

# Temporal Evolution of Bacterial Community Structure, Co-Occurrence Patterns, and Community Assembly Process in Epiphytic Biofilms of Submerged Plants in a Plateau Lake

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## Research Article

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# Abstract

In shallow macrophytic lakes, epiphytic biofilms are formed on the surface of submerged plant stems and leaves because of the accumulation of algae and bacteria. Epiphytic biofilms have a significant impact on the health of the host vegetation and the biogeochemical cycling of lake elements. However, there is a lack of understanding of the community diversity, species interactions, and community assembly mechanisms in epiphytic bacterial communities (EBC) of plants during different growth periods. We investigated the successional dynamics, co-occurrence patterns, and community building processes of epiphytic biofilm bacterial communities of typical submerged plants, *Najas marina* and *Potamogeton lucens* from July, 2020 to November, 2020. The results showed that EBC diversity and abundance varied significantly among seasons, community diversity and abundance increased gradually from July to November, and temperature was the best explanatory factor for predicting seasonal changes in EBC community structure. Co-occurrence network analysis revealed that the average degree and plot density of the network gradually increased from July to November, indicating that the complexity of the EBC network gradually increased. The bacterial community co-occurrence network is mainly limited by temperature, pH, and transparency. The phylogeny-based null model analysis showed that microbial community construction was dominated by deterministic processes in different periods, and their contribution tended to increase gradually. Additionally, we found that as the dominant role of deterministic processes gradually increased, the microbial co-occurrence links also increased and the potential interrelationships between species became stronger. Altogether, these findings provide insights into the seasonal variability of EBC assemblage and co-occurrence patterns in lacustrine ecosystems.

## 1. Introduction

About 40-80 % of the earth's cells exist in biofilms; these cells drive all biogeochemical processes and represent the primary means of survival for active bacterial and archaeal life (Flemming and Wuertz, 2019). Epiphytic biofilms are usually complex matrix-enclosed communities composed of a variety of microorganisms, such as bacteria, fungi, algae, protozoa, and epifauna, that play an important role in the biogeochemical cycles of aquatic systems (Liu et al., 2020); participate in the nutrient cycle; purify water; decompose, transform, and absorb pollutants (Sepehri and Sarrafzadeh, 2018; Zhou, 2018). According to the attached substrate, the water body epiphytic biofilm can be classified into different types such as attached rock biofilm, attached mud biofilm, and attached plant biofilm. In this study, epiphytic biofilm refers to the biofilm attached to the surface of the stem and leaves of submerged plants. In shallow macrophytic lakes, where submerged plants are widely distributed, epiphytic biofilms are widely present. Epiphytic biofilms have an important impact on the health of host plants and the biogeochemical cycling of lake elements. (He et al., 2020; Levi et al., 2015).

The biodiversity of microbial communities includes the number of taxa, their abundance, and the ecological links between different bacterial taxa. We now have a more comprehensive and thorough understanding of the relationship between microbial community structure and environmental physicochemical factors (Boulétreau et al., 2012; Hao et al., 2017). However, the understanding of the

biological interactions of the microbial community is limited owing to the limitations of research tools. Recently, the association network analysis methods have provided access to complex microbial communities and key populations, and have been widely used to decipher the symbiotic patterns of various groups in microbial communities and their complex interactions. (Barberan et al., 2012; Deng et al., 2012; Deng et al., 2016; Ju et al., 2014). Network analysis allows for the statistical identification of taxa (i.e., keystone species of bacteria) within the constructed network that has a considerable influence on microbial structure and function (Jiao et al., 2020).

The generation and maintenance of microbial diversity is often defined as a community assembly process. Understanding the ecological processes (community assembly process) underlying the microbial community assembly has become a central topic in microbial ecology. Deterministic processes and stochastic processes are the two main modes of microbial community construction (Zhou and Ning, 2017); most studies emphasize the importance of determining factors in community construction such as changes in the environmental conditions (Smith et al., 2015; Xue et al., 2016), habitat conditions of the host (Brune and Dietrich, 2015; Delgado-Baquerizo et al., 2017), heterogeneity of carbon and nutrient resources (Vályi et al., 2016), species characteristics and/or interspecies interactions (Fischbach and Segre, 2016; HilleRisLambers et al., 2012), and plant diversity (Lange et al., 2015; Shi et al., 2015). However, stochastic processes play a major role in the assembly of microbial communities in specific environments or at specific stages (Chen et al., 2019; He et al., 2020; Sun et al., 2021a). Therefore, elucidating the relative contributions of both these processes is important in understanding the microbial community assembly process. Multivariate analysis, neutral-theory-based process models, and null modeling analysis are the main methods used to infer ecological randomness (Zhou and Ning, 2017). Specifically, the null model analysis allows inference of the ecological processes that control changes in community composition in time or space by calculating the phylogenetic distance between OTUs in two different communities. Ecological process is quantified using the  $\beta$ -nearest interspecific affinity index ( $\beta$ -NTI). Null models generate statistically expected stochastic patterns through random permutations of ecological data, it is different from process-oriented mechanism models (e.g., neutral models), and they are widely used in ecological research (Gotelli, 2010). Null model analysis providing a viable method for evaluating deterministic and stochastic processes.

