



Microbial community structure and diversity attached to the periphyton in different urban aquatic habitats

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Received: 14 December 2023 / Accepted: 4 April 2024 / Published online: 12 April 2024
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Abstract Periphyton is a complex community composed of diverse prokaryotes and eukaryotes; understanding the characteristics of microbial communities within periphyton becomes crucial for biogeochemical cycles and energy dynamics of aquatic ecosystems. To further elucidate the community characteristics of periphyton across varied aquatic habitats, including unpolluted ecologically restored lakes, aquaculture ponds, and areas adjacent to domestic and industrial wastewater treatment plant outfalls, we explored the composition and diversity of prokaryotic and eukaryotic communities in periphyton by employing Illumina MiSeq sequencing. Our findings indicated that the prokaryotic communities were predominantly composed of Proteobacteria (40.92%), Bacteroidota (21.01%), and Cyanobacteria (10.12%), whereas the eukaryotic communities were primarily

characterized by the dominance of Bacillariophyta (24.09%), Chlorophyta (20.83%), and Annelida (15.31%). Notably, *Flavobacterium* emerged as a widely distributed genus among the prokaryotic community. *Unclassified_Tobrilidae* exhibited higher abundance in unpolluted ecologically restored lakes. *Chaetogaster* and *Nais* were enriched in aquaculture ponds and domestic wastewater treatment plant outfall area, respectively, while *Surirella* and *Gomphonema* dominated industrial sewage treatment plant outfall area. The alpha diversity of eukaryotes was higher in unpolluted ecologically restored lakes. pH and nitrogen content (NO_2^- -N, NO_3^- -N, and TN) significantly explained the variations for prokaryotic and eukaryotic community structures, respectively. Eukaryotic communities exhibited a more pronounced response to habitat variations compared to prokaryotic communities. Moreover, the association networks revealed an intensive positive correlation between dominant Bacillariophyta and Bacteroidota. This study provided useful data for identifying keystone species and understanding their ecological functions.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10661-024-12599-x>.

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Keywords Periphyton · Prokaryotes · Eukaryotes ·
Community structure · Microbial diversity

Introduction

Aquatic ecosystems are essential for supporting agricultural, industrial, and human production activities.

Freshwater environments are not only crucial for providing freshwater resources for societal development but also serve as vital habitats for diverse microbial communities (Wang et al., 2018). Periphyton, consisting of bacteria, archaea, protozoa, fungi, algae, and exopolysaccharide matrices, is extensively distributed in aquatic ecosystems (Carles et al., 2022; Wang et al., 2010). It plays a fundamental role in biogeochemical cycling, pollutant degradation, and sustaining the dynamics of aquatic ecosystems (Huang et al., 2018; Mai et al., 2020; Stelzer & Lamberti, 2001). For instance, periphyton accounted for up to 80% of primary productivity in some aquatic systems and was a critical food source in aquaculture (van Dam et al., 2002). Furthermore, periphyton has shown the capacity to reduce nutrient levels in wastewater and remove organic pollutants, heavy metals, and antibiotics, indicating a significant potential for biological pollutant remediation (Tamminen et al., 2022; Yang et al., 2020). Due to its sensitivity to environmental changes, periphyton has been identified as a bioindicator of aquatic ecosystem health (Chen et al., 2022; Wang et al., 2010). Therefore, studying the community structure and diversity of periphyton is essential for enhancing ecosystem stability and understanding ecological characteristics.

Rapid urbanization has led to a notable decline in water quality and diminished ecological functions in natural water bodies in industrial cities (Zhang et al., 2021). The discharge of sewage from anthropogenic activities and urban development significantly altered water physicochemical properties (e.g., pH, dissolved oxygen, nutrient levels, and light penetration), adversely affecting microbial communities and aquatic ecosystems (Liao et al., 2018; Esser et al., 2023; Zhang et al., 2021). Wastewater containing nutrients, metals, organic/inorganic compounds, and significant quantities of microorganisms can lead to modifications in the community structures and ecological functions of periphyton (Carles et al., 2022; Tamminen et al., 2022). For example, the diversity of prokaryotic and eukaryotic communities was markedly lower in wastewater-impacted streams, with core prokaryotic communities comprising Proteobacteria, Bacteroidota, and Chlorophyta (Esser et al., 2023). Additionally, aquaculture systems, being highly eutrophic, favor certain microorganisms. Alphaproteobacteria and Bacteroidetes were more enriched in periphyton within these ecosystems, whereas

Chlorophyta predominated in eukaryotic communities (Zhang et al., 2023). Effluent from wastewater treatment plants diminished sulfur bacteria populations, impeding complete denitrification activities (Esser et al., 2023). However, microorganisms in wastewater enhanced the transformation of pollutants and nutrients, positively impacting water purification and ecological stabilization (Desiante et al., 2022). Organic micropollutants have been shown to increase the tolerance of phototrophic microorganisms in periphyton, with Cyanobacteria's relative abundance rising with the percentage of wastewater effluent, while diatoms showed the opposite trend (Carles et al., 2021). These studies have demonstrated that anthropogenic activities can lead to environmental pollution sources, which in turn can modify the physicochemical properties of water bodies and indirectly augment the heterogeneity of aquatic ecosystems.

Prokaryotes and eukaryotes constitute the primary microbial communities in periphyton, playing a crucial role in regulating biological structure and ecosystem function (Zhang et al., 2023; Guo et al., 2021). The carbon source required for the reproduction of prokaryotes came mainly from the decomposition of algal secretions or residues, while the elements required for photosynthesis and the growth of eukaryotes (algae) were supplied by the metabolism and decomposition of bacteria, respectively (Krohn-Molt et al., 2013). Guo et al. (2021) found that the diversity of prokaryotic and eukaryotic communities varied over time, with complex interactions between the dominant phyla (Guo et al., 2021). Eukaryotes, especially diatoms and green algae, as primary producers, significantly contributed organic matter for the growth of the periphyton community, underscoring their importance in matter cycles and energy flux (Hu et al., 2022; Battin et al., 2016; Kamjunke et al., 2015). While current research on periphyton has largely focused on single water ecosystems or microbial communities (Carles et al., 2022; Lin et al., 2019; Zhang et al., 2023), the community structures of prokaryotes and eukaryotes across different aquatic habitats remain insufficiently understood.

In this study, Illumina MiSeq sequencing of 16S rRNA and 18S rRNA was employed to investigate the composition of prokaryotic and eukaryotic communities associated with periphyton in unpolluted ecologically restored lakes, aquaculture ponds, domestic wastewater treatment plant outfall area, and industrial

wastewater treatment plant outfall area in urban areas (Changzhou, China). We aim to (1) explore the structure of the prokaryotic and eukaryotic communities attached to the periphyton under different water environments and (2) identify the primary environmental factors influencing variations in microbial community and analyze potential relationships between prokaryotes and eukaryotes within periphyton. This study can provide fundamental data regarding the community structure of prokaryotes and eukaryotes, as well as enhance our understanding of the availability of periphyton in ecological management.

Materials and methods

Study area and sampling

Changzhou is an industrial city, located in the south of Jiangsu Province (N 31.09°~32.04° and E 119.08°~120.12°), China, with a population of approximately 5.28 million. The Beijing-Hangzhou River Grand Canal and Zaogang River flow through urban areas and join the Guan River in the city center of Changzhou (Cui et al., 2019). Surface water and periphyton samples were collected from four diverse habitats in January 2023. The four distinct types of aquatic ecological environments include unpolluted ecologically restored lakes (U, U1-U3), aquaculture ponds (A, A1-A3), Wunan Domestic Sewage Treatment Plant outfall area (WN, WN1-WN3), and Mahang Industrial Sewage Treatment Plant outfall area (MH, MH1-MH3). The treated wastewater from the wastewater treatment plants (WN and MH) was discharged directly through drain lines into the nearby rivers, and the sampling sites were located in the rivers near the wastewater outlets. The sampling sites are shown in Fig. 1. Detailed information regarding the wastewater treatment plants and sampling sites can be referred to Supplementary Material (Table S1, S2).

