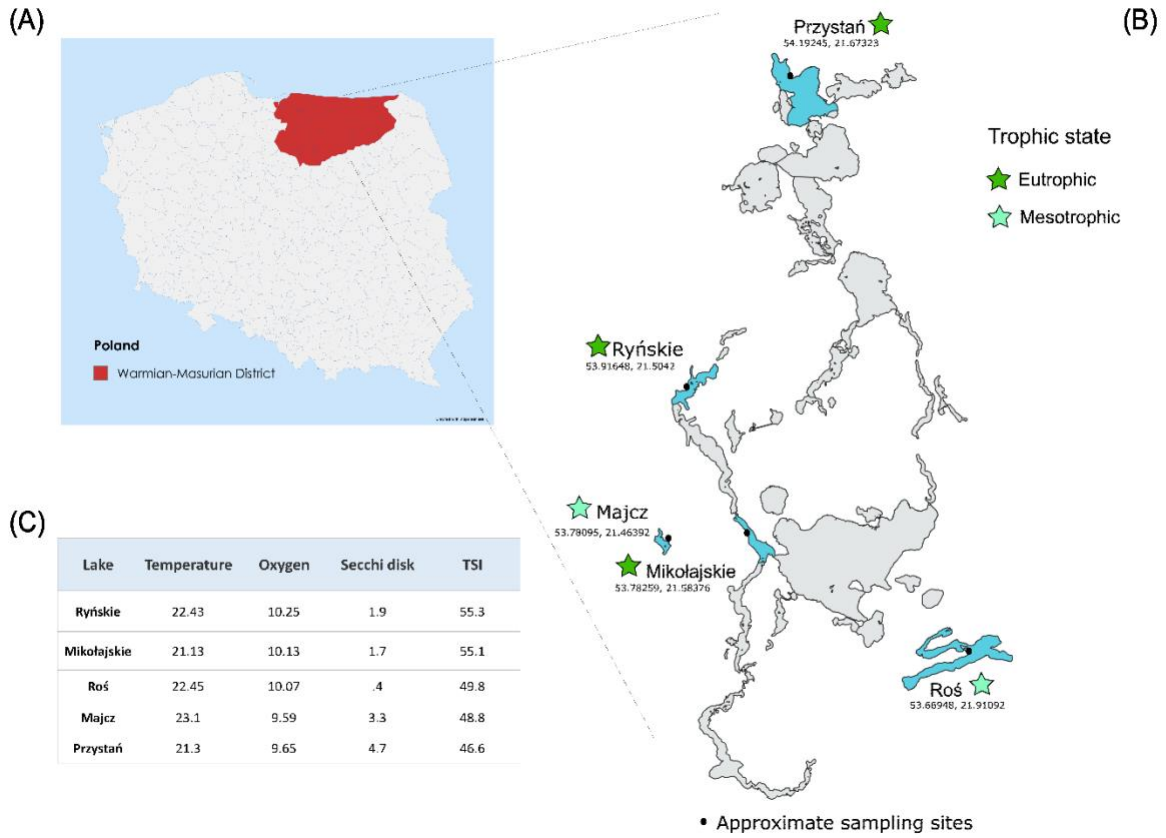
community structure; and (3) assess bacterial functional diversity and physiological activity to reveal habitat-specific physiological traits.

2. Materials and methods

2.1 Research area: site description.

For this study, five lakes within the Great Masurian Lake District (GMLD) in northeastern Poland were selected as sampling sites (Fig. 1). Four of these lakes—Ryńskie, Roś, Mikołajskie, and Przystań—are part of the Great Masurian Lake System (GMLS), a network of interconnected lakes linked by natural and artificial channels. Although Lake Majcz, the fifth lake, is not directly connected to the GMLS, it lies within the same district and shares a common geological origin. Moreover, its waters experience similar environmental conditions and moderate anthropogenic influences, making it a relevant inclusion as a mesotrophic lake subject to relatively weak anthropopressure compared to the other studied lakes. Though the lakes in the GMLD share similarities in origin, geological setting, catchment characteristics, and climate conditions, they exhibit varying degrees of eutrophication.

According to Carlson's trophic classification, Lake Ryńskie is the most eutrophic, with a mean Trophic State Index (TSI) of 55.3 over the last three years (2021–2023) (Carlson 1977). This is attributed to factors such as slow water exchange and high urbanization in the surrounding area, and the presence of a large sewage treatment plant, whose effluent flows directly into the lake. Similarly, lakes Mikołajskie and Roś, both located in highly urbanized zones, are classified as eutrophic, with mean TSI values of 55.1 and 49.8, respectively (Table S1). In contrast, Majcz and Przystań are categorized as mesotrophic, consistent with data from previous years (Bowszys et al. 2014; Grabowska et al. 2019). Among the studied lakes, Przystań has the lowest mean TSI of 46.6, making it the least trophic. Situated in the northern part of the district, Przystań was one of the last lakes to transition from oligotrophic to mesotrophic status between 2005 and 2015, as reported by Siuda et al. (2020b).



(C)

Lake	Temperature	Oxygen	Secchi disk	TSI
Ryńskie	22.43	10.25	1.9	55.3
Mikołajskie	21.13	10.13	1.7	55.1
Roś	22.45	10.07	.4	49.8
Majcz	23.1	9.59	3.3	48.8
Przystań	21.3	9.65	4.7	46.6

Fig. 1. Geographic location of the studied lakes. (A) The map shows the Great Masurian Lake District (GMLD) (northeastern Poland), where the sampled lakes are located (highlighted in red, upper left inset). (B) The five sampled lakes are displayed with marked sampling sites. Lakes are classified as eutrophic (green stars) or mesotrophic (light green stars) based on their Trophic State Index (TSI). (C) The table summarizes key environmental parameters: temperature (°C), dissolved oxygen (mg/L), Secchi disk depth (m), and TSI. The map of the district (upper left inset) was created through the Mapchart website (MapChart), while the lakes' map on the right using QGIS program version 3.40 (QGIS Development Team 2025)

2.2 Samples collection

From each lake, two types of samples were collected: aquatic plants for biofilm analysis and water samples. Three water samples were taken at increasing distance from the reed stands within each lake: (1) the littoral zone within the *P. australis* field, (2) the littoral zone outside the *P. australis* field, and (3) the pelagic zone. Subsamples from the littoral zones outside *P. australis* fields were collected from areas at least 10 m away from the nearest *P. australis* stand, while pelagic zone samples were taken from locations at least 200 m from the littoral zone. A total of 5 L of surface water was collected from each sampling site. Biofilm samples from *P. australis* stems were collected following the method described by Tsuchiya et al. (2011), with modifications as follows: stems of *P. australis* were cut into 30 cm fragments, starting 5 cm below the water surface and extending to a depth of 35 cm. The uppermost 10 cm was discarded, and the remaining 20 cm long stem section was placed into a sterile plastic bottle filled with lake water from the corresponding *P. australis* field. A total of 10 stem fragments were collected from each lake.

All samples were transported to the laboratory within two hours of collection. In the laboratory, biofilm was removed from the surface of each stem using a sterile brush and suspended in 0.2 µm-filtered water collected from the corresponding sampling site. For each biofilm sample, biofilm resuspension was performed using all ten collected stems. The biofilm from each 10 cm-long stem section was suspended in a known volume of water (typically 100 mL). The diameter of each of the 10 stems was recorded. The prepared samples were then analysed using Biolog® EcoPlates™ (Biolog Inc., Hayward, CA, USA; see section 2.4 for details) and for leucine aminopeptidase activity.

For taxonomic composition analysis, each water and biofilm sample was filtered under low air pressure (up to 200 hPa above atmospheric pressure) using Nucleopore filters (Whatman, Maidstone, UK) with a 0.2 µm pore size. The filters were stored at -80°C until DNA extraction.

Each studied lake had a unique sample identifier assigned: RYN (Ryńskie), ROS (Roś), MIK (Mikołajskie), PRZ (Przystań), and MAJ (Majcz). From each lake, four distinct subsamples were collected: B (biofilm microorganisms from the *P. australis* stem surface), LP (littoral lake water within the *P. australis* field), L (littoral lake water outside the *P. australis* field), and P (pelagic lake water). When analysing or referring to the three water samples (LP, L, and P) collectively, we use the term 'water column.' Each sample type was prepared in two forms: as untreated water and as a suspension in sterile 0.2 µm-filtered lake water, depending on the specific requirements of further analyses.

2.3 Physical and chemical metadata

For each of the three sampling points, temperature and oxygen concentration were measured *in situ* using a YSI ProODO multiparametric probe directly during the sample collection (Yellow Springs, YSI Inc./Xylem Inc., USA). The depth of the photic zone was determined using a portable light meter (LiCor LI-250A with a spherical underwater PAR quantum sensor LI-193R) and a Secchi disk.

Water samples for chemical analysis were collected and frozen until laboratory processing. Dissolved organic carbon (DOC) was quantified using a Shimadzu TOC 5050 carbon analyser. Total phosphorus (TP) and phosphate (PO_4^{3-}) concentrations were measured using the molybdate method with a Shimadzu UV-VIS 1201 spectrophotometer, following the protocol described by (Grasshoff et al. 1983). Total nitrogen (TN) was determined using commercially certified Merck-Millipore cell tests (Spectroquant Nitrogen (Total) Cell Test, 114537), according to the manufacturer's instructions, and analysed with a Merck Spectroquant Pharo 300 spectrophotometer.

The Trophic State Index (TSI) was calculated as the arithmetic mean of TSI values derived from the total phosphorus concentration measurement taken across the three consecutive summer seasons of the years 2021, 2022, and 2023 (Table S1) following the Carslon formula:

$TSI_{TP}=14.42\times\ln(TP)+4.15$

where TP is in µg/L.

2.4 Physiological fingerprint diversity of microbial communities

The Biolog® EcoPlates™ (Biolog Inc., Hayward, CA, USA) method was used to determine community-level physiological profiles, with modifications as described below. The EcoPlate is a 96-well microplate containing triplicate of 31 different carbon sources, enabling the assessment of bacterial community substrate utilization. Each environmental sample (biofilm

or water) was tested in three independent biological replicates to ensure reproducibility of the results. The carbon sources included: Carbohydrates — D-cellobiose, α-D-lactose, β-methyl-D-glucoside, D-xylose, erythritol, D-mannitol, N-acetyl-D-glucosamine, D-galactonic acid γ-lactone; Phosphorylated compounds — Glucose-1-phosphate, D,L-α-glycerol phosphate; Amines — Phenylethylamine, putrescine; Carboxylic acids — D-glucosaminic acid, D-galacturonic acid, γ-hydroxybutyric acid, itaconic acid, α-ketobutyric acid, D-malic acid, pyruvic acid methyl ester, 2-hydroxybenzoic acid, 4-hydroxybenzoic acid; Complex carbon sources — Tween 40, Tween 80, α-cyclodextrin, glycogen, Amino acids — L-arginine, L-asparagine, L-phenylalanine, L-serine, L-threonine, glycyl-L-glutamic acid.

The untreated water and biofilm samples were processed following the method described by Kiersztyn et al. (2019b). Each well was filled with 150 µL of sample, and plates were incubated in the dark at 22 °C for 72 hours. Absorbance at 590 nm was measured every 4 hours using a Biotek Synergy H1 plate reader (Biotek Corporation, USA). The observed reduction of tetrazolium chloride, indicative of microbial respiration, increased over time.

For analyses, we used the Vmax of respiration rate, calculated using Gen 5 software (Biotek Corporation, USA). Vmax was determined by identifying the steepest slope of the absorbance of tetrazolium chloride reduced during respiration across every four consecutive readings during the 72-hour incubation period, using a linear regression model. The maximal rate of colour development (Vmax, mOD/min) was considered proportional to microbial respiration for each carbon source. Control wells (without added carbon sources) were used to determine baseline respiration.