The study of microbial community co-occurrence patterns and community assembly mechanisms is important for understanding biological ecology (Ren et al., 2019). Submerged plants serve as a special medium for biofilm attachment in water bodies, and plant growth and development stages have a significant impact on the biofilm community (Xia et al., 2020), which may affect the growth of bacteria (Buchan et al., 2014). However, the relative contributions of deterministic and stochastic processes in the assembly of epiphytic biofilm bacterial communities are still poorly understood, and the co-occurrence patterns of EBC networks are not yet clear.

In this study, we specifically raised the following questions: (1) Does the EBC structure differ among months? (2) How do EBC co-occurrence patterns differ over time? What are keystone species? (3) What ecological processes dominate the community construction in this process? Consequently, we analyzed

the structure and diversity of epiphytic bacteria by high-throughput sequencing of 16S rRNA genes in a typical large plateau lake with different submerged plant epiphytic biofilms. The co-occurrence patterns and ecological processes of EBC were analyzed by phylogenetic molecular ecological network analysis based on statistical methods of the null model. The study aimed to reveal the evolutionary characteristics of the community structure of EBCs over time, the patterns of interactions, and to elucidate the relative importance of deterministic and stochastic factors in EBCs. Overall, this work is expected to improve our understanding of the mechanisms underlying the construction of biofilm EBC communities.

## 2. Materials And Methods

### 2.1 Study area and Sampling

The Caohai National Nature Reserve (104°12'-104°18' E, 26°49'-26°53'N), located in Guizhou Province, southwest China, is a typical clear water grass-type lake, which is one of the main wintering sites of *Grus nigricollis*, an endemic plateau bird in China. Caohai has a subtropical plateau monsoon climate with an annual average temperature of 10.9 °C; the hottest month (July) has an average temperature of 17.3 °C, whereas the coldest month (January) has an average temperature of 2.1 °C. Caohai is characterized by a distinct dry and wet season; the normal water area is 22.39 km<sup>2</sup>, and the maximum water depth is about 5 m. The main aquatic vegetation in this lake includes the emergent aquatic plants *Scirpus validus Vahl* and *Phragmites communis* and the submerged plants *Najas marina*, *Potamogeton lucens*, and *Ceratophyllum demersum L.* For five months, from July to November 2020, we collected water and a total of 59 biofilm samples from six sites in the Caohai Lake, and the sampling targets were divided into two plant species, *Najas marina* (29 samples) named N, and *Potamogeton lucens* (30 samples) named P. The six sampling sites were located in two different areas with different physicochemical conditions, upstream of the lake (L1, L2, and L3) and downstream of the lake (L4, L5, and L6). Submerged plants at a depth of 0.5 m were collected with fish hooks and washed with in situ water. Approximately 10 g fresh weight leaf samples were cut from three to five replicate plants and transferred to sterile 500 mL polyethylene bottles containing 400 mL of 50 mM phosphate buffer (PBS, pH = 7.4) for EBC analysis (Xia et al., 2020; Yan et al., 2019). All samples were preserved in incubators containing ice packs and promptly returned to the laboratory. Furthermore, 1.5 L water samples (n=30) were collected from the macrophyte sampling area for physicochemical analysis. Dissolved oxygen and pH were measured using a portable instrument (HQ30D, Hach, Loveland, CO, USA). The physicochemical properties of the overlying water were measured according to the Chinese standard method for surface water (GB3838-2002). Water transparency (SD) was measured using a Secchi Disc. Total nitrogen (TN) was measured by alkaline persulfate digestion and ultraviolet spectrophotometry (UV mini-1240, Shimadzu, Japan). The total phosphorus (TP) content was determined spectrophotometrically. Ammonia nitrogen (NH<sub>4</sub><sup>+</sup>-N) was determined spectrophotometrically using a nano reagent, chemical oxygen demand (COD) was determined using the potassium permanganate index method, and chlorophyll *a* (Chl *a*) was determined spectrophotometrically after extraction with 90 % ethanol (Yan et al., 2019).