Water samples were collected into 5-L PET sterile plastic bottles pre-washed with water. Representative rocks (one to two rocks) were obtained from the water at depths ranging from 0.2 to 0.5 m, and periphyton attached to the top surface of the rocks (approximately 200 cm²) was softly scraped manually per sampling site using a sterilized soft brush and suspended in 120 mL of distilled water (Huang et al., 2018; Roberto et al., 2019; Yan et al., 2019).

Both water samples and periphyton suspensions were transported to the laboratory for subsequent physicochemical analysis. Then, the periphyton suspensions were filtered through a 0.22- μ m pore-size polycarbonate filter (Millipore, USA) using a vacuum pump after sampling. The filters were placed in a vehicle-mounted refrigerator during transportation and then stored at -80 °C in the laboratory until DNA extraction.

Analysis of physicochemical variables and periphyton biomass

Water temperature (*T*) and pH were measured *in situ* using a portable multi-parameter meter (HACH, USA). The concentrations of total phosphorus (TP) and soluble reactive phosphorus (SRP), the total nitrogen (TN), ammonium-nitrogen (NH₄⁺-N), nitrate-nitrogen (NO₃⁻-N), and nitrite-nitrogen (NO₂⁻-N) were determined in the laboratory according to the previous studies (Huang et al., 2018; Xia et al., 2020). Dissolved organic carbon (DOC) was determined by using a Multi N/C 3100 analyzer (Analytik Jena, Germany). The Chlorophyll-a (Chl-a) was measured after the extraction of 0.45- μ m pore size glass fiber membranes in 90% acetone for 24 h in the dark at 4°C, and the content of Chl-a was determined using a UV-9000 spectrophotometer (METASH, China) according to the previous study (Xia et al., 2020). Three subsamples of each periphyton suspension (10 ml) were filtered through 0.22- μ m pore size pre-combusted glass fiber membranes. Each membrane was weighed after drying for 24 h at 105°C to constant weight (dry weight). The membranes were then combusted in a muffle furnace at 500°C for 2 h and weighed again (ash weight), and the ash-free dry weight (AFDW) was calculated by subtracting the ash weight from the total dry weight (Wang et al., 2010; Xia et al., 2020). All physicochemical measurements of the water samples and periphyton biomass were determined in triplicate.

DNA extraction, PCR amplification, and sequencing for prokaryotes and eukaryotes

The DNA was extracted from the periphyton samples using the HiPure Soil DNA Kits (Magen, Guangzhou, China) according to the manufacturer's protocol. The DNA extracts were mixed and the quality

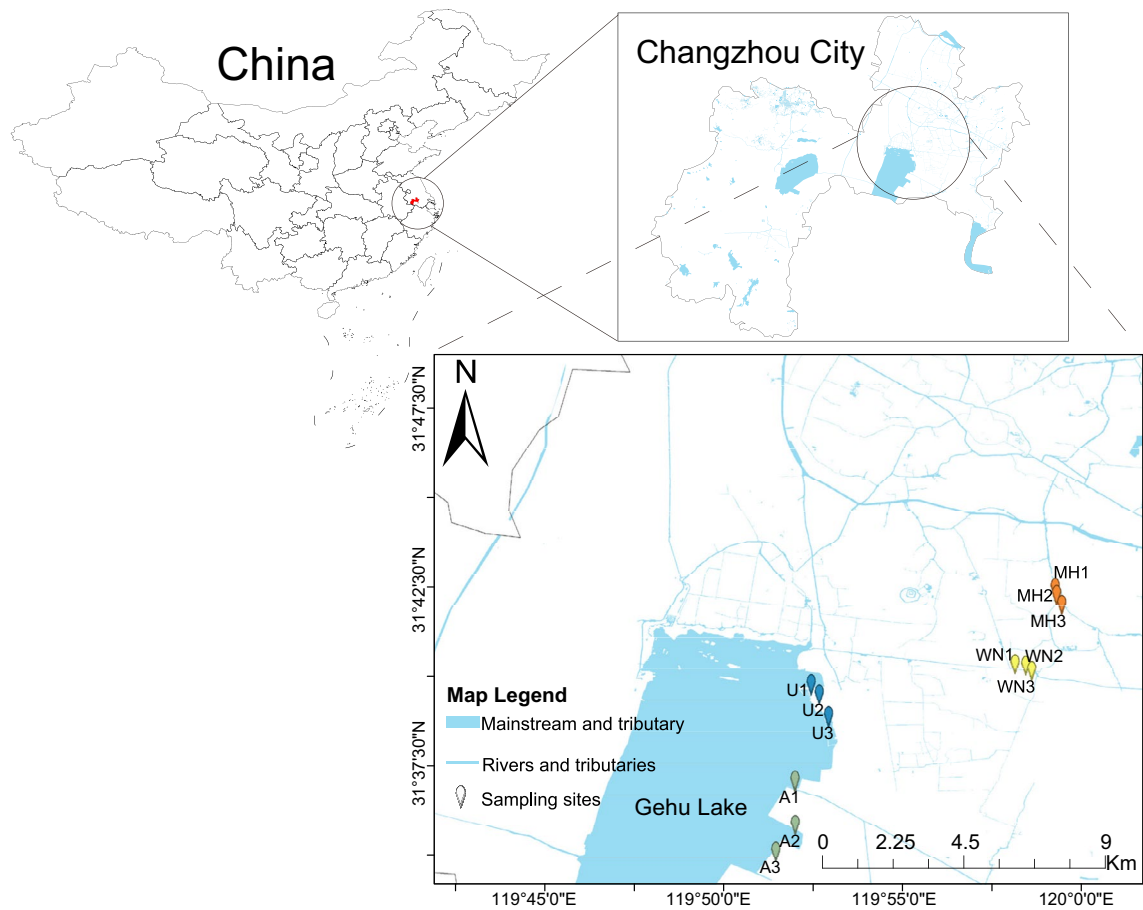


Fig. 1 Map and regional locations of different sampling sites. Specific latitude and longitude information for each sampling site can be found in the supplementary material

and concentrations of DNA were estimated by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). For PCR amplification, a forward primer 341F (5'-CCTACGGGNGGCWGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used for V3-V4 region of 16S rRNA gene (Cui et al., 2019). A forward primer 528F (5'-GCGGTA ATTCCAGCTCCAA-3') and a reverse primer 706R (5'-AATCCRAGAATTTACCTCCAA-3') were provided for the V4 region of the 18S rRNA gene (Manirakiza et al., 2022). Two-step PCRs were conducted. A 50- μ L reaction mixture contained 10 μ L of 5 \times Q5@ Reaction Buffer, 10 μ L of 5 \times Q5@ High GC Enhancer, 1.5 μ L of 2.5 mM dNTPs, 1.5 μ L of each primer (10 μ M), 0.2 μ L of Q5@ High-Fidelity DNA Polymerase, and 50 ng of template DNA. The target regions of the ribosomal RNA gene were amplified

by PCR (95 $^{\circ}$ C for 5 min, followed by 30 cycles at 95 $^{\circ}$ C for 1 min, 60 $^{\circ}$ C for 1 min, and 72 $^{\circ}$ C for 1 min and a final extension at 72 $^{\circ}$ C for 7 min) using related regions' primers. Universal PCR primers took the place of primers F and R for the second PCR amplification in the same volume reaction systems under the following reaction conditions: 95 $^{\circ}$ C for 5 min; followed by 12 cycles of 95 $^{\circ}$ C for 1 min, 60 $^{\circ}$ C for 1 min, and 72 $^{\circ}$ C for 1 min; and finally 72 $^{\circ}$ C for 7 min. The amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA), then quantified with the ABI StepOnePlus Real-Time PCR System (Life Technologies, USA). Subsequently, the purified amplicons were equimolarly pooled and subjected to paired-end sequencing on the Illumina platform by Guangzhou Gene-Denovo Biotechnology Co., Ltd.