To assess the effect of each carbon source on respiration, the difference (ΔVmax) between carbon-containing wells and control wells was calculated. If ΔVmax was greater than zero, the substrate was considered utilized, and the value was included in further analyses. If ΔVmax was zero or negative, the substrate was deemed ineffectively utilized, and its Vmax was set to zero. For community-level physiological fingerprinting, the relative respiration rate for each carbon source was calculated as its percentage contribution to the total Vmax across all wells on the plate. To ensure comparability between biofilm and water column results, a volumetric coefficient was applied to biofilm measurements; however, data were not normalised to cell density or biomass. The coefficient was calculated as follows: Volume coef. for biofilms = (100 [ml]/ mean volume [ml] of single 10 cm long *P. australis* stem). This adjustment enabled volumetric comparison between the activity of planktonic microorganisms in water and that of biofilm-associated microorganisms on an equivalent stem volume. However, the observed functional differences may reflect apparent activity per inoculum volume and could be influenced by inoculum density and matrix composition (e.g., EPS or exudates) due to the lack of normalization.

2.5 Leucine-aminopeptidase activity

Maximal potential leucine-aminopeptidase activity (Vmax_LAP) was measured fluorometrically following the methods of Chróst and Siuda (2006) and Kiersztyn et al. (2019b). For each assay, 3.9 mL of water sample was mixed with 0.1 mL of L-leucine-4-methyl-coumarinylamide hydrochloride (Leu-MCA) substrate solution prepared in ethanol, resulting in final Leu-MCA concentrations of 1.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 25.0, and 50.0 µM. Separate stock solutions were prepared for each Leu-MCA concentration. To ensure consistency, calibration standard solutions for fluorescence product measurement (7-amino-4-methylcoumarin, AMC) were prepared with the same ethanol concentrations.

Fluorescence of AMC was measured spectrofluorometrically (excitation: 380 nm, emission: 460 nm) using a microplate reader (Biotek Corporation, USA) at time zero and after 0.5–1 hours of incubation at 20 °C. The enzyme-substrate system followed first-order Michaelis–Menten kinetics, described by the equation:

$$v = V_{max} * [S] / (K_m + [S])$$

where v is the reaction velocity, Vmax is the maximum reaction rate, [S] is the substrate concentration, and Km is the Michaelis constant. Nonlinear regression analysis was performed to determine the kinetic parameters using Origin 6.1 software (OriginLab Corporation, Northampton, USA). To ensure comparability between biofilm and water results, the volumetric coefficient was applied to biofilm measurements.

2.6 DNA Extraction, DNA Amplification, and Sequencing

DNA was isolated using the GeneMATRIX Soil DNA Purification Kit (EURx) from 20 samples taken from each of the collected 0.2-um filters following the manufacturer's protocol with minor modifications: depending on the biomass recovered on the filters, one-quarter or one-half of the filter was used as the starting material instead of soil, and after the addition of 60 µL of lysis buffer the samples were homogenised for 10 min with a “bead beater” (vertical homogenizer), stored at -80℃ for 1h, and homogenised again for 5 min at maximum speed. For the final elution, 30 µL of DNase/RNase-free water was used instead of elution buffer for the recovery of the DNA.

Extracted environmental DNA was quantified using Implen NanoDrop (Thermo Scientific), and the fragmentation size of DNA was checked with a 1% agarose gel. Samples were sent to the University of Warsaw Sequencing Facility (CeNT’s Genomics Core Facility) for the subsequent amplification, library preparation, and sequencing on the NovaSeq 6000 next-generation sequencing platform. The prokaryotic V4-V5 hypervariable region of the 16S rRNA gene was amplified using universal prokaryotic primers 515F-Y (5’- GTGYCAGCMGCCGCGGTAA -3’) and 926R (5’- CCGYCAATTYMTTTRAGTTT -3) (Parada et al. 2016). The forward primer was tagged with 24 different Multiplex Identifier (MID) to enable the pooling of multiple samples in a single microtube, as described in the protocol of Romac (2022). Amplifications were performed in triplicate with Phusion High-Fidelity DNA polymerase (ThermoFisher); 25 µl of the reaction mixture was done as follows: 1 ng of DNA, 1X of 5X Phusion™ HF Buffer (ThermoFisher), 0.25 µM barcoded forward and reverse primer, 0.02 U/µL of polymerase, and dNTPs (200 µM each). For cycling conditions, a 4 min heating step at 98°C was followed by 20 cycles of 98°C for 20 s, 50°C for 30 s, 72°C for 10 s, and a final extension of 72°C for 5 min. All amplifications were done in triplicate to balance the variance within samples while obtaining adequate amounts of amplified DNA, then combined, and finally purified using a PCR clean-up kit (Syngen).

2.7 Sequence analysis

Sequence quality checks were performed on raw sequencing data using FastQC (Andrews S. 2010). The sequencing of samples RYN_P, RYN_B, and RYN_L (16S) and ROS_P and ROS_B was repeated, and in some cases, the datasets were merged. The resulting sequences were deposited in the EMBL-EBI European Nucleotide Archive (ENA) under Project ID PRJEB90292. A detailed description of the processing steps is available in the corresponding Zenodo repository (10.5281/zenodo.17552625). Reads were then imported into the QIIME2 environment for demultiplexing and adapter trimming using the cutadapt demux-paired

function. Sequencing primers were subsequently removed with Cutadapt (Martin M. 2011; Bolyen E et al. 2019). Denoising was conducted using the DADA2 algorithm in QIIME2 with the dada2 denoise-paired function (minimum overlap = 10; max mismatches = 0; consensus method for chimera removal (Callahan et al. 2016). Following denoising, amplicon sequencing variant (ASV) tables and feature tables were merged.

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). The SILVA 138.1 database required formatting to be compatible with VSEARCH what was performed using the Python script provided by McNichol and Hernández Limón (2022). All taxonomic and diversity analyses were conducted in RStudio IDE using the phyloseq package (Allaire J. 2012; McMurdie and Holmes 2013). Before analysis, ASVs classified as Eukaryota, chloroplasts, or mitochondria were removed from the 16S dataset, while ASVs assigned to Metazoa, Embryophyta, Bacteria, or Archaea were filtered out from the 18S dataset. Whenever the alignment could not assign an exact taxonomy, the sequence was classified to the lowest reliable taxonomic rank for both protists and bacteria. ASVs tables derived for DADA2 denoising, fasta files and taxonomy assignation tables are available in the Zenodo repository (10.5281/zenodo.17552625).

2.8 Statistical analyses

Statistical analyses, including alpha diversity, beta diversity, Principal Coordinates Analysis (PCoA), Non-metric Multidimensional Scaling (NMDS), ADONIS, ANOVA, and envfit were conducted in R (version 4.2.0) within RStudio 2023.09.0 (Allaire J. 2012). The following packages were used: *vegan* (Oksanen et al. 2023), *qiime2R* (Bisanz 2018), *ggplot2* (Wickham 2016), *phyloseq* (McMurdie and Holmes 2013), *ape* (Paradis and Schliep 2019), *microViz* (Barnett and et al. 2021), and *microbiome* (Lahti and Shetty 2017). The analytical workflow was based on the code published by Karlicki et al. (2024) with modifications adapted to this study. The code used with the implemented modification is available in the Zenodo repository under DOI 10.5281/zenodo.17552625.

Eukaryotic and prokaryotic prevalence analyses were performed using the *microbiome* and *microViz* packages. General taxonomic analyses were conducted on non-normalized data, transformed to relative abundances of ASVs for sampling sites B, LP, L, and P across the five lakes (20 samples in total). For diversity analyses and core microbiome identification, samples were categorized into biofilm-associated and water-associated groups. To account for differences in sequencing depth, datasets were normalized using Scaling with Ranked Subsampling (SRS) (Beule and Karlovsky 2020), and all subsequent statistical analyses were performed on normalized 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 as those with at least 0.001% relative abundance in at least 90% of the samples within each group.

2.9 Feature Selection by Random Forests

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 the 20 samples, we applied a

supervised machine learning algorithm—Random Forest (RF) (Karlicki et al. 2024). This method has been demonstrated to be effective in classifying amplicon data (Hermans et al. 2020; Fang et al. 2022; Karlicki et al. 2024). Given the substantial environmental differences between sessile and planktonic communities, only ASVs contributing more than 0.1% of their relative abundance were retained. Following this filtration step, ASVs were renormalized and transformed into scoring units using the scale function. The refined dataset was then used to train RF models, and Out-Of-Bag (OOB) error was estimated. For each dataset, we selected the top 60 ASVs with the highest mean decrease in the Gini coefficient index, representing those with the greatest impact on sample classification. These ASVs were subsequently visualized using heatmaps and ordination plots.

2.10 Core ASVs in Microbial Database for Activated Sludge (MiDAS)

To assess the potential role of specific amplicon sequence variants (ASVs) from *P. australis* biofilm in bioremediation, we investigated their presence in wastewater treatment plants using the Microbial Database for Activated Sludge (MiDAS). A total of 250 prokaryotic ASVs forming the core structure of the biofilm—comprising both taxa unique to the biofilm and those shared with the surrounding water—were selected for analysis. In addition, three prokaryotic ASVs identified by RF analysis, which did not correspond to either unique or shared taxa, were included. Overall, the core biofilm community consisted of 253 prokaryotic and 110 eukaryotic ASVs, which were analysed to assess their functional relevance within these systems. The unique sequences of each ASV were extracted from the post-denoising FASTA file using their respective unique IDs. Comparison of the core ASVs with the MiDAS taxonomic database was conducted using the BLAST tool provided in MiDAS Field Guide webpage (Table S2) did not yield any matches for the protist ASVs, likely due to the limited representation of eukaryotic taxa in the database (only three genera from the Nucletmycea group, comprising 17 species).

To determine species-level classification, the sequences were queried against the MiDAS database using the BLAST tool (Dueholm et al. 2022; Dueholm et al. 2024). The MiDAS database includes microbial species identified from over 740 activated sludge plants and 280 anaerobic digesters worldwide, with data contributed by wastewater treatment facilities participating in the global database initiative. It provides detailed insights into in situ microbial metabolism (where known), morphotypes, and relative abundance across various wastewater treatment systems, including activated sludge, anaerobic digesters, and influent wastewater.

For each ASV, the corresponding sequence ID from the MiDAS database was recorded, along with the identified species and genus. Relevant metadata—such as database entry links and alignment statistics including sequence coverage, number of mismatches, and percentage identity (% ID)—were also documented. Only sequences with a % ID ≥99.5% were considered for downstream analysis. In cases where no match met this threshold, only the highest-scoring result (with the best % ID) was reported. For ASVs with confirmed matches in the MiDAS database, their known or predicted metabolic roles within activated sludge systems were also annotated. This information contributes to understanding the ecological function of biofilm-associated prokaryotes in wastewater treatment environments.

2.11 Specific Pollution Sensitivity Index (IPS) calculation

The Specific Pollution Sensitivity Index (IPS) was calculated for the 5 biofilm samples (RYN_B, ROS_B, MIK_B, MAJ_B, and PRZ_B). From the latter half of the 20th century, various methods

have been developed to assess water quality using diatoms, including the Diatom-Based Eutrophication/Pollution Index (IPE) (Dell'Uomo and Torrisi), the Biological Diatom Index (IDB) (Prygiel' et al. 1993), the Trophic Diatom Index (TDI) (Kelly and Whitton 1995), and Watanabe's Index (WAT) (Watanabe et al. 1986), but the Specific Pollution Sensitivity Index (IPS) (Coste and Ricard 1982) is the most widely applied across Europe. For each sample a CSV file with the absolute abundances of each species in the samples was created and used as an input file for the R package *DiaThor* (Gelis et al. 2022) and uploaded by the *diat_loadData()* function which checks and converts the input data into the necessary format. As the first output, we obtained a match of the input taxa names with their value for each metric (Table S3). *DiaThor* assigns the metrics based on the DiatBarcode Database (Rimet et al. 2019), a database specific for Diatoms. The output of *diat_loadData()* was then used to obtain the IPS by the *diat_ips()* function (Table S4). The IPS is based on the weighted averaging equation developed by Zelinka and Marvan (1961):

$$SPI = \sum A_j v_j s_j / \sum A_j v_j$$

where A_j represents the relative abundance of species j, v_j is its indicator value (ranging from 1 for ubiquitous taxa to 3 for highly specific taxa), and s_j is its sensitivity value (ranging from 1 for species indicative of highly polluted waters to 5 for those typical of pristine habitats) (Prygiel' et al. 1993). Based on the SPI score, water quality at a given site is classified as follows: Bad (1–5), Poor (5–9), Moderate (9–13), Good (13–17), and High quality (17–20) (Blanco 2024).