## 2.2 DNA extraction, PCR amplification, and high-throughput sequencing

The epiphytic bacteria were isolated as follows: sonicating the leaf samples in polyethylene bottles for 3 min (KQ5200DE, Kunshan), shaking for 30 min (225r/min, SHZ-82A, Changzhou), followed by sonication for another 3 min. After complete isolation, 100 mL of the mixture was filtered through a 0.22- $\mu$ m membrane filter (Millipore Ireland Ltd., Ireland) to collect the epiphytic bacteria. The membranes were stored at -20°C and used for bacterial DNA extraction. Bacterial DNA was extracted using a Fast DNA® Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. DNA concentration and purity were assessed using a NanoDrop2000 (Thermo Fisher Scientific, Waltham, MA, USA), and DNA quality was assessed through electrophoresis on a 1 % agarose gel. The V3–V4 regions of the bacterial 16S rRNA gene were amplified using primers 338F (5-ACTCCTACGGGAGGCAGCAG-3) and 806R (5-GGACTACHVGGGTWTCTAAT-3). Using 20  $\mu$ L of purified DNA as a template, PCR was performed using the ABI Gene Amp 9700 (Thermo Fisher Scientific, Waltham, MA, USA). In the PCR cycler, the DNA was first pre-denatured at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s. After the denaturation had been completed, annealing was performed at 55°C for 30 s, followed by extension at 72°C for 45 s, 72°C for 10 min, and then at 10°C until halted by the user. The PCR and Illumina sequencing methods were conducted according to the protocol described in our previous report (Yan et al., 2019). Raw sequences were quality-filtered using Trimmomatic and merged using Flash (<https://ccb.jhu.edu/software/FLASH/index.shtml>) version 1.2.11. The 16SrRNA gene sequences were analyzed using the RDP Classifier algorithm (<https://sourceforge.net/projects/rdp-classifier/>) against the Silva (SSU128) 16S rRNA database with a confidence threshold of 70 % (Yan et al., 2019).

## 2.3 Alpha and beta diversity analyses

A sampling diagram was drawn using ArcGIS (version10.6). Data were visualized using R software (version 4.0.4; <https://www.r-project.org/>). The physicochemical properties and bacterial diversity index of the water column were visualized by boxplots, which were drawn using 'ggplot2' package. The Mothur V. 1.30.1 software package and QIIME approach were used to analyze the alpha diversity, and the Chao1 index and Shannon diversity index were used to evaluate the community richness and diversity. The Wilcoxon rank-sum test was used to test the differences in alpha diversity among samples at different time points. Qiime was used to calculate the beta diversity distance matrix, and R 'vegan' package was used for NMDS analysis. ANOSIM analysis was used to determine whether the differences between groups (two or more groups) were significantly greater than the differences within groups, to determine whether the grouping was meaningful. Additionally, redundancy discriminant analysis (RDA) was performed using 'vegan' package.

## 2.4 Co-occurrence network analysis

We divided 59 samples into 5 groups (A, B, C, D, and E) according to month and constructed a network of each month. Network topology properties such as modularity, average degree, average path length, average clustering coefficient, and graph density were calculated using the "igraph" package in R (V4.0.4).

Network analysis was performed by calculating pairwise Spearman rank correlations based on the relative abundance of genera (Peng et al., 2014). These constructed networks were visualized using Gephi (v0.9.2) (Xu et al., 2019) and Cytoscape (v3.8.2). Nodes in the network represent OTUs, and the edges connecting two nodes represent positive or negative correlations between them. Among these network topology parameters, graph density and averageness represent network complexity (Shi et al., 2016). In co-existing networks, network nodes (OTUs) are divided into four groups. Peripherals mainly represent specialists in the microbial network, while module hubs and connectors mainly represent species close to generalists. Network hubs mainly represent super-generalists in microbial networks (Olesen et al., 2007).

## 2.5 Estimating community assembly processes with the null model analysis

According to the method of (Brislaw et al., 2019; Stegen et al., 2012), we used MEGA 7 to calculate the interspecific phylogenetic distance using the maximum likelihood method. Then, R 'Picante' package and the function "comdistnt" were used to quantify the phylogenetic replacement degree of each pair of communities as abundance-weighted  $\beta$ -mean-nearest taxon distance ( $\beta\text{MNTD