(Guangzhou, China). Unfortunately, one of the periphyton samples (A1) failed to construct a library due to the low DNA concentrations for 18S rRNA gene sequencing. The raw sequences were deposited into the NCBI Sequence Read Archive with the accession number SRP454379.

Bioinformatic analysis

The DADA2 R package (version 1.14) implements a complete pipeline to turn paired-end Fastq files from the sequencer into merged, denoised, chimera-free, inferred sample sequences. Raw reads were filtered and truncated by removing reads containing unknown nucleotides and primer sequences (Esser et al., 2023). Subsequently, paired-end denoised reads were merged as raw ASVs (amplicon sequence variants) with a minimum overlap of 12bp (Callahan et al., 2017). Chimera sequences were identified and deleted by the UCHIME algorithm (Edgar et al., 2011), and the denoised and chimera-free ASV sequences were output. The representative ASV sequences were classified into organisms by a naive Bayesian model using an RDP classifier (version 2.2) based on the SILVA database (version 138.1) with a confidence threshold value of 0.8 (Esser et al., 2023; Yu et al., 2022). The ASV tables were randomly subsampled to ensure the same sequencing depth and an equal number of sequences per sample (Wang et al., 2019). The values with a total relative abundance below 0.01% were subjected to quality filtering to ensure the statistical reliability and biological validity for all microbial analysis, as documented in the literature (Liu et al., 2018).

Statistical analysis

The variations of the diversity index were carried out using a one-way analysis of variance (ANOVA) followed by a Turkey HSD test. For data that did not satisfy normality and heteroscedasticity assumptions, Kruskal-Wallis and Dunn's test were used to identify the differences in physicochemical parameter of water and periphyton biomass (Roberto et al., 2019). To estimate the beta diversity of microbial communities, principal coordinates analysis (PCoA) was conducted based on the Bray-Curtis distance matrix, and the analysis of similarity statistics (ANOSIM) was also performed to evaluate the differences using the

'vegan' package (Xia et al., 2020). Spearman's correlation coefficients were calculated for the top ten phyla of each of the prokaryotic and eukaryotic communities. Only significant correlations ($p < 0.05$, abs (correlations coefficients) < 0.6) were used for the network visualization, which is implemented with 'igraph' package (Guo et al., 2021; Xia et al., 2020). A Mantel test was conducted to assess the relationships between the standardized physicochemical parameters and the microbial communities (Xia et al., 2020) (at the phylum level). Redundancy analysis (RDA) was conducted to explore environment-community relationships, and the significance was tested using function *envfit* with 999 permutations. All statistical analyses were carried out by using R and graphical representations were conducted by using the 'ggplot2' package.

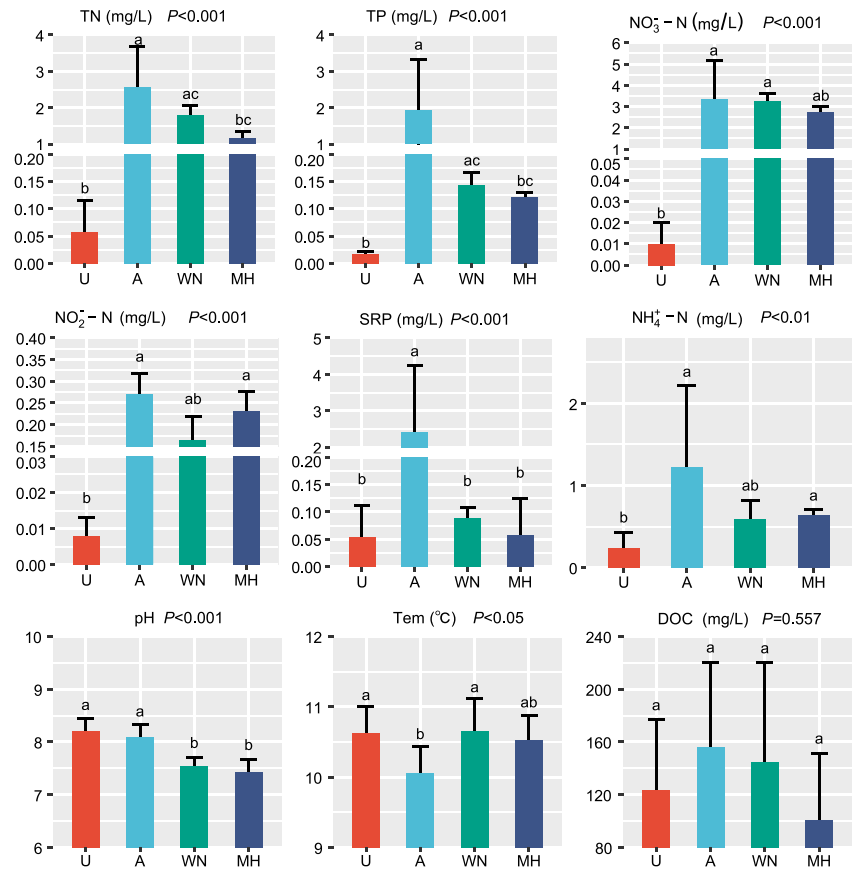
Results

Physicochemical parameters and periphyton biomass

Variations in physicochemical parameter were observed across the four aquatic ecosystems, with temperature and pH ranging from 9.5 to 11.2°C and 7.12 to 8.4, respectively (Table S3). Noticeably, significant differences in environmental variables were observed. The highest concentrations of TN, NO₃⁻-N, NO₂⁻-N, NH₄⁺-N, TP, and SRP were observed in aquaculture ponds (A), whereas the lowest concentrations were detected in unpolluted ecologically restored lakes (U). The pH levels were significantly elevated in the U and A areas compared to the WN and MH areas ($p < 0.001$). Dissolved organic carbon (DOC) levels did not significantly vary across the different areas (Fig. 2).

The levels of AFDW and Chl-a associated with the periphyton exhibited statistically significant variations ($p < 0.05$). As depicted in Fig. 3, the AFDW values varied between 0.46 and 11.29 mg/cm², the Chl-a values ranged from 0.74 to 16.28 µg/cm². The AFDW was notably higher in aquaculture ponds (A), significantly exceeding values observed in both the unpolluted lakes (U) and the Wunan Domestic Sewage Treatment Plant outfall area (WN) (Fig. 3a, $p < 0.05$). Conversely, the lowest Chl-a content was recorded in the U area, significantly lower than that in the other studied areas (Fig. 3b, $p < 0.05$).

Fig. 2 The major physicochemical properties of the water were analyzed in unpolluted ecologically restored lakes (U), aquaculture ponds (A), domestic wastewater treatment plant outfall area (WN), and industrial wastewater treatment plant outfall area (MH). Tem, temperature; TN, total nitrogen; TP, total phosphorus; SRP, soluble reactive phosphorus; DOC, dissolved organic carbon



Alpha and beta diversity of prokaryotes and eukaryotes in periphyton

A total of 599,921 clean sequences were generated for prokaryotes, while 938,223 clean sequences were generated for eukaryotes in periphyton. Rarefaction curves suggested sufficient sequencing depth for analysis, with Good's coverage exceeding 0.9999 (Fig. S1).