3. Results

3.1. Protist and bacterial diversity in biofilm and water samples

Genomic DNA from the biofilm of reed stalks *Phragmites australis* and water samples collected from five selected lakes within the Great Masurian Lake District of NE Poland were sequenced to study bacterial and protist communities. For bacterial community analysis, the V4-V5 region of the 16S ribosomal gene was sequenced, while for protist community analysis the V4 region of the 18S ribosomal subunit gene was used. A total of 2,963,123 reads were obtained for the 16S bacterial gene using NovaSeq Illumina sequencing, with an average of 64,054 to 2,963,123 reads per sample; 43.84% (1,299,072) of reads completed the merging and chimera elimination filtering procedures (Table S5). For the 18S gene, 3,348,426 reads were acquired, with per-sample counts ranging from 87,668 to 227,016 reads (Table S6). After filtering, 2,080,910 reads remained, accounting for 62.15% of the total number of reads. A total of 6,336 ASVs were inferred from the 16S dataset, while 4,971 ASVs were inferred from the 18S dataset. The rarefaction curves for both datasets indicated that the sequencing depth was adequate (Fig. S1). In the bacterial amplicon dataset, ASVs identified as eukaryotes were excluded, resulting in 5,999 ASVs (88.88% of the initial count). Similarly, in the V4 18S rDNA dataset, sequences identified as prokaryotes and metazoans were removed, yielding 4,509 ASVs (90.71% of the initial count) (Tables S7 and S8).

The most prevalent bacterial phyla in both biofilm and water samples were Proteobacteria (21-67%), Actinobacteriota (0.2-42.8%), Bacteroidetes (15.2-41.4%), and Cyanobacteria (2.5-14.6%) (Fig. 2a). Within the Proteobacteria group, Gammaproteobacteria accounted for approximately 12 to 22% of reads, and Alphaproteobacteria accounted for 6 to 45%, depending on the specific sample (Fig. S2). Actinobacteria and Acidimicrobia were the primary

representatives of the Actinobacteriota phylum. Other notable phyla included Planctomycetota, Verrucomicrobiota, and Chloroflexi. Notably, the biofilm of Lake Majcz (MAJ_B sample) exhibited a higher abundance of Firmicutes communities compared to other samples (Fig. 2a). The dominant members of the protist community consist of Dinoflagellata (3.4-89.4%), Gyrista (1.5-38%), Perkinsea (0.7-28%), Ciliophora (2.5-24%), Chlorophyta (0.9-25.7%), Cryptophyta (0.5-20.5%), Fungi (0.6-39.2%) and Cercozoa (0.16-30.9%) (Fig. 2b). While the dominant members remain consistent across samples, their relative abundances vary between biofilm and water samples of the five lakes. For instance, Dinoflagellata is overrepresented in the waters of Lake Mikołajskie inside the *Phragmites* field (MIK_LP), as well as in the pelagic waters of Lakes Ryńskie and Mikołajskie (RYN_P and MIK_P), compared to other lake waters. Perkinsea shows higher abundance in Ryńskie (except for sample RYN_P), Roś, and Przystań. The biofilms of Mikołajskie, Przystań, and Roś exhibit great similarities, characterized by the prevalence of Gyrista, particularly diatoms, less abundant in the planktonic community. Additionally, Cercozoa account for 30.94% and 17.87% of the biofilm communities in Lakes Majcz and Roś, respectively.

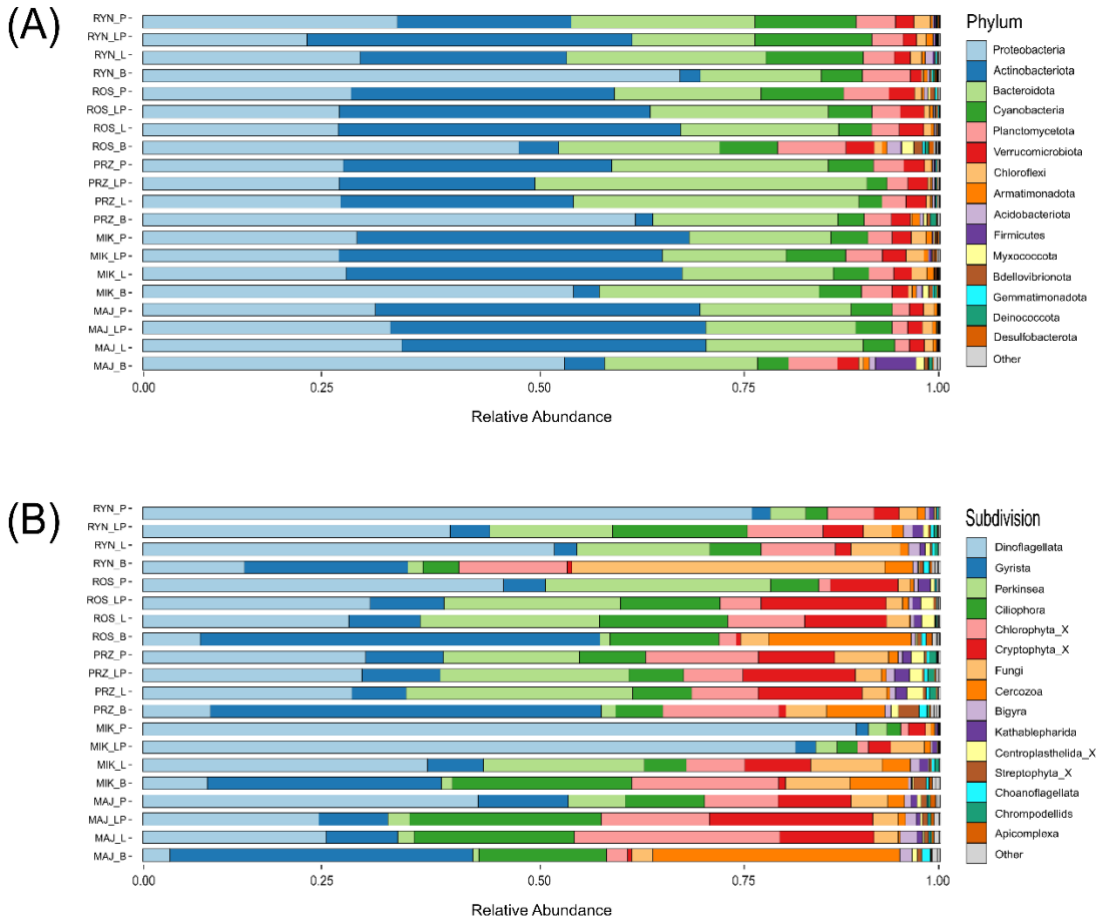


Fig. 2. Taxonomic composition of the bacteria and protists communities. (A) Bar plot showing the relative abundances of the most abundant bacterial taxa at the phylum level across biofilm and water samples from the five studied lakes. (B) Bar plot showing the relative abundances of the 15 most abundant protist taxa at the subdivision level. All remaining taxa are grouped as "Other".

While dominant phyla remain consistent across samples, notable differences emerge at lower taxonomic levels, particularly in biofilm samples which exhibit greater taxonomic diversity than planktonic communities (Fig. 3). In the lake water samples, prokaryotic communities were dominated by members of Actinobacteriota (Frankiales), followed by members of Proteobacteria (Burkholderiales, Microtrichales), Bacteroidota (Chitinophagales, Flavobacteriales, Cytophagales), and Cyanobacteria (Synechococcales), together with the SAR11 clade (Alphaproteobacteria) (Fig 3a). In contrast, in biofilms, the relative abundance of these groups decreases, allowing for the growth of Proteobacteria orders such as Rhizobiales, Sphingomonadales, Pseudomonadales, and Rhodobacterales. Though bacterial communities are relatively stable, protist communities show greater variability between lakes. For instance, within Fungi (Basidiomycota), Agaricomycotina — mainly represented by Tremellomycetes — was found only in the biofilms of Ryńskie and Mikołajskie. Among Alveolata (Dinoflagellata and Ciliophora), Gonyaulacales were present in all samples, but were particularly abundant in the pelagic zones of the two aforementioned lakes, whereas *Peritrichia* occurred predominantly in biofilms near Lake Mikołajskie shoreline. Biofilms from Mikołajskie also hosted *Cymbellales* (Diatomea, Ochrophyta), *Vampirellida* (Rhizaria), and *Sphaeropleales* (Chlorophyta), all of which were rare in planktonic communities and decreased in abundance with increased distance from the shore. Biofilms from the less eutrophicated Majcz and Przystań lakes shared similar profiles, showing higher proportions of *Vampirellida* (Rhizaria), *Ochromonadales* (Chrysophyceae, Ochrophyta), and *Cymbellales* (Diatomea, Ochrophyta). In these lakes, protist communities are more consistent across zones, whereas in other lakes, species abundance shifts with distance from the shore (Fig. 3b). Based on the general taxonomic annotation of dominant taxa, it is evident that biofilm microbial communities and planktonic ecosystems differ substantially. The distribution of the most common phyla in biofilms differs from their occurrence in the water, suggesting a preference for either a planktonic or an attached lifestyle.

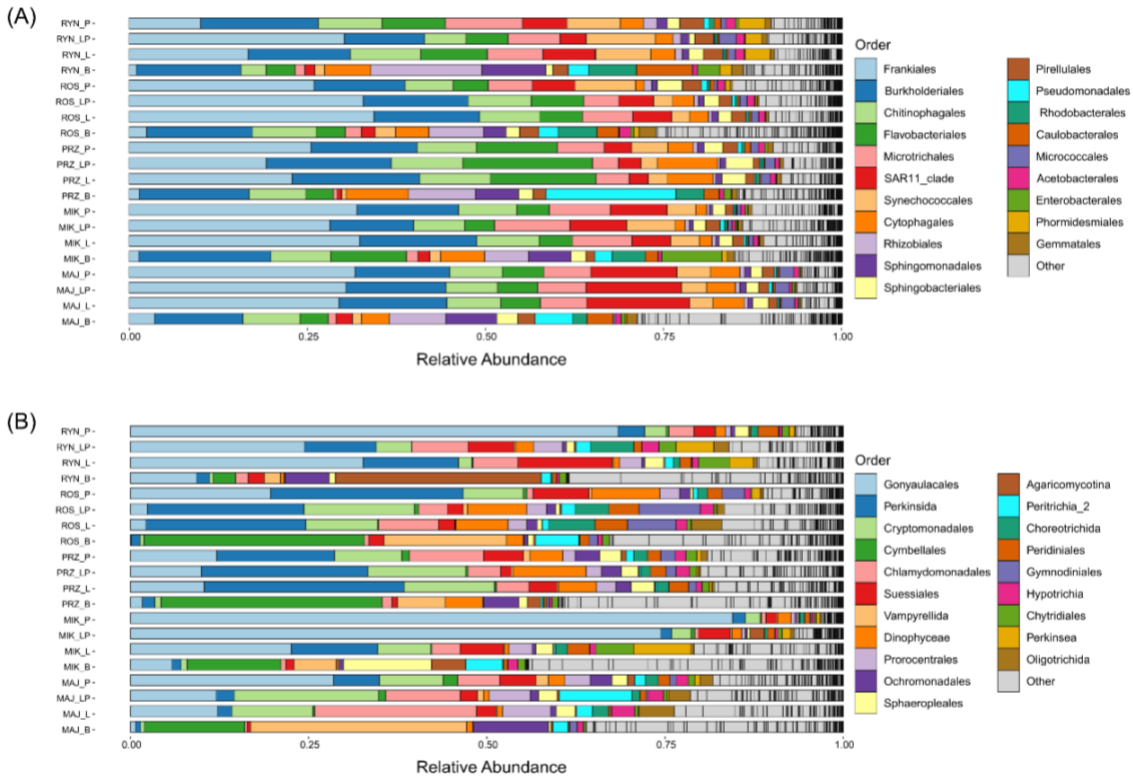


Fig. 3. Taxonomic composition of the bacteria and protists communities. (A) Bar plot showing the relative abundances of the most abundant bacterial taxa at the order level across biofilm and water samples from the five studied lakes. (B) Bar plot showing the relative abundances of the 15 most abundant protist taxa at the order level. All remaining taxa are grouped as "Other".