The average ASV number was determined to be 1786 for prokaryotes and 996 for eukaryotes in periphyton. Regarding alpha diversity, the diversity indices, including Shannon and Chao1, revealed that prokaryotic communities exhibited greater richness and diversity compared to eukaryotic communities (Fig. 3c, d). For prokaryotes, the U area exhibited higher Shannon index values compared to other areas (A, WN, and MH), although these differences were not statistically significant (Fig. 3c). Eukaryotic communities in the U area showed significantly higher diversity compared to other aquatic environments, as

indicated by both Shannon (5.63 ± 0.33) and Chao1 (1265.99 ± 132.03) indices (Fig. 3d).

The PCoA and ANOSIM analyses were performed using Bray-Curtis dissimilarity to investigate the variations in microbial community structures within periphyton. Both the PCoA and ANOSIM analysis indicated that the prokaryotic (Fig. 4a) and eukaryotic (Fig. 4b) communities from four distinct areas exhibited clustering patterns. The prokaryotes from the A area and the MH area were separated primarily along the first coordinate axis, while the prokaryotic communities from the A area and the U area were separated mainly along the second coordinate axis. The eukaryotic community from the U area and the WN area were conspicuously separated primarily along the second coordinate axis. Additionally, the eukaryotic community exhibited lower similarity compared to the prokaryotic community ($R_{\text{eukaryotes}} = 0.9378 > R_{\text{prokaryotes}} = 0.7901$, Fig. 4), as determined by the ANOSIM analysis. The ANOSIM analysis confirmed significant differences between the microbial

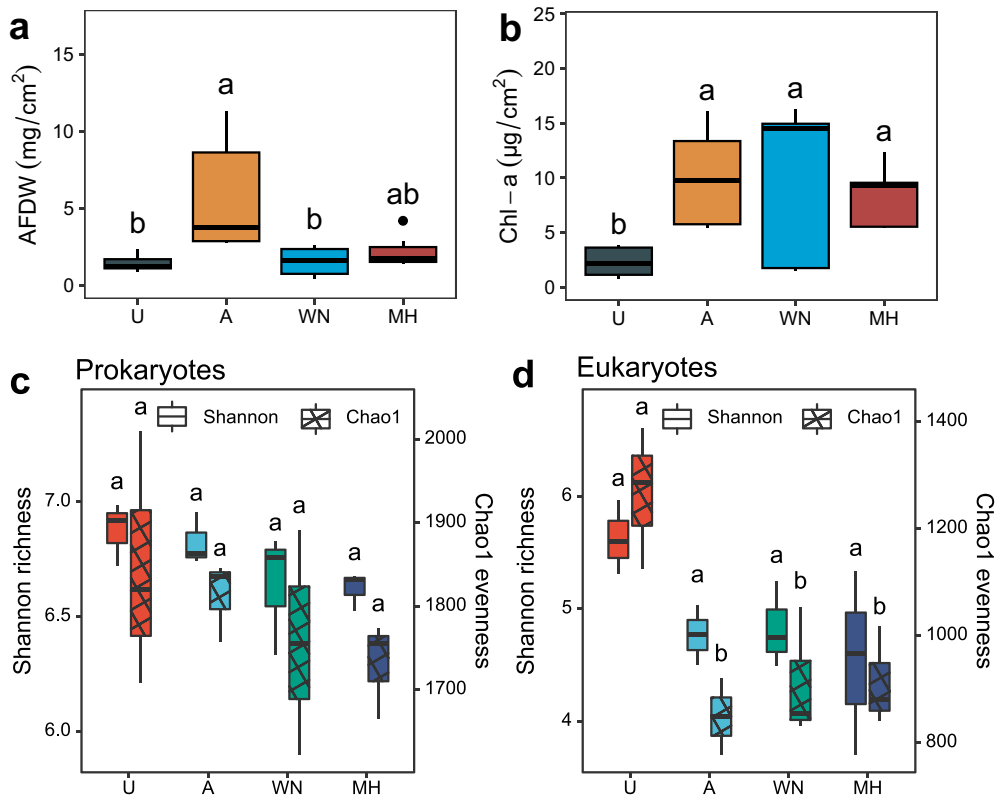


Fig. 3 The content of AFDW (a) and Chl-a (b) of periphyton in the unpolluted ecologically restored lakes (U), aquaculture ponds (A), domestic wastewater treatment plant outfall area

(WN), and industrial wastewater treatment plant outfall area (MH). Diversity index of Shannon and Chao1 about prokaryotes (c) and eukaryotes (d)

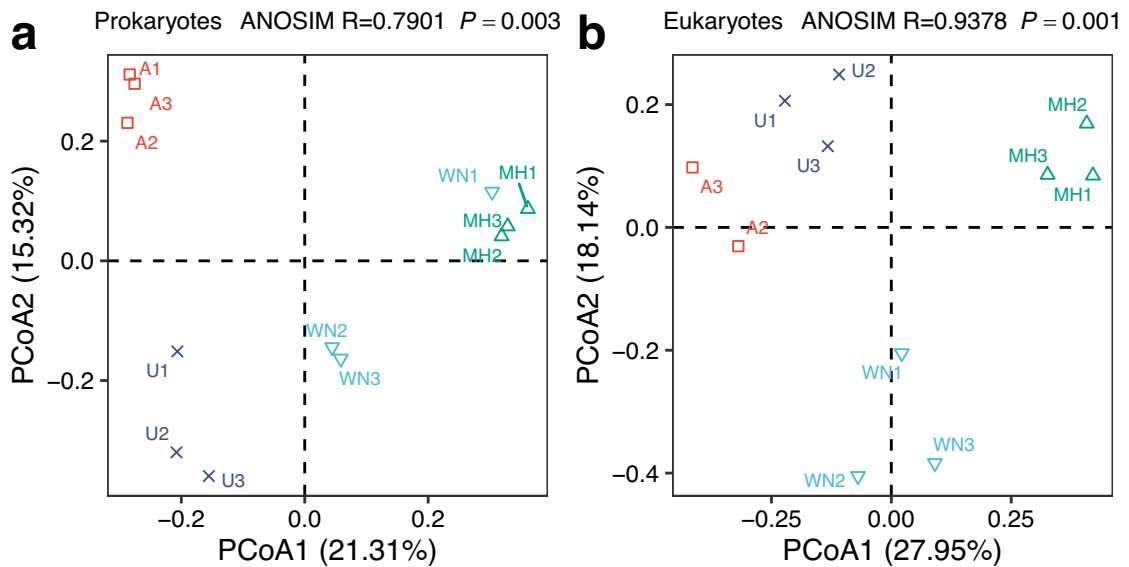


Fig. 4 Principal Coordinates Analysis (PCoA) of the microbial community structure in prokaryotes (a) and eukaryotes (b) within periphyton

communities in the U area and the MH area ($p < 0.01$, Fig. S2).

Microbial community composition of periphyton

In total, 13,650 Amplicon Sequence Variants (ASVs) were taxonomically classified into 453 genera across 38 phyla, 104 classes, 223 orders, and 279 families within prokaryotes. Moreover, a total of 4036 ASVs were classified into 419 genera across 40 phyla, 102 classes, 206 orders, and 319 families within eukaryotes.

For prokaryotes, the periphyton sampled from four distinct aquatic environments exhibited comparable patterns of prokaryotic community compositions

(Fig. 5a). At the phylum level, the prokaryotic communities of all aquatic environments were dominated by Proteobacteria (33.28–45.82%), Bacteroidota (15.01–32.96%), Cyanobacteria (7.03–15.08%), and Verrucomicrobiota (3.12–15.39%). The relative abundance of Proteobacteria in the U area was notably lower compared to other areas (A, WN, and MH). Furthermore, it was observed that the relative abundance of Bacteroidota in the MH area surpassed that of the other areas. At the genus level (Fig. 5c), *Flavobacterium* was the major genus that contributed to Bacteroidota abundance in the four aquatic habitats. *Luteolibacter* (17.80%) and *Flavobacterium* (12.09%) were identified as the predominant genera in the U area. The relative abundances of *Flavobacterium*

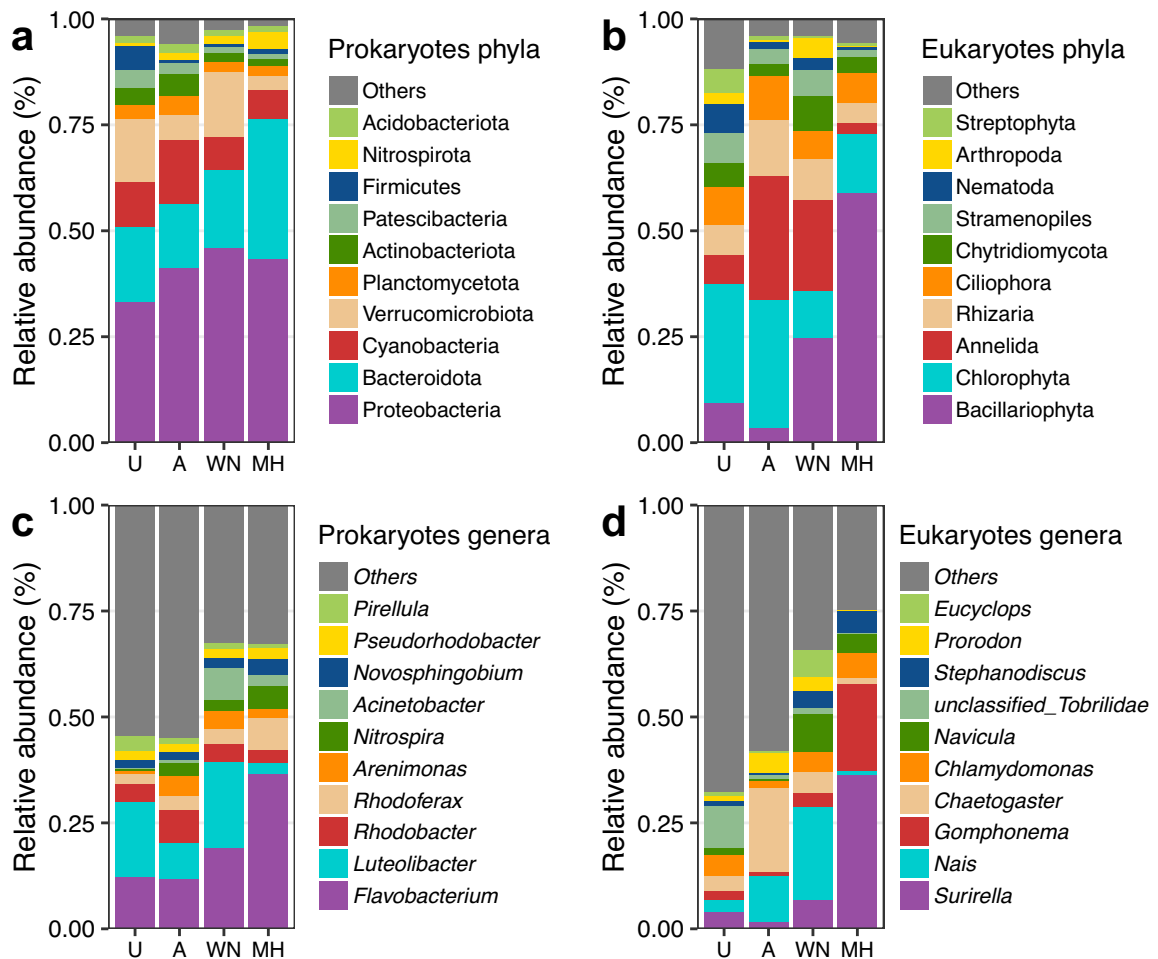


Fig. 5 The community compositions of periphyton encompass the phyla of prokaryotes (a) and eukaryotes (b), as well as the genera of prokaryotes (c) and eukaryotes (d). “Others” repre-

sents the different taxa except for the top ten phyla/genera with the highest average relative abundance among all samples

(11.79%), *Luteolibacter* (8.30%), and *Rhodobacter* (7.95%) were identified to be significantly elevated in the A area. *Flavobacterium* (18.92%), *Luteolibacter* (20.37%), and *Acinetobacter* (7.48%) displayed a higher relative abundance in the WN area. *Flavobacterium* (36.47%), *Rhodoferrax* (7.33%), and *Nitrospira* (5.61%) were more abundant in the MH area.

In terms of eukaryotic community, the community compositions of four distinct areas exhibited varying distributions (Fig. 5b). At the phylum level, the eukaryotic communities in the four aquatic habitats were dominated by Bacillariophyta (3.45–58.90%), Chlorophyta (11.07–30.16%), Annelida (2.42–29.36%), Rhizaria (4.92–13.01%), Ciliophora (6.64–10.54%), Chytridiomycota (2.84–8.06%), and Stramenopiles (1.86–7.04%).

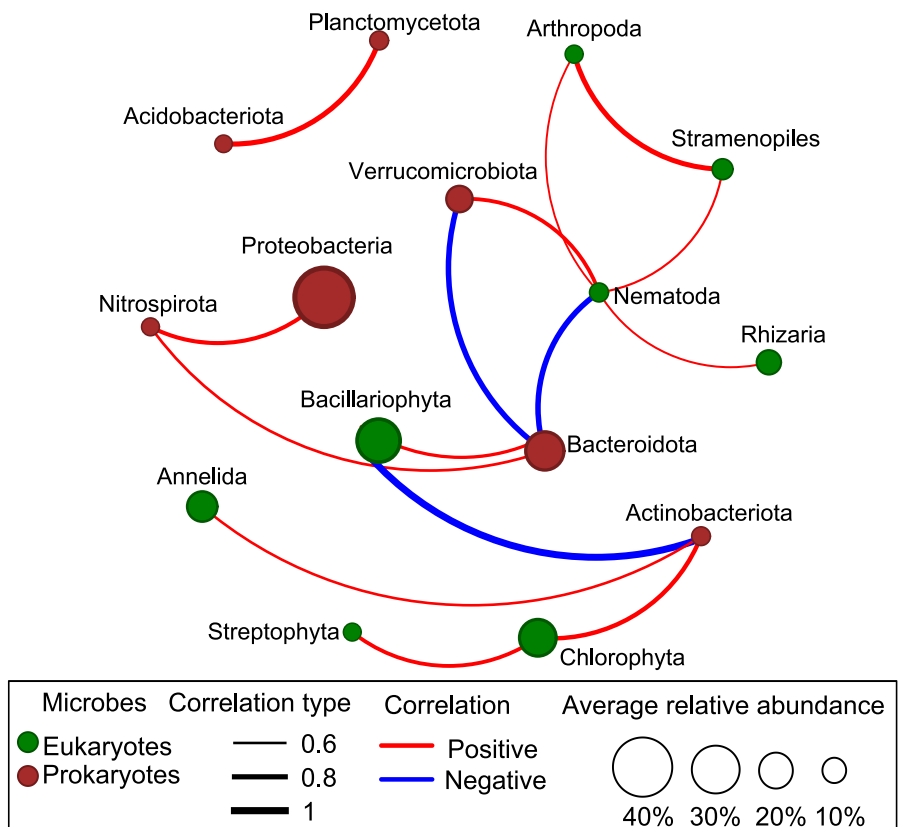
At the genus level, *unclassified_Tobrillidae* (9.83%) and *Chlamydomonas* (5.07%) were found to be abundantly distributed in the U area. *Chaetogaster* (19.85%) and *Nais* (10.85%), classified as Annelida, were more abundant in the A area. Relatively higher abundances of *Nais* (10.85%), *Navicula* (9.07%), and

Surirella (6.80%) genera were found in the WN area. In comparison to the remaining three aquatic habitats, the MH area demonstrated the highest relative abundances of *Surirella* (36.24%) and *Gomphonema* (20.40%), both classified under Bacillariophyta. Significantly, the compositions of dominant genera varied across four distinct aquatic environments.