3.2 Protist and bacterial community structure

To further investigate the diversity and similarities among communities, we performed alpha and beta diversity analyses of both bacterial and protist communities. Biofilm prokaryotic communities exhibit significantly greater diversity compared to planktonic communities across all parts of the lake (Fig. 4a). The higher bacterial diversity in biofilms is confirmed by the Wilcoxon test ($p < 0.005$) (Supplementary Data 1). Alpha diversity of samples was calculated using the Shannon metric. It ranges from ~5.7 to ~6.7 for biofilm and ~4.2 to ~5.1 in water samples (Table S9). These findings indicate that compared to free-living fractions, biofilm-associated communities contain a greater variety of ASVs and exhibit a more even distribution of their abundances. In contrast, protist biofilm communities are neither richer nor more even than plankton communities (Fig. 4b). For the 18S data, the Wilcoxon test did not reveal any significant differences (Supplementary Data 2).

To determine whether the biofilm communities are more similar to each other or the surrounding water, we conducted a beta diversity analysis using Bray-Curtis dissimilarity metrics, visualized using Principal Coordinates Analysis (PCoA). Biofilm and planktonic communities were distinctly separated across all *three* water zones in the five lakes, for both prokaryotes and protists. This separation is evident along Axis 1, which explains 49.6% of the total variance for prokaryotes and 25% for protists.

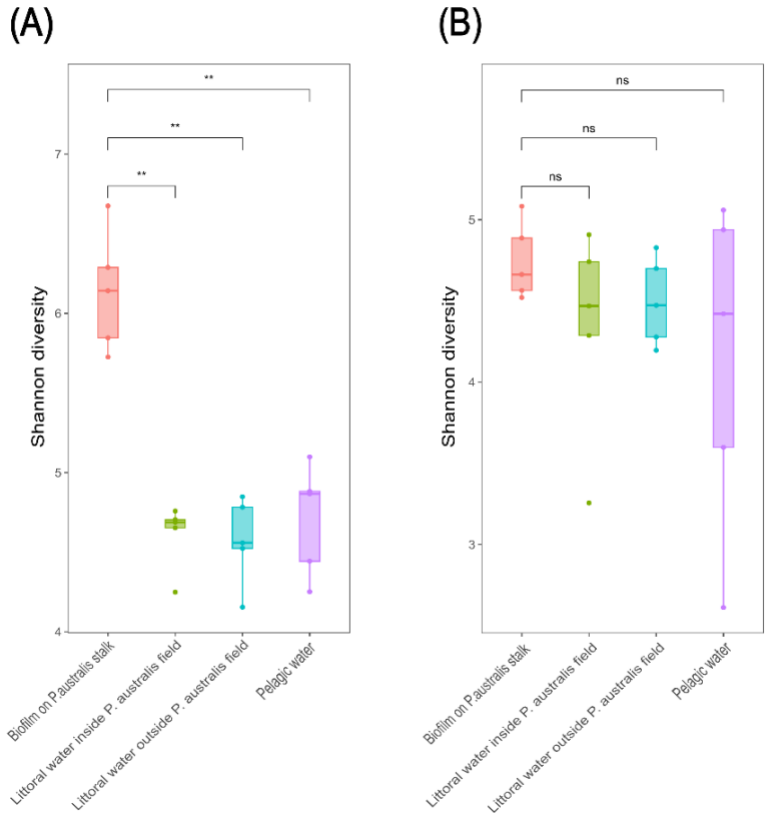


Fig 4. Alpha diversity of prokaryotic and eukaryotic communities. Boxplots showing the alpha diversity of biofilm and water samples based on (A) prokaryotic 16S V4–V5 rDNA and (B) eukaryotic 18S V4 rDNA, calculated using the Shannon index. Significant differences between sample types were assessed with the Wilcoxon test. Statistical comparisons are displayed only between biofilm and each of the three water fractions.

Furthermore, biofilm samples clustered together across lakes, although single biofilm replicates per lake limit within-lake inference (Fig. 5a). The compositional difference was confirmed by PERMANOVA (adonis) analysis (p-value < 0.001) and beta-dispersion analysis (p-value > 0.05) for both datasets (Supplementary Data 1 and 2). To ensure that the observed differences were not influenced by unequal group sizes (5 biofilm vs. 15 water samples), PERMANOVA tests were performed using balanced pairwise comparisons between biofilm samples and each water zone. These analyses consistently confirmed significant compositional divergence between biofilm and water communities across all zones. Notably, bacterial communities within biofilm seem to differ between the less eutrophic (Majcz and Przystań) and the more eutrophic lakes (Ryńskie, Roś, and Mikołajskie) as visible along Axis 2 of Fig. 5a. To further investigate whether the free-living communities share similarities between lakes, we assessed beta-dispersion metrics among samples from different lake zones, excluding biofilm samples. This analysis revealed significant differences in community composition between lakes across all zones in both datasets (Fig. 5b).No significant variation was detected between zones within individual lakes (Supplementary Data 1 and 2). For bacterial communities (Fig. 5b, left), Majcz and Przystań stood out as the most dissimilar, with Ryńskie, Mikołajskie, and Roś forming a tighter cluster. Protist communities (Fig. 5b, right) showed a similar pattern, though Majcz and Przystań were more alike to each other than they

were for bacteria. Mikołajskie and Ryńskie were again the most similar, whereas Roś displayed the most distinct protist composition.

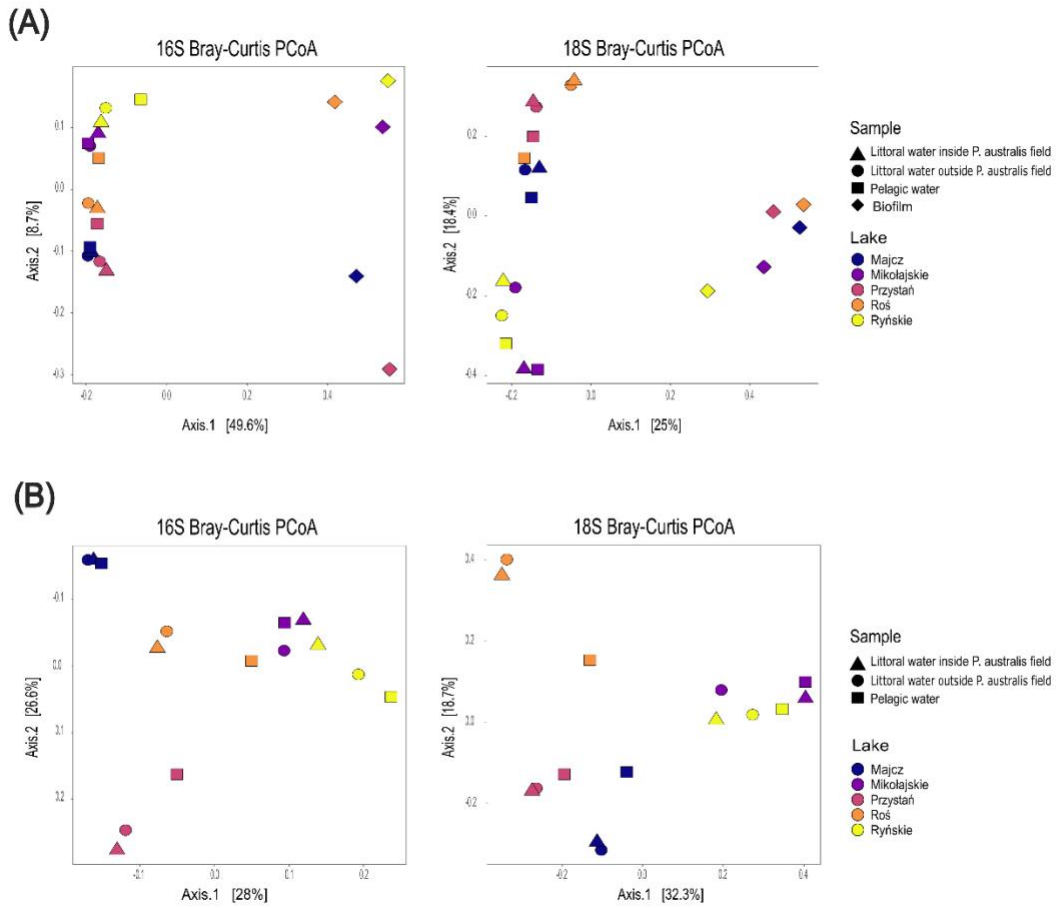


Fig. 5. Beta diversity of prokaryotic and eukaryotic communities. Ordination plots based on Bray–Curtis PCoA illustrating beta-dispersion for (A) all samples, including water ("littoral inside/outside" and "pelagic" zones) and biofilm ("Phragmites surface"), and (B) water samples only. The left panels display 16S rRNA data, while the right panels show 18S rRNA data. Shapes represent the sample type, and colours indicate different lakes.

To assess whether environmental factors influence community composition, both in biofilm and free-living fractions, we used the envfit function, which fits environmental vectors onto the NMDS ordination plot. A total of nine environmental parameters were included in the analysis. For prokaryotic and protist communities in the water column, environmental influence was evident, whereas no significant effects were observed for biofilm-associated communities (Table S10). Specifically, six environmental variables were significantly correlated with the ordination of free-living bacterial communities (P-value < 0.05) (Fig. S3A), and only temperature showed a significant correlation for protists (Fig. S3B). In contrast, none of the environmental parameters showed a significant association with the composition of biofilm communities (Table S10), although the absence of within-lake replication limits the ability to fully disentangle habitat and lake effects.

3.3 Biofilm and Water Column Core Community

The taxonomic analysis presented in paragraph 2.1 does not fully explain the diversity of microbial communities within the biofilm and water column, likely because the most abundant high-level taxa are similar across the samples. However, alpha diversity and beta-dispersion analyses indicate that biofilm communities are distinct from free-living communities. Therefore, to assess the contribution of specific ASVs to this diversity, we first examined the proportion of ASVs forming the core of each fraction (i.e. biofilms or free-living) and the extent to which these core ASVs are shared between them. This approach allowed us to identify ASVs that are consistently present and likely play a significant role in shaping community structure within each habitat. Additionally, we examined the overlap of ASVs between the biofilm and water samples, offering deeper insights into the microbial dynamics of these two distinct environments.