Correlations between prokaryotic phyla and eukaryotic phyla

The Spearman correlation coefficients between the top ten phyla of prokaryotes and eukaryotes ranged from -0.95 (correlation between Bacillariophyta and Actinobacteriota) to 0.85 (correlation between Planctomycetota and Acidobacteriota). The association networks generated from the coefficients (Fig. 6) revealed 15 edges, the majority of which demonstrated positive correlations, with 12 edges accounting for 80% of the total correlations. Out of the three negative edges, they were linked to the prevalent prokaryotic phyla (e.g., Bacteroidota

Fig. 6 Association network of microbial communities at phylum level within periphyton. The top ten phyla for each of the prokaryotic (brown nodes) and eukaryotic (green nodes) communities are shown. The size of nodes corresponds to the average relative abundance of their phyla. The network's nodes are connected by edges, where red and blue edges represent significant positive and negative connections, respectively. The estimated connections were determined through Spearman correlation analysis, with a significance level set at 0.05. Isolated nodes were excluded. The magnitude of correlation coefficients is indicative of the strength of the correlation, with thicker edges representing stronger correlations



and Verrucomicrobiota) and eukaryotic phyla (e.g., Bacillariophyta).

Relationships between microbial communities of periphyton and environmental factors

The study investigated the correlation between physicochemical factors and periphyton communities (including prokaryotes and eukaryotes) using the Mantel test. The results revealed significant correlations between four phyla with higher relative abundance and water parameters (Fig. 7a, b). Within the prokaryotic community, there were significant positive correlations between pH and dominant taxa (Proteobacteria and Bacteroidota)

(Mantel's $R > 0.2, p < 0.05$). For eukaryotes, the Mantel test demonstrated that Bacillariophyta and Annelida showed positive correlations with environmental factors (TP, SRP, and TN) (Mantel's $R > 0.2, p < 0.05$). As indicated by the redundancy analysis (RDA), the significant variables accounted for 52.38% and 49.92% of the total variations in the prokaryotic and eukaryotic communities, respectively (Fig. 7c, d). The pH was found to have a significant impact on the prokaryotic communities of periphyton (Table S4; permutation *envfit*, $p < 0.01$). Environmental factors such as total nitrogen (TN), NO_3^- -N, and NO_2^- -N were identified as playing crucial roles in shaping eukaryotic communities (Table S4, permutation *envfit*, $p < 0.01$).

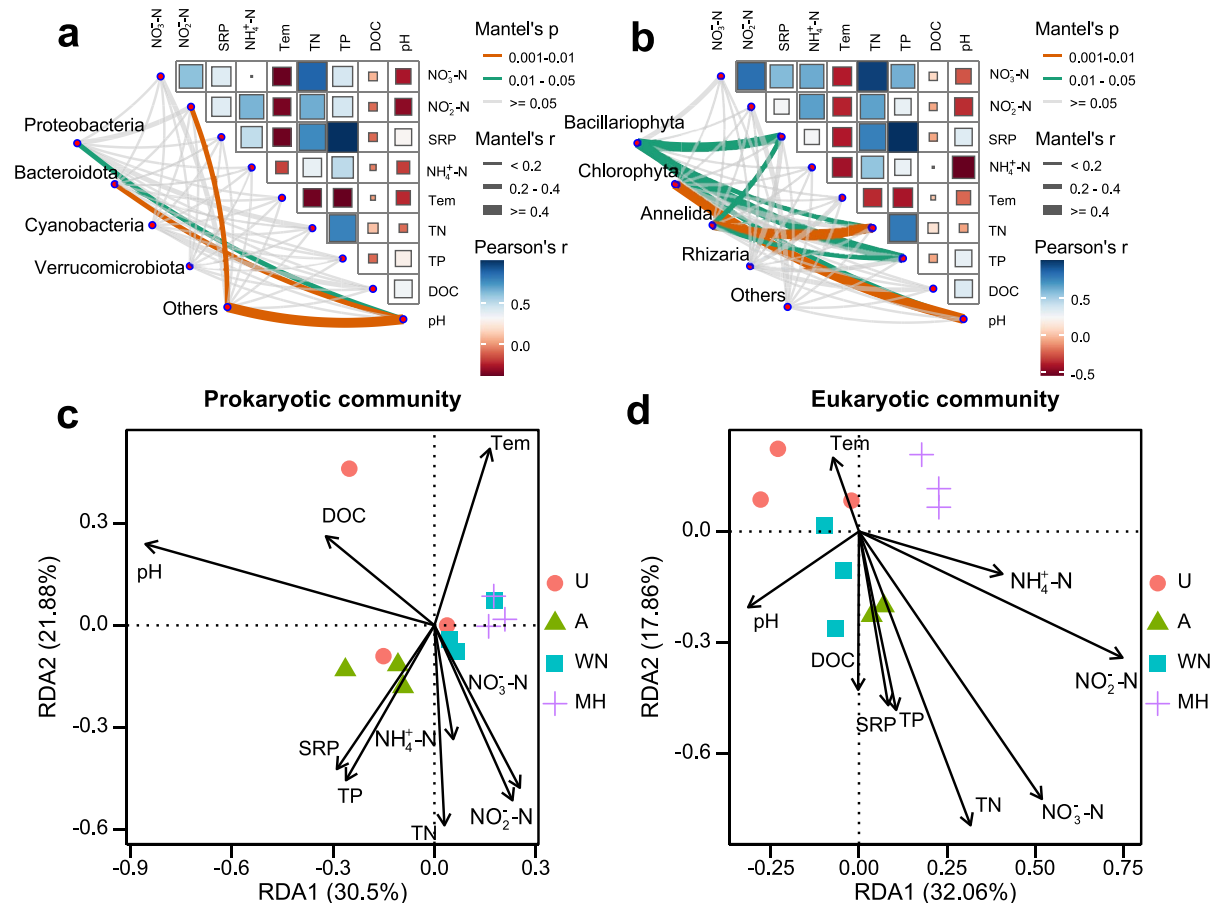


Fig. 7 The Mantel test of dominant prokaryotic phyla (a) and eukaryotic phyla (b) (top four phyla). Taxonomic groups (genera) were related to each environmental parameter. Pairwise comparisons of physicochemical properties are displayed with a color gradient to denote Spearman's correlation coefficients.