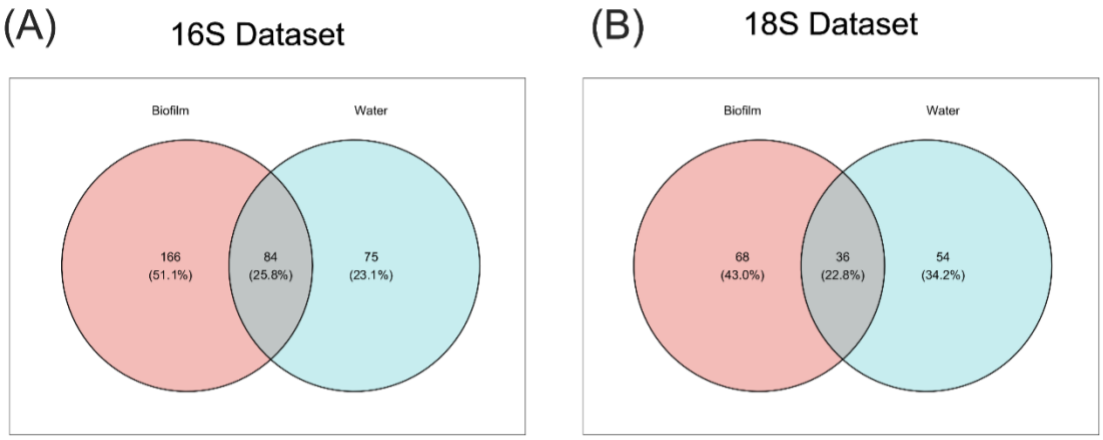


Fig. 6. Core microbiome of biofilm and planktonic communities. Venn diagrams showing the ASVs constituting the core structures of biofilm and water-column communities and their intersection for (A) prokaryotic 16S V4–V5 rDNA and (B) eukaryotic 18S V4 rDNA. The "biofilm" samples refer to the microbial communities attached to the surface of Phragmites australis stalks, whereas the "water" samples represent pooled water-column communities from the three lake zones. The core microbiome was defined by filtering total ASVs according to sample association (biofilm or water) and selecting those with a relative abundance ≥0.001% in at least 90% of the samples within each group.

Among bacterial ASVs forming the core of both communities, 166 (51.1%) are specific to the biofilm and 75 (23.1%) for water column (Fig. 6a). Similarly for protists, 68 (43.0%) ASVs are exclusive for the biofilm core and 54 (34.2%) to the water column core (Fig. 6b). Because core size can be influenced by group size and prevalence thresholds (water samples, n = 15; biofilm, n = 5), we additionally 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), supporting that this pattern is not an artefact of unequal group sizes. The bacterial ASVs specific to the biofilm core were primarily affiliated with Proteobacteria, including *Aeromonas* and *Acinetobacter* (Gammaproteobacteria), *Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium* (Rhizobiaceae) and *Sphingomonas* (Sphingomonadales, Alphaproteobacteria), and *Comamonadaceae* family (Betaproteobacteria), as well as with Bacteroidota (*Flavobacterium*) (Table S11). For protists, the ASVs exclusive for biofilm core consisted of Chlorophyta *(Enallax, Pseudendoclonium, Ulvella),* Diatoms *(Cymbella, Navicula, Encyonema),* Chrysophyceae

(Ochromonadales clade-XII_X), Fungi *(Chytridiales, Agaricomycetes, Cryptococcus, Nowakowskiella, Cladochytriales),* and Ciliophora *(Rimostrombidium)* (Table S12). Most bacterial ASVs specific to the water column were affiliated with Actinobacteriota (*hgcI_clade*, *Rhodoluna*), Cyanobacteria (*Nodosilinea*, *Cyanobium*), and Bacteroidota (*Kapabacteriales*, *Fluvicola*). Among the protist ASVs of the water column, dominant groups include Dinoflagellates (e.g., Ceratium*, Yihiella),* Chlorophyta *(e.g., Volvox)* and Ciliates *(e.g., Sessilida, Tintinnopsis, Oligotrichida).* For shared ASVs, the most abundant prokaryotes include the *hgcI_clade, Clade III of the SAR11_clade, Sporichthyaceae, Limnohabitans and* the *CL500-29 marine group.* Among protists, the dominant shared ASVs belong mostly to the same groups as the unique ones, but are represented by different ASVs, namely Alveolates *(e.g., Ceratium* ASV_3*, Perkinsida* ASV_5*),* Chlorophytes *(e.g., Volvox* ASV_19*),* and Cryptophytes *(*ASV_4, 6, 11, and 28).

3.4. Identification of characteristic taxa with the Random Forest approach

We employed a RF algorithm to identify ASVs characteristic of biofilm and planktonic communities. For each dataset, we focused on the top 60 ASVs ranked by the RF model using the mean decrease in Gini coefficient indices (Fig. S5). The classification achieved an out-of-bag (OOB) error rate of 0% for the 18S rDNA dataset and 5% for the 16S rDNA dataset. Out of 60 selected ASVs in the 16S rDNA dataset, 26 ASVs were characteristic for the biofilm group, while 44 ASVs to the water column (Fig. 7a). ASVs from the families *Rhizobiaceae, Rhodobacteraceae, Spirosomaceae, Saprospiraceae, Aeromonadaceae, Alteromonadaceae, Moraxellaceae, Pseudomonadaceae, Microscillaceae, Leptolyngbyaceae,* and *Sphingomonadaceae* define the characteristic prokaryotic biofilm community (Table S13). While biofilms may appear similar when considering only the most abundant taxa (Fig. 2a), a more detailed examination of characteristic ASVs (Fig. 7a) revealed distinct taxonomic signatures often linked to lake trophic status (TSI). For instance, in Ryńskie, the most eutrophic lake in the dataset (TSI=55.3) - the ASVs associated with the genus *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (ASV_68, ASV_172, ASV_140, and ASV_126) - are the most characteristic of the biofilm. Other significant ASVs defining the biofilm in Ryńskie include *C2U/Pseudoxanthobacter sp.* (ASV_143), *Ohtaekwangia* (ASV_169) and *Acquabacterium* (ASV_190). In eutrophic lakes such as Roś and Mikołajskie, *Rhodobacteraceae* (ASV_78 and ASV_193) and *Sphingomonadaceae* (ASV_74) are the most characteristic. Furthermore, specific ASVs belonging to Aeromonas and Rheinheimera are also among the most characteristic ASVs in Lake Mikołajskie. The genera *Pseudomonas* (ASV_131), *Acinetobacter* (ASV_43), *Hydrogenofaga* (ASV_119) and *Acquabacterium* (ASV_139) are the most characteristic of the biofilms in lakes Majcz and Przystań, with lower TSI (48.8 and 46.6, respectively). *Rhizorhapis* (ASV_145) and *Sphingomonas* (ASV_120), albeit more widespread across biofilm samples of different lakes, are more characteristic for the less eutrophic lakes. Several other ASVs, including *Flectobacillus* (ASV_163), *Comamonadaeae* (ASV_104), *Porphyrobacter* (ASV_166), *Pirellula* (ASV_152), *Leptolyngbya_SAG_2411* (ASV_125) and *Haliscomenobacter* (ASV_127) were identified as characteristic to all five biofilms, regardless of the trophic status of the lakes to which they belong.

Interesting results were noted for ASV_92 of the uncultured *Rhizobiales* and ASV_109 of Rhodoferax (Fig. 7a). ASV_92 is found to be characteristic for the biofilm of the three most nutrient-rich lakes: Ryńskie, Roś and Mikołajskie, but appears to be characteristic also for some plankton communities, especially in Lake Ryńskie (RYN_LP). A similar pattern is observed in Roś, Mikołajskie, and Przystań, where *Rhodoferax* (ASV_109) appears to be

characteristic for both biofilm and water column samples. Additionally, *Comamonadaceae* and *Pirellula* ASVs are shared between biofilm and water, although distinct ASVs are more characteristic for each habitat (ASV_104, 152 for biofilm; ASV_111, 51, 40, 21 for water). 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. 7b).

The RF model for the 18S dataset identifies diatoms as the ASVs that are the most characteristic for protist biofilms. Among ASVs characteristic for biofilms, more than half (16) represent 9 different genera of diatoms (Fig. 7b). Other characteristic protist groups in biofilm samples include Chlorophytes, Ochrophytes, Cercozoa, Fungi and Ciliophora. A few genera are characteristic across all five biofilm samples, notably *Leptophrys* (ASV_166 and 170), *Achnanthidium* (ASV_47) and *Epithemia* (ASV_154). However, similarly to prokaryotic biofilms, the protist component also exhibits lake-specific ASVs. In Ryńskie, a few ASVs such as *Ochromonadales* (ASV_61), *Sporobolomyces* (ASV_62) and *Tremellomycetes* (ASV_134) characterise the biofilm community. The biofilm in Mikołajskie includes *Enallax* (ASV_31) as the only genus unique to this lake. However, most of the characteristic ASVs in Mikołajskie, such as *Leptophrys* (ASV_166 and 170), *Vampyrellida* (ASV_145), and the diatoms *Gomphonema* (ASV_76), *Navicula* (ASV_174 and 79), and *Encyonema* (ASV_110) are also shared with the biofilm samples from Roś and, to some extent, Ryńskie. In Roś, characteristic ASVs belong to diatoms, particularly *Cymbella* (ASV_132, 86, 29, 74, 115) and *Fragilaria* (ASV_136 and 165). The biofilm community in Majcz is characterized by ASVs such as *Platyophrya* (ASV_144), *Leptophrys* (ASV_7), *Hyalodiscus* (ASV_59) and *Chlorochromonas* (ASV_53). In Przystań, prominent ASVs include *Cymbella* (ASV_10 and 36), *Ulvella* (ASV_93), *Naegeliella* (ASV_155), and *Encyonema* (ASV_110).

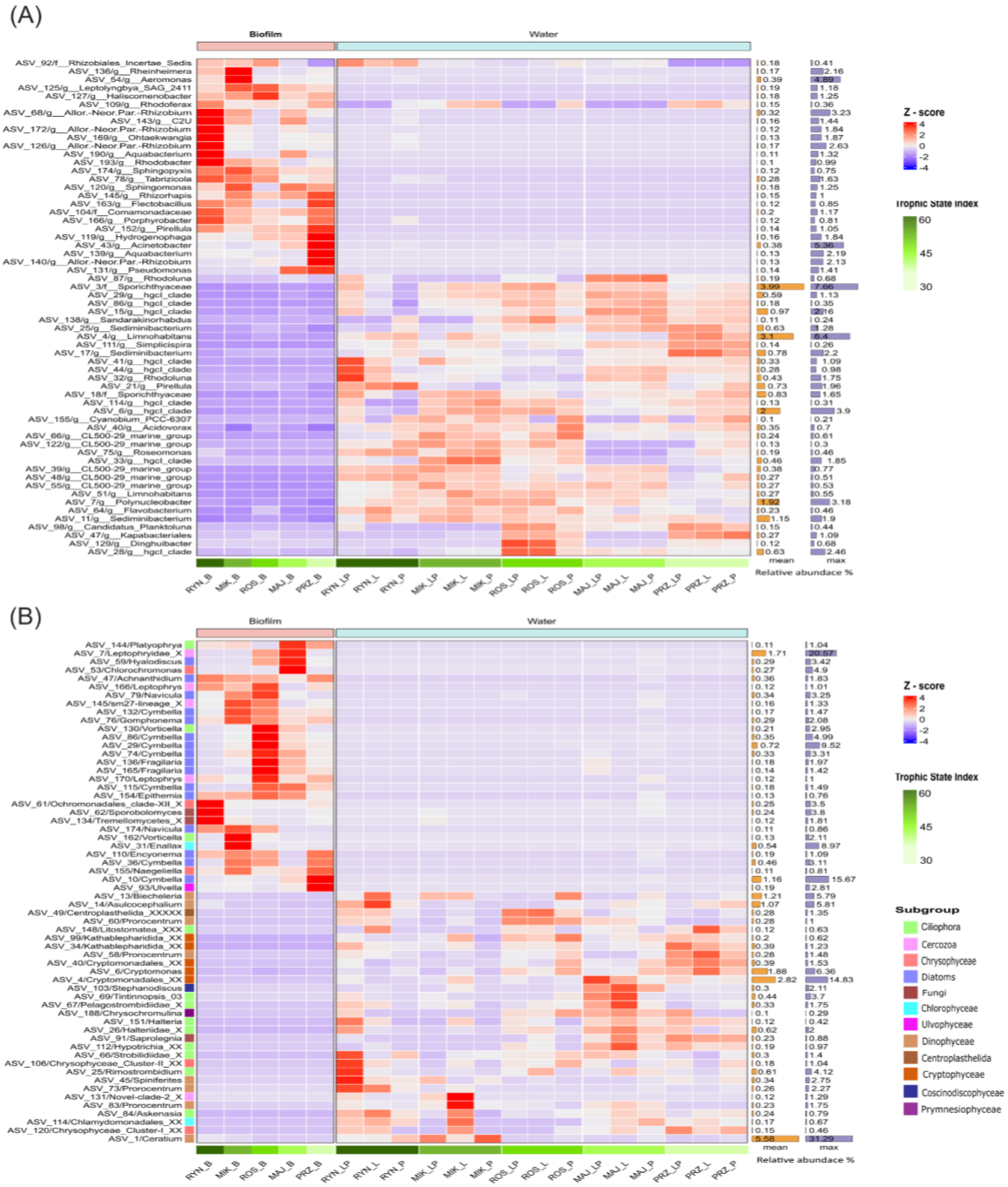


Fig. 7. ASVs characteristic for biofilm and water communities identified by machine learning. Characteristic ASVs for each investigated sample were identified using a RF algorithm. (A) Heatmap showing the abundance of the 60 ASVs selected by the RF model across the 20 samples for prokaryotes. (B) Heatmap showing the abundance of the 60 ASVs selected by the model for protists. The first five samples correspond to 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 on the right of each heatmap display the overall relative abundance of each ASV across the total dataset.