Redundancy analysis of prokaryotes (c) and eukaryotes (d) at phylum level and selected environmental variables. Tem, temperature; TN, total nitrogen; TP, total phosphorus; NO_3^- -N, nitrate; NO_2^- -N, nitrite; NH_4^+ -N, ammonia; SRP, soluble reactive phosphorus; DOC, dissolved organic carbon

Discussion

Biomass varied across aquatic ecological habitats

Significant variations in periphyton biomass, as indicated by ash-free dry weight (AFDW) and Chlorophyll-a (Chl-a), were observed across the study habitats. The Chl-a in periphyton serves as an indicator of algal biomass and also provides quantitative methods in relation to resource utilization (Smeti et al., 2019). The Chl-a content was lower in the U area but higher in the A area, indicating a potential constraint on Chl-a levels by low nutrient concentrations, specifically nitrogen and phosphorus (Weitere et al., 2021; Bengtsson et al., 2018). The peak Chl-a contents in the A area were noted to approach $15 \mu\text{g}/\text{cm}^2$, a level near the threshold for nuisance conditions (Welch et al., 1988). This phenomenon can be attributed to the promotion of filamentous periphytic algae growth by elevated Chl-a levels, with filamentous species potentially contributing to water deterioration (Huang et al., 2018; Welch et al., 1988). Thus, the Chl-a present in periphyton may serve as a critical indicator for assessing water eutrophication. In addition, uneaten feed and sewage discharge increased the organic matter and nutrient levels, and the stable water flow further promoted the accumulation of organic matter by the periphyton (Wang et al., 2010; Huang et al., 2018; Smeti et al., 2019) and periphyton biomass (AFDW and Chl-a) thus increased significantly in aquaculture ponds.

Community structure and diversity differed among aquatic environments

The alpha diversity indexes of the prokaryotic community in periphyton were notably higher than those of the eukaryotic community. Specifically, the number of prokaryotic amplicon sequence variants (ASVs) was nearly double that of the eukaryotes. These differences were attributed to some bacteria secreting extracellular polymers to create microhabitats for microorganism adhesion in periphyton, which is also in agreement with previous studies showing a generally higher diversity of prokaryotes than eukaryotes (Stelzer & Lamberti, 2001; Yu et al., 2022; Yan et al., 2019). The ecological restoration of habitats enhanced the colonization of periphyton and the diversity of aquatic biotas, with the U area showing

higher diversity indices for both prokaryotes and eukaryotes. This highlights the habitat complexity generated after ecological restoration (restored rivers or lakes) promoted the colonization of periphyton and improved the community diversity of aquatic biotas (Lin et al., 2019; Huang et al., 2018); higher diversity in eukaryotes (algae) also implied a positive effect on nitrate uptake in aquatic ecosystems (Baker et al., 2009). In contrast, reduced eukaryotic diversity was observable in aquatic environments impacted by human activities, such as aquaculture ponds and wastewater treatment plant outfall areas (Fig. 3c, d). This phenomenon may be attributed to the heightened sensitivity of eukaryotic communities to environmental stressors, including nutrients and micropollutants present in wastewater (Carles et al., 2021; Esser et al., 2023). Consequently, the eukaryotic diversity in these aquatic settings was lower compared to that in ecologically restored lakes. It implied that the loss of biodiversity may lead to adverse consequences for ecosystem functions and biogeochemical cycles in aquatic ecosystems.

In our study, we observed that differences in eukaryotic communities surpassed those in prokaryotic communities ($R_{\text{prokaryotes}}=0.7901 < R_{\text{eukaryotes}}=0.9378$) across all aquatic environments. This finding implies that dissimilarity within prokaryotic communities may exhibit a comparatively slower rate of increase in response to environmental changes than dissimilarity within eukaryotic communities, which is consistent with previous research (Xia et al., 2020; Liu et al., 2015). The results suggest that eukaryotes may display a stronger sensitivity to environmental changes. Studies have found that the bacterial communities showed higher dispersal ability than phytoplankton, and the similarity of the planktonic bacterial community was higher than that of the micro-eukaryotic community (Soininen et al., 2011). This is due to that smaller species may have greater niche plasticity than larger species, and small microorganisms were easier to disappear than larger microorganisms during colonization and extinction (Wang et al., 2015).

Prokaryotic and eukaryotic community composition in periphyton differed by habitats

Variations in the taxonomic composition and spatial distribution of periphyton communities can be

observed in diverse river systems (Esser et al., 2023; Guo et al., 2021). However, the community compositions of periphyton, encompassing both prokaryotes and eukaryotes, in different aquatic environments have not been extensively investigated. In this study, the predominant prokaryotic phyla, including Proteobacteria, Bacteroidota, Cyanobacteria, and Verrucomicrobiota, were consistent with results reported in previous studies (Wang et al., 2019; Wang et al., 2022; Guo et al., 2021). These phyla and their fundamental elements metabolism (carbon, nitrogen, phosphorus, and sulfur) were the main drivers of the material cycle in aquatic environments (Yu et al., 2022). Proteobacteria was commonly found in water and sediments, and it played a key role in the decomposition of organic matter; this result is consistent with numerous studies (Esser et al., 2023; Wang et al., 2019; Yu et al., 2022). In the anthropogenically polluted aquatic environments (A, WN, and MH areas), the relative abundance of Proteobacteria was notably higher compared to the unpolluted ecological restored lakes (U area). This suggests that wastewater influx may serve as a nutrient source (Fig. 2, nitrate and nitrite) facilitating the proliferation of Proteobacteria, particularly due to the presence of certain taxa within Proteobacteria known for their efficient nitrite-oxidizing capabilities (Han et al., 2018). The study reported that Bacteroidetes came from different animal intestines (fish, earthworms) and entered the water through excretion, the gene of surface adhesion protein made it more likely to attach to the substrates (Fernandez-Gomez et al., 2013); they also have been recognized as valuable alternative fecal biomarkers (Wery et al., 2008). Furthermore, certain species within the Bacteroidota phylum have been found to contain genes associated with ammonium assimilation, suggesting their potential role in regulating total ammonia levels (Kim et al., 2017).

At the prokaryotic genus level, the relative abundance of *Flavobacterium* in the MH area (36.47%) was approximately three times greater than that observed in the U area (12.09%) and A area (11.79%). *Flavobacterium* was identified as the predominant heterotroph related to biogeochemical processes and carbon cycling; it was also widely found in freshwater environments and has been isolated from environmental samples such as the biofilms of diseased fishes and domestic drain conduits (Esser et al., 2023; Carles et al., 2022; Basson et al., 2008). Organic

compounds from wastewater in the WN and MH area may explain the higher abundance of *Flavobacterium*, because *Flavobacterium* possessed the ability to form biofilms or flocs, and utilized a wide range of organic compounds as carbon and energy sources (Jo et al., 2016). *Luteolibacter*, a heterotrophic bacterium, was found to be present in both the A and WN areas. *Luteolibacter* has been isolated from various sources including red algae, leeches, driftwood, seawater, tundra soil, forest soil, and sludge samples (Dahal et al., 2021), and it can utilize monosaccharides and complex proteins and promote carbon cycling (Pascual et al., 2017). We note that *Luteolibacter* exhibited a higher relative abundance in periphyton, which offered insights for separating *Luteolibacter* from natural water columns to further understand its ecological role in aquatic environments. *Acinetobacter*, which had a relatively high abundance in the outfall areas of wastewater treatment plants (WN and MH), was a core genus and indicator species of the wastewater bacterial community. It was considered to be one of the dominant bacteria of the biofilms in wastewater treatment systems due to its strong biofilm-forming ability (Kim et al., 2013).

Eukaryotes exhibited notable community compositional variations across different ecological environments (Fig. 5b, d). The relative abundance of Chlorophyta was notably higher in both the U and A areas. Chlorophyta has been linked to eutrophication (Nozaki et al., 2003) and was recognized as a significant autotrophic organism (Esser et al., 2023). Despite the significant differences in nitrogen and phosphorus (Fig. 2), the relative abundance of Chlorophyta was similar in the two aquatic habitats. This suggests that nutrients were not the main factors affecting Chlorophyta distribution. It was reported that the concentrations of Ca^{2+} and HCO_3^- can affect Chlorophyta; Chlorophyta was found to play a key role in promoting HCO_3^- for photosynthetic fixation (Yan et al., 2020). Annelida (a member of Metazoa) was found to be dominant in A area, some agricultural practices (e.g., the use of organic feed) also were thought to promote eukaryotic diversity (Lentendu et al., 2014), and Annelida has been considered an indicator species in some agricultural environments (Xie et al., 2017; Carles et al., 2021). A higher abundance of Bacillariophyta (diatoms) in the MH area in comparison to the WM area, they were also found the downstream of wastewater treatment plants

(Tamminen et al., 2022). Diatoms survived through photosynthesis and promoted ecosystem functions by exuding carbohydrates and amino acids (Battin et al., 2016). Furthermore, diatoms were sensitive indicators to pollutants and were usually used for ecosystem biomonitoring as well as ecotoxicological studies (Keck et al., 2016). Exposure of periphyton to wastewater led to heightened tolerance of phototrophs (e.g., diatoms and green algae) to micropollutants (Carles et al., 2021); this increased tolerance may have potential implications for essential ecosystem functions. In contrast to prokaryotes, eukaryotes community exhibit a greater taxa divergence, including green algae and diatoms, which may demonstrate distinct response patterns to environmental changes. These distinctive community structures in periphyton may provide a foundation for further research on the biological functions (biogeochemical cycling and ecological indicators) of microorganisms within aquatic systems.

Significant variations were observed in the composition of eukaryotic genera, with some unique eukaryotic genera identified across the four aquatic habitats. *Unclassified_Tobriliidae* (belonging to Nematoda) in the U area were the dominant taxa; this is consistent with the result that *Tobriliidae* were predominant taxa in alkaline lakes (Gattoni et al., 2022). Following the restoration of the aquatic habitats, there was a gradual increase in both the abundance and diversity of the microbial community. The enrichment of soft-bodied invertebrates and diatoms may provide a food source for *Tobriliidae*, making it a dominant taxa in the lake ecosystems (Traunspurger, 2000). The predominant genus observed in the A area were *Chaetogaster*. *Chaetogaster* were common commensals of gastropods and bivalves in aquaculture systems and they generally did not affect the aquatic ecosystems (Madsen & Jr, 2024). The dominance of *Nais* in the WN area suggested that the nutrient-rich substrates from the effluents may foster favorable conditions for their growth (Juget et al., 1989). Multiple genera (*Surirella*, *Gomphonema*, and *Navicula*) dominated the outfalls area of the industrial wastewater treatment plant (MH area). Indicator species analysis showed that *Surirella* was associated with poor water quality in the highly impaired sites (Minerovic et al., 2020). Previous studies also uncovered positive correlations between *Surirella* and the accumulation of heavy metals (copper) (Djaoudi et al., 2022; Duong et al., 2008). *Gomphonema* can secrete

lipids and display deformities in water bodies contaminated with heavy metals, making it a recognized indicator species for metal pollution (Gautam et al., 2017). Wastewater from industrial wastewater treatment plants may promote the colonization of these dominant genera and cause the accumulation of heavy metals; the utilization of these advantageous eukaryotic taxa to indicate metal pollution holds great significance. These findings emphasize that habitat differences probably shaped the community composition of periphyton, resulting in the certain species proliferation of particular microbial taxa.

The association networks analysis unveiled complex microbial interrelationships between prokaryotes and eukaryotes. The multitude of positive correlations suggests that bacteria and algae can coexist in specific environmental conditions, implying potential functional interdependencies between these two biotic groups (Koedooder et al., 2019). Our results exhibited a notable correlation between Bacillariophyta and Bacteroidota in periphyton. The positive relationships observed may be attributed to the synergistic effect of algae and bacteria in aquatic ecosystems (Liu et al., 2014). The release of dissolved organic matter by algae has been widely proposed as a significant carbon source that can sustain bacterial survival (Xia et al., 2020; Liu et al., 2014). Given that most algae (diatoms) cannot directly synthesize nitrogen and rely on nitrate or ammonium delivered by nitrogen-fixing bacteria (Foster et al., 2011), algal growth thus was likely to benefit from bacteria that supply and deliver nitrogen and phosphorus substances.

Water physiochemical factors can affect community compositions of periphyton

Significant changes in the periphyton community were found in response to environmental variables. The present study revealed significant impacts of pH and nutrients on both prokaryotic and eukaryotic microorganisms (Fig. 7, Table S4). Previous studies have shown that abiotic factors, such as pH and nutrients, can influence the distributions of microbial communities (Guo et al., 2021; Li et al., 2022). In prokaryotes, dominant taxa (Proteobacteria and Bacteroidota) were found to be positively correlated with pH. This relationship may be attributed to the pH range of 7 to 9, which likely created an optimal environment conducive to the growth and functional

maturation of microorganisms (Lindström et al., 2005). Moreover, the respiration of algae (green algae, diatoms) likely improved the pH of water and provided a weak alkaline environment for the growth of bacteria. As reported in another study (Sanchez-Andrea et al., 2011), Bacteroidota exhibited habitat preference in relatively neutral aquatic environments. In eukaryotes, the RDA analysis revealed that nitrogen content (NO_3^- -N, NO_2^- -N, and TN) significantly influenced the variations of the eukaryotic community (Table S4), indicating that nitrogen played a pivotal role in shaping the eukaryotes (Hayden & Beman, 2016). Nitrogen was the preferred nitrogen source of some diatoms and green algae; the higher nitrosynuclease activity and transporters of eukaryotes promoted their utilization of nitrogen sources (Glibert et al., 2016). The earlier study also indicated that the proliferation of eukaryotic microorganisms, especially algae, is heavily dependent on the presence of phosphorus (Liu et al., 2014), a finding that aligns with our results.

Conclusions

This study investigated the community characteristics of prokaryotes and eukaryotes within periphyton across unpolluted ecologically restored lakes, aquaculture ponds, domestic wastewater treatment plant outfall area, and industrial wastewater treatment plant outfall area. The results revealed significant variations in biomass, diversity, abundance, and composition of periphyton communities among these habitats. Eukaryotic diversity and community composition responded more strongly to habitat differences compared with prokaryotes, indicating a potentially slower adaptation of prokaryotes to environmental changes. The variations in the community structure of prokaryotes and eukaryotes can be explained by pH and nitrogen content (NO_2^- -N, NO_3^- -N, and TN), respectively. Furthermore, the association networks revealed intensive interspecific correlations between prokaryotic and eukaryotic phyla. However, more environmental factors and long-term sampling strategies should be considered to comprehensively understand the community structures of periphyton. Considering the importance of periphyton in different aquatic habitats, our findings may offer valuable information on microbial distribution patterns and

have practical implications for ecosystem management strategies.

Author contributions Songnan Yang: Methodology, Data curation, Software, Formal analysis, and Writing-original draft; Huiping Zhou: Writing-review & editing, Conceptualization, Funding acquisition, and Supervision; Zhongzheng Pang: Data curation, Investigation, and Formal analysis; Yiqun Wang: Data curation, Validation, and Formal analysis; Jianying Chao: Resources, Project administration and Supervision; All authors reviewed and approved the manuscript.

Funding This research work was financially supported by the Innovation team project of Nanjing Institute of Environmental Sciences, Ministry of Ecology and Environment (ZX2023QT017).

Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate The authors declare that the manuscript is original and has not been published in any journal. All authors have read, understood, and have complied as applicable with the statement on “Ethical responsibilities of Authors” as found in the Instructions for Authors.

Consent to publish Not applicable.

Competing interests The authors declare no competing interests.

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