3.5 Assessment of core and Random Forest-identified taxa for their environmental relevance

The unique and characteristic taxa of biofilms identified were further investigated for their potential roles in bioremediation and as bioindicators. Among the prokaryotic ASVs, 253 ASVs were identified in the MiDAS database, indicating that they are present in at least one of the 740 activated sludge plants in the dataset (Table S2). Of these, 147 ASVs were classified as unique for biofilm while 80 were shared with water samples and 26 were identified as characteristic ASVs by the RF analysis. Notably, 80.77% of the characteristic ASVs selected by the RF analysis and present in the MiDAS database exhibited more than 99.5% species-level identity with their corresponding MiDAS matches. Similarly, 66.67% of the unique ASVs also showed positive matches, whereas only 17.5% of the core ASVs shared with water samples were identified in the database (Table S2).

Among the eukaryotic taxa present in the biofilm, particular attention was given to ASVs identified through RF analysis. This analysis highlighted benthic or epiphytic pennate diatoms as the primary taxa characteristic for the biofilm, suggesting their significant role in its microbial community structure and function (Fig. 7b). Among the studied sites, the biofilm in Ryńskie exhibited the highest proportion of diatom species tolerant to environmental change, with an IPS of 14.46732 (Tables S3 and S4). Within them, some of the most abundant pennate diatoms included *Nitzschia inconspicua, Achnanthidium pyrenaicum, Epithemia sorex, Cocconeis pediculus, Encyonema caespitosum, Navicula cryptotenella, Gomphonema truncatum* and *Navicula radiosa*. In contrast, lakes Mikołajskie and Przystań showed IPS values of 16.59644 and 16.76647, respectively, indicating the presence of more sensitive diatom species, like *Epithemia turgida, Cymbella helvetica and Cymbella proxima*. The water of Majcz and, in particular, of Roś appeared to possess high-quality conditions with IPS values of 17.23158 and 18.2174, respectively.

3.6 Community physiological fingerprint and enzymatic activity

While bacterial biofilm and planktonic communities differ in taxonomic richness and composition (Fig. 4 and Fig. 5a), they may show great similarity in terms of their physiological activity. On the contrary, bacterial activity in biofilm could reveal some features characteristic of *P. australis* biofilm communities. To gain a deeper understanding of the physiological potential of prokaryotic communities, we measured leucine-aminopeptidase (LAP) activity to evaluate the proteolytic capacity of bacteria in the samples and assessed the physiological profile of the bacterial communities using Biolog EcoPlate analysis. The maximal potential leucine-aminopeptidase activity in bacterial biofilms was 10 to 100 times higher than in the water column communities (Table S14). The highest LAP activity was observed in the biofilms of lakes Przystań, Mikołajskie, and Roś, indicating significantly elevated proteolytic activity of these communities. Conversely, Lake Majcz exhibited the lowest LAP activity (3297.07 nM l^{-1} h^{-1}) among the biofilm samples. Despite varying levels of LAP activity, biofilm bacterial communities consistently demonstrate a much higher substrate utilisation rate than bacterioplankton from both littoral and pelagic zones, highlighting their greater proteolytic potential. The respiration rate analysis obtained from the Biolog EcoPlate supports these findings. We evaluated the bacterial community's ability to utilise 31 different carbon sources, testing each environmental sample in three independent biological replicates to ensure reproducibility. As observed with LAP activity, biofilm communities exhibited higher average respiration rates of available carbon sources than planktonic communities (Fig. 8a), and this

trend is further detailed in Figure 8b which shows the percentage respiration rate of the main organic compound groups respired by the microbial communities (Fig. 8b and Table S15). Amines were the least utilised carbon source in the waters of Mikołajskie and Ryńskie (2-8%). However, within the biofilm communities of these lakes, amine utilisation increased to 20%, comparable to the utilisation of other carbon source groups. In these two lakes, carbohydrates were the most utilised carbon sources for both biofilm and planktonic communities, while complex carbon sources dominated in littoral bacterioplankton. In contrast, amines were the least utilised in Lake Przystań, but unlike the other two lakes, biofilm communities here showed a low rate of amine utilisation. Interestingly, littoral plankton outside the *P. australis* zone exhibited a significant increase in amine usage (19.55%). Carbohydrates and complex carbon sources consistently emerged as the preferred carbon sources. However, in Lake Przystań, both biofilm and surrounding water communities showed a shared preference for complex compounds, differing from the pelagic bacterioplankton. An interesting case was observed in the littoral zone inside the *P. australis* field of Przystań, where we found the only communities unable to utilise phosphate carbon. Furthermore, in lakes Majcz and Roś, both free-living and sessile bacteria showed an increased preference for amines which were the preferred carbon source in several samples (e.g., MAJ_L, LP, P, and ROS_LT, B, P). In Majcz biofilm communities, no specific carbon source was preferred, with all sources utilised at rates ranging from 11-18%. For the littoral zone of Roś, carboxylic acids were the least utilised source, while complex compounds were the least utilised in both the biofilm and pelagic zones.

To further investigate these observations, we applied Bray-Curtis dissimilarity metrics based on the relative respiration rate of the 31 carbon sources and visualized the results using NMDS analysis. Biofilm samples clustered together at the centre of the graph, a grouping confirmed by PERMANOVA (adonis) analysis (p-value < 0.001) and beta-dispersion analysis (p-value > 0.05) (Supplementary Data 3). Despite the cumulative barplot in Fig. 8b showing similar carbon source utilisation patterns to free-living communities in the same lake, biofilm communities exhibited a distinct physiological fingerprint. Notably, the biofilm in Mikołajskie Lake stands out as the most distinct in its carbon source utilisation, as shown in Figs. 8c and 8d where it is separated from the main biofilm cluster.

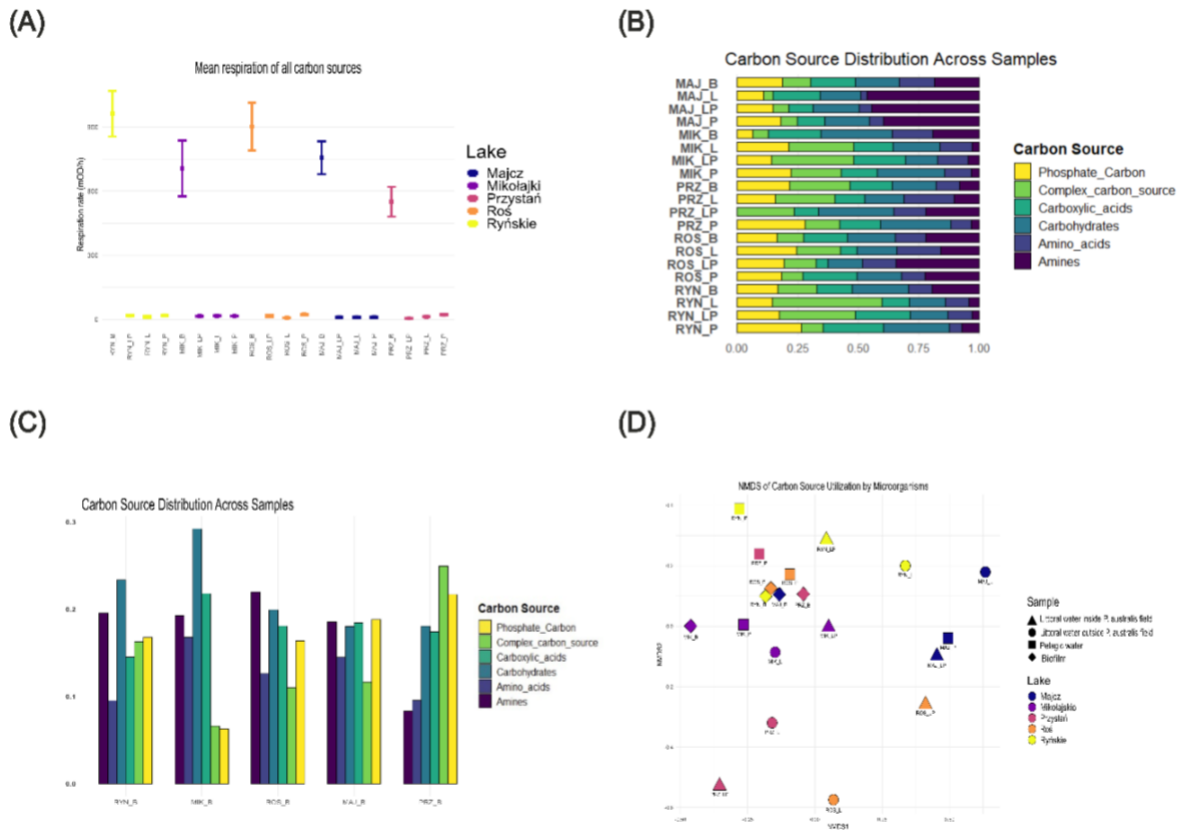


Fig. 8. Visualization of Biolog EcoPlate Results. (A) Boxplot illustrates the average respiration rate of 31 carbon sources per sample. (B) Bar plot displays the average relative utilization of carbon sources, categorized by compound type for each sample. (C) A focused comparison of carbon sources used 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.

4. Discussion

4.1. Epiphytic biofilm communities in freshwater lakes are distinct from planktonic ones

Metabarcoding analyses of 16S and 18S rDNA datasets from investigated lakes revealed that microbial communities forming biofilms on *P. australis* stalks were clearly distinct from the surrounding planktonic ones, both for bacteria (Figs. 2a and 3a) and for protists (Figs. 2b and 3b). In all biofilm samples, the most abundant bacterial classes were Alphaproteobacteria, Gammaproteobacteria, Bacteroidia, Cyanobacteria, Planctomycetes, and Bacilli (Fig. S2), what is consistent with previous studies (Hempel et al. 2008; Hempel et al. 2009; Levi et al. 2017; Wijewardene et al. 2022). At lower taxonomic levels, biofilms were specifically enriched in Rhizobiales, Sphingomonadales, Rhodobacterales, Cytophagales, Burkholderiales, Enterobacterales, and Pseudomonadales, in line with patterns reported in earlier studies (Hempel et al. 2008; Borsodi et al. 2020; Lennert et al. 2024). The surrounding planktonic community showed only some taxonomic similarity with biofilm samples, though with notable shifts in relative abundance: Alphaproteobacteria, - dominant in biofilms - were underrepresented, while Actinobacteria were more prominent (Figs. 2a, 3a, and S2). Although bacterial biofilm communities appeared more stable and consistent across different lakes (Fig. 3a), protist biofilm communities were more variable, yet remained clearly distinct from

their planktonic counterparts (Fig. 3b). The dominant protist taxa in biofilms included Ciliophora, Ochrophytes (particularly Bacillariophyceae and Chrysophyceae), Chlorophyta (*Ennalax*), Basidiomycota (Tremellomycetes, *Cryptococcus*), and Cercozoa (e.g., amoeboid taxa such as Vampyrellidea) (Figs. 2b and 3b), reflecting patterns reported in the literature on freshwater plant-associated biofilms (Wijewardene et al. 2022; Carvalho Da Silva and Fernandes 2023; Freudenthal et al. 2024; Zhang et al. 2024). Planktonic samples were enriched with different taxa, such as Dinoflagellata, Perkinsea, Cryptophyta and Spirotrichea (Fig. 3b) consistent with previous observations of planktonic protists in temperate lakes (Cruaud et al. 2019; Karlicki et al. 2024). Overall, biofilm communities were more diverse than the surrounding free-living ones, particularly for bacteria (Fig. 4a). The separation between biofilm and planktonic assemblages was observed for both prokaryotes and protists, evident in their taxonomic compositions and confirmed by beta diversity analyses of community structure (Fig. 5a, right and left panels, respectively). These patterns were consistently replicated across five independent lakes and for both marker genes, with homogeneity of dispersion confirming that centroid differences were not driven by variability within groups (betadisper test, $p > 0.05$).

To further explore the complexity of biofilm communities, we sought to identify taxa that were uniquely associated with biofilms. We first identified the core taxa that consistently dominated biofilm communities, providing a baseline of the most stable members, followed by RF analysis to detect taxa that were particularly characteristic of biofilms. Core community analysis showed that biofilms harboured a larger core represented by a greater number of unique ASVs than planktonic communities (Fig. 6). The high number of unique taxa in lake biofilms in comparison to planktonic ones suggests that biofilms support a more complex and functionally diverse microbial assemblage (Besemer et al. 2012; Robinson et al. 2025). The larger core likely reflects the higher richness observed in biofilms (Fig. 4) and may be attributed to the heterogeneous microenvironments within them, which promote the coexistence of diverse and stable microbial lineages (Battin et al. 2007).

RF analysis of both 16S and 18S rDNA datasets not only further supported a clear separation of biofilm and planktonic clusters, but also highlighted the taxa most strongly driving this differentiation (Fig. 7). The majority of ASVs identified overlapped with the biofilm core, underlining the central role of these taxa in sustaining biofilm structure and function (Fig. 7, Tables S11, S12 and S13). The bacterial ASVs selected by RF analysis as characteristic for biofilms included representatives of *Rhizobium*, *Rhodobacter*, *Flectobacillus*, *Comamonadaceae*, *Porphyrobacter*, *Pirellula*, *Leptolyngbya_SAG_2411* and *Haliscomenobacter* (Fig. 7a; Table S13). The core and RF-selected bacterial ASVs (Tables S11 and S13) comprised of persistent freshwater biofilm colonizers (Battin et al., 2007; Besemer et al., 2012), characterized by high surface affinity, EPS production, and nutrient cycling, all promoting resilient assemblages (Besemer et al., 2012; Newton et al., 2011, Costerton et al., 1995; Flemming & Wingender, 2010). Their ability to adapt to life on surfaces enables them to rapidly initiate biofilm formation, shape community structure and support its development, and create stable conditions that promote similar ecological settings, regardless of the location (Battin et al., 2016; Flemming & Wuertz, 2019). The presence of *Aeromonas*, *Acinetobacter* and *Flavobacterium* can contribute to organic matter degradation and biofilm formation under variable conditions, while *Sphingomonas* and *Comamonadaceae* support biofilm structure and carbon cycling (Czieborowski et al. 2020; Grilo et al. 2021; Lu et al. 2022; Pandolfo et al. 2023). The presence of the *Allorhizobium–Neorhizobium–*

Pararhizobium–Rhizobium complex suggests macrophyte associations enhancing nitrogen availability and stability. Several of these genera are also recognised as plant growth-promoting bacteria (PGPB) like select *Sphingomonas* strains that produce the phytohormone indole-3-acetic acid (IAA) which enhances root development while other strains inhibit plant pathogens via antimicrobial activity (Vega-Celedón et al. 2024). Other core members such as *Sphingomonas*, *Hydrogenophaga*, *Pseudomonas*, *Rhodoferax*, and *Flavobacterium* (Table S11), are linked to diatom biofilms, abundant in our samples (Grossart et al. 2005; Bruckner et al. 2008).

The eukaryotic core (Table S12) highlighted further structural complexity, with phototrophic chlorophytes and benthic diatoms (*Cymbellales*, *Gomphonema*, *Navicula*) forming mats that stabilize surfaces and promote colonisation (Fig. 2b) (Fernández et al. 2019, Wetzel, 2001; Jan., 1984). The detected fungal taxa (Chytridiales, Nowakowskiella, Cladochytriales) likely acted as decomposers, contributing to carbon and nutrient recycling within the biofilm (Gleason et al. 2008; Jobard et al. 2010; Grossart et al. 2016; Klawonn et al. 2023). In addition, parasitic or saprotrophic chytrids may influence algal dynamics by altering phototroph biomass or releasing metabolites, thus enriching the trophic web and microbial diversity through resource provisioning (Roberts et al. 2020). Among potential bacterial grazers, we identified Ciliates (*Rimostrombidium)* (Table S12); their grazing activities help balance bacterial populations, promote community turnover and prevent overgrowth of opportunistic taxa as well as favour reorganisation of the biofilm (Wey et al. 2012; Hoque et al. 2023). RF analysis of the 18S rDNA dataset identified protists ASVs representing *Leptophrys*, *Epithemia* and *Achnanthidium*. Diatoms emerged as the most characteristic biofilm-associated group, with genera like *Cymbella*, *Gomphonema*, *Navicula* and *Epithemia* present consistently (Fig. 7b), in line with their widely recognised role as key contributors to biofilm structure (Flemming and Wingender 2010a; Ramanan et al. 2016; Battin et al. 2016; Fernández et al. 2019).

Together, these results provide compelling evidence that epiphytic biofilm communities developing on *P. australis* stalks are not only taxonomically distinct from surrounding planktonic assemblages but also harbour a unique and diverse set of microbial taxa, including both bacteria and protists (Figs. 6 and 7, Tables S11, S12 and S13). While previous studies have primarily focused on bacterial differences between biofilm and planktonic communities (Souffreau et al. 2018; Fedorov et al. 2022), our findings significantly extend this perspective by showing that protist communities are also clearly and consistently differentiated between these two niches Our findings provide further evidence (Figs 2, 3, 5a, 6 and 7) for the distinction between sessile and free-living communities (Brablcová et al. 2013; Mou et al. 2013; Robinson et al. 2025, Sorokovikova et al. 2013), moreover, this study adds new insight into the composition of epiphytic biofilms in comparison to lake planktonic communities, a topic that has gained increasing attention in recent years (Souffreau et al. 2018; Yan et al. 2019; Borsodi et al. 2020) yet remains less explored than rhizosphere-associated microbiota. A better understanding of the diversity of biofilms might also be crucial for sustaining a lake's resilience. As demonstrated in marine systems (Zhang et al. 2019), biofilms can serve as reservoirs of microbial diversity, preserving rare, dormant, or otherwise undetectable taxa that are absent from planktonic surveys but may become active under changing environmental conditions. This role reflects the protective nature of biofilms which not only buffer environmental fluctuations, but also enhance microbial diversity and stability.

4.2 Potential of *Phragmites australis*-associated biofilms in bioremediation

When evaluating the importance of unique and characteristic taxa identified in biofilm communities, our analysis highlighted their role as active agents in natural bioremediation and environmental restoration. Remarkably, over half of the bacterial ASVs (52.57%) showed >99.5% species-level identity with taxa listed in the MiDAS database which compiles microorganisms frequently occurring in activated sludge systems worldwide (Table S2). This overlap suggests functional continuity between engineered and natural environments, indicating that biofilm-associated bacteria may similarly contribute to pollutant degradation and nutrient cycling *in situ*. Several matched genera are well-documented for their metabolic versatility; for instance, *Rhodobacter* species exhibit diverse metabolisms, including photoorganoheterotrophy, chemolithoautotrophy and aerobic respiration on substrates like pyruvate and lactate, and are often used in soil remediation, hydrogen production, and nutrient removal in wastewater systems (Osaka et al. 2006; Rosenberg et al. 2014; Hülsen et al. 2016; Peng et al. 2018; Mohsin et al. 2019). Similarly, *Sphingopyxis*, though less studied *in situ*, includes strains capable of degrading chlorophenols and accumulating polyhydroxyalkanoates (PHA), pointing to a role in biodegradation of recalcitrant pollutants (Godoy et al. 2003). Other key genera include *Porphyrobacter* which metabolises simple organic compounds aerobically, aiding organic matter turnover (Coil et al. 2015), and *Pseudomonas* which is widely recognised for denitrification and degradation of a broad range of pollutants (Palleroni 2015). *Rhodoferax*, *Flavobacterium*, *Aeromonas,* and *Hyphomicrobium* also contribute to nitrogen cycling and organic matter degradation (McBride et al. 2009; Martineau et al. 2013; McIlroy et al. 2016; Wang et al. 2018; Jin et al. 2020, Chen et al. 2014). The *Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium* group is of particular note; these metabolically flexible bacteria utilise a wide array of carbon and nitrogen sources, and some can form root nodules and fix nitrogen, highlighting their potential role in nutrient transformation within biofilms (Kuykendall et al. 2005; Kuykendall and Dazzo 2015).

These findings gain further significance when considered in the context of biofilms associated with *P. australis*, the aquatic macrophyte selected as the biofilm substrate in our study. *Phragmites australis* is widely recognized for its use in constructed wetlands and phytodepuration systems due to its structural support for biofilm development and its active role in pollutant uptake (Scholz and Xu 2002; Stottmeister et al. 2003; Zhao et al. 2012; Srivastava et al. 2014; Milke et al. 2020). The presence of microbial taxa with proven wastewater treatment functions, coupled with the natural phytodepuration capabilities of *P. australis*, emphasizes the potential of these plant-biofilm systems to serve as active agents in sustaining lake ecosystem health.

4.3 Environmental conditions have limited influence on the biofilm composition

Biofilm communities exhibited apparent similarity across lakes (Figs. 2, 3, and 5a), whereas planktonic assemblages were considerably more variable between lakes (Fig. 5b and Supplementary Data 1 and 2), likely reflecting lake-specific environmental conditions, including distinct physical and chemical parameters (Fig. S3 and Table S10). What is more, within each lake, water column community composition remained relatively stable across spatial zones, regardless of the proximity to biofilms on *P. australis* stems (Figs. 5a and 5b). These results strongly support the conclusion that habitat characteristics exert a stronger influence on planktonic community structure than any influence exerted by nearby biofilm communities (Yan et al. 2019; Iniesto et al. 2022).

Biofilm samples clustered across lakes (Fig. 5a), harboured a larger core (Fig. 6) and shared physiological fingerprints (Fig. 8d) consistent with lifestyle structuring. The remarkably consistent biofilm composition (Figs. 2, 3) and a high number of unique ASVs (Fig. 6) suggest that biofilms are only marginally shaped by surrounding planktonic communities or lake-specific environmental conditions (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 S10); however, this interpretation is limited by the small number of biofilm samples (n = 5) and possible scale mismatches. Given one biofilm sequence per lake (n = 5) and single-timepoint sampling, envfit for biofilms has low power, and bulk-water variables may not reflect reed-surface microenvironments. Instead, they are more strongly structured by internal dynamics - driven by a stable core of bacterial and protist taxa that play central roles in establishing and maintaining the biofilm homeostasis (Tables S11 and S12, see also Discussion 4.1).

Our findings reinforce existing evidence that biofilm communities are not strongly shaped by the surrounding environment, particularly in macrophyte-associated habitats where the host plant exerts a stronger influence than external conditions (Wijewardene et al. 2022; Sentenac et al. 2022; Sentenac et al. 2023), possibly by selecting core microbial taxa that contribute to both biofilm stability and plant health (Bais et al. 2006; Berendsen et al. 2012; Vandenkoornhuyse et al. 2015). At the same time, taxa within the biofilm itself act as a primary force structuring community composition. Central to this self-organization is the extracellular polymeric substance (EPS) matrix produced by the microbes which creates steep gradients of oxygen, pH, and nutrients (Flemming and Wingender 2010b). These microscale gradients foster spatial heterogeneity and tightly coordinated metabolic interactions that define the internal structure of the community (Battin et al. 2007). Such internal dynamics often outweigh the influence of the external aquatic environment on biofilm composition (Besemer et al. 2012). The consistency we observed across biofilm communities, despite environmental and spatial differences, aligns with the well-established view of biofilms as stable, self-regulating systems that protect microorganisms from external perturbations (Flemming and Wingender 2010a; Flemming and Wuertz 2019). Our results provide further support for the notion that biofilms remain largely unperturbed by external factors, maintaining microbial assemblages that are resilient, host-associated, and functionally buffered from variability in surrounding lake conditions (Dang and Lovell 2016).

Although our analyses did not reveal a significant environmental influence on biofilm structure, the examination of characteristic taxa showed that subtle differences between lakes may still emerge. In our dataset, the distribution of specific ASVs showed some lake-specific patterns. In the most eutrophic lake, Ryńskie (TSI = 55.3), characteristic ASVs represented genera linked to nutrient-rich conditions and involved in nitrogen cycling or complex organic matter degradation (Wang et al. 2017). Similarly, eutrophic lakes Mikołajskie and Roś were enriched in ASVs (Fig. 7a) from taxa associated with phototrophic or photoheterotrophic metabolisms advantageous in high-nutrient environments. *Aeromonas* and *Rheinheimera*, characteristic for Mikołajskie, are known opportunists in nutrient-enriched or anthropogenically impacted waters (Janda and Abbott 2010; Zhao et al. 2023b; Lennert et al. 2024). Conversely, biofilms in less eutrophic lakes Majcz and Przystań (TSI = 48.8 and 46.6) were characterised by taxa typical of meso- to oligotrophic environments, showing metabolic flexibility under nutrient limitation. Interestingly, in Ryńskie, ASV_92 (uncultured *Rhizobiales*) was also detected in the adjacent water, particularly near the biofilm. This may

indicate dispersal from biofilms under high trophic conditions, possibly driven by detachment events. Given Ryńskie's frequent wave action from wind, boats and jet skis, such physical disturbance may facilitate microbial dispersion. Beyond nutrient levels, other physical factors also influenced biofilm composition (Table S1). For example, the reduced light penetration in Roś may favour *Rhodobacteraceae* with photoheterotrophic potential, whereas Przystań, characterised by the highest water clarity (Secchi depth = 4.7 m), may support taxa such as *Rhizorhapis* and *Sphingomonas* which are likely adapted to intense light conditions and stable macrophyte substrates (Kopejtka et al. 2020; Villena-Alemany et al. 2024). In addition to bacterial ASVs, diatoms assemblages also reflected lake-specific conditions, consistent with their use as bioindicators of water quality (Poulíčková et al. 2004; Arumugham et al. 2023; Blanco 2024). IPS scores derived from epiphytic diatoms broadly matched trophic status assessments, though some discrepancies (e.g. high IPS in Roś despite elevated TSI) underline the limitations of this index which can be influenced by light, hydrodynamics, or substrate type. These results support the potential of macrophyte-associated biofilms as ecological indicators but also highlight the need for integrated approaches combining molecular, taxonomic, and physicochemical data. Beyond diatoms, other protists also reflected local conditions. In less eutrophic lakes such as Majcz and Przystań, phototrophic *Chlorochromonas*, *Naegeliella* and *Ulvella* were characteristic, indicating an autotrophic or mixotrophic biofilm layer supporting primary production under nutrient limitation (Nicholls and Wujek 2015; Zhu et al. 2015; Nunes et al. 2022). In more eutrophic lakes such as Ryńskie, fungal ASVs (*Sporobolomyces*, *Tremellomycetes*) and heterotrophic flagellates (*Ochromonadales*, *Vorticella*) dominated, contributing to decomposition, nutrient scavenging, and bacterial predation. Lake-specific taxa further reflected environmental filtering: photo(hetero)trophic *Platyophrya* and *Chlorochromonas* typified Majcz, whereas predatory cercozoans such as *Leptophrys* and *Vampyrellida* were more common in Mikołajskie, Roś and Ryńskie, likely supported by higher bacterial abundance and trophic complexity. Despite some lake-specific taxa, the overall structure of biofilm communities remained remarkably stable and largely unaffected by environmental factors. The observed variations in specific taxa do not reflect a direct influence of factors such as trophic status, light, or turbulence but rather adaptive potential of certain taxa to thrive under specific local conditions, without altering the core structure of the biofilm.

4.4 Functional diversity of biofilm and planktonic community

The distinction between biofilm and water column communities extends beyond taxonomic composition, encompassing notable differences in microbial communities' physiology (Fig. 8c). Biofilm communities consistently demonstrate a much higher substrate utilisation rate than plankton from the water of littoral and pelagic zones, underscoring their greater proteolytic activity and higher average respiration rates (Fig. 8a and Table S14). Across all five lakes, biofilm suspensions consistently exhibited elevated Vmax and LAP signals relative to water column inocula, and LAP followed Michaelis–Menten kinetics, supporting biological activity under our assay conditions. This is consistent with the fact that bacteria tend to prioritise metabolic processes for nutrient acquisition when attached to a surface rather than focusing on cell division (Fiebig et al. 2014; Van Gestel et al. 2015; Zhao et al. 2023a). Notably, carbon source utilisation patterns appear consistent across biofilm-associated communities but differ markedly from those observed in planktonic (free-living) communities (Fig. 8c). This shared physiological profile among biofilm communities suggests the existence of common active metabolic pathways and may indicate a certain degree of functional redundancy or

functional similarity (Loreau 2004; Eisenhauer et al. 2023). In contrast, protist communities are unlikely to contribute to this functional redundancy, as their ecological roles tend to be more tightly linked to specific functions, unlike the broader metabolic flexibility often observed in bacterial taxa (Ramond et al. 2025).

The physiological profiles of the biofilm-associated microbial communities in lakes Ryńskie, Majcz, Roś and Przystań, as determined by the Ecoplate method, suggest the presence of ecologically stable and functionally well-established microbial consortia (Fig. 8b). These communities showed high and broad metabolic activity across diverse carbon sources: amines, carbohydrates, phosphate carbon, and complex carbon sources. Such broad substrate utilisation is indicative of functionally diverse, well-structured microbial communities, typically associated with late-stage or stable biofilm development and the presence of cooperative enzymatic interactions (Battin et al. 2007; Flemming and Wingender 2010a). In contrast, the biofilm from Lake Mikołajskie exhibited a markedly different profile, with high utilisation of labile substrates (carbohydrates, amino acids, amines) and poor usage of more complex carbon or phosphate sources (Fig. 8c). This pattern points towards an opportunistic, early-stage community dominated by fast-growing r-strategists thriving in environments enriched in labile organic matter, such as detritus or decaying biomass - conditions typical of eutrophic systems or areas with nutrient loading (Marshall 1986; Newton et al. 2011; Shade et al. 2012). Nonetheless, all biofilms showed high amine usage, indicating exposure to nitrogen-rich organic matter, possibly from senescent plant tissues or root exudates of *P. australis*.

By contrast, planktonic communities across lakes showed marked metabolic divergence, reflecting adaptation to local conditions such as nutrient availability, DOM (dissolved organic matter) composition, and microbial interactions (Zwart et al. 2002). Moreover, a clear physiological divergence between pelagic and littoral profiles across lakes suggests strong spatial structuring of microbial function. Littoral communities in Lakes Mikołajskie and Ryńskie specialised in complex carbon utilisation, suggesting adaptation to complex DOM, potentially derived from plant material (Stets and Cotner 2008; Lambert and Perga 2019). While pelagic communities in Lake Mikołajskie exhibited similar trends of the forementioned littoral zones, in Ryńskie's pelagic zone a decrease in complex carbon utilisation was observed, suggesting the presence of more generalist microbial assemblages. Altogether, the data support the classification of both lakes as medium-to-high eutrophicated systems, inhabited by communities capable of degrading both labile and refractory organic matter. In Roś, broad metabolic activity across compartments, including amine and complex carbon use is present; this implies the presence of both labile and structurally diverse DOM which may be derived from internal aquatic microbial turnover, the excretion of organic matter by algae, or from allochthonous terrestrial vegetation (Kajan et al. 2023). The pelagic zone is particularly metabolically versatile, which aligns with literature highlighting the central role of DOM availability and quality in shaping bacterial functional diversity (Sala et al. 2020; Hu et al. 2022). Additionally, the littoral zone within *Phragmites* stands in Roś showed a distinct metabolic signature, likely reflecting interactions with plant exudates, detritus, or micro-scale oxygen gradients (Wang et al. 2022). Overall, the data support the interpretation of Roś as a mesotrophic system, functionally structured and productive, with spatially diversified microbial assemblages (Kiersztyn et al. 2019a; Wu et al. 2024). In contrast, Lake Majcz presented a unique physiological profile in its water column: metabolic activity was dominated by amine substrate utilisation while other carbon sources were minimally

processed, indicating high availability of nitrogen-rich organic matter, likely derived from protein degradation and/or zooplankton excretion (Barrett & Kwan, 1985; Tamm, 1991). Such dominance of amine metabolism reflects a community adapted to nitrogen-rich conditions and may suggest nutrient imbalance or episodic nitrogen enrichment (Preston-Mafham et al., 2002; Mills & Garland, 2004). The absence of phosphate, combined with moderate DOC levels (7.8–7.84 mg/L) and a trophic state index (TSI) of ~48.8, supports the hypothesis of phosphorus limitation, reinforcing the idea of a mesotrophic, nitrogen-enriched system.

Finally, the planktonic communities in Lake Przystań displayed broad carbon source utilisation, with particularly high activity on carbohydrates and complex carbon compounds and comparatively lower activity on amines and amino acids. The prominent consumption of polymeric and structurally diverse carbon sources may indicate a microbial assemblage specialised in degrading plant-derived or recalcitrant organic matter, potentially from terrestrial runoff or benthic resuspension (Morris & Monier, 2003; Mills & Garland, 2004). The low reliance on amines indicates limited nitrogen-rich substrates, contrasting with more protein-enriched systems. (Tamm, 1991; Barrett & Kwan, 1985). Such metabolic versatility may reflect a mature, stable community structured to exploit diverse carbon sources under moderate nutrient conditions (Preston-Mafham et al., 2002). Overall, the contrasting metabolic profiles of Lakes Majcz and Przystań highlight the value of functional assays in revealing microbial adaptations and key environmental drivers in aquatic ecosystems.

SUMMARY

Our study revealed that *P. australis* stem biofilms host remarkably diverse bacterial and protist communities compared to the surrounding plankton. While planktonic communities are largely shaped by local lake conditions, biofilms maintain a consistent microbial core, reflecting ecological interdependencies among bacteria, protists, and aquatic plants that underpin resilient and functionally stable biofilms across freshwater systems.

Biofilm communities exhibited also a distinct functional profile compared to planktonic ones, with higher rate of substrate utilisation, proteolytic activity, and respiration, reflecting shared metabolic pathways and functional redundancy, underscoring their role as metabolically versatile and stable consortia in freshwater ecosystems. Integrating community profiling with functional databases (e.g. MiDAS) suggests that biofilms associated with *P. australis* harbour bacteria capable of key ecosystem processes, such as denitrification, xenobiotic degradation and organic matter transformation. This highlights their metabolic versatility and their potential for use in ecosystem monitoring and nature-based water purification.

Macrophyte-associated biofilms, such as those on *P. australis*, harbour uniquely structured and compositionally rich microbial assemblages that contribute substantially to freshwater ecosystem functioning. Their high proportion of unique taxa highlights the need to include biofilm bacterial and protistan communities in broader surveys to capture the full extent of microbial diversity and reveal their ecological roles in freshwater systems.

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Conflicts of Interest

The authors have declared that no competing interests exist.

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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.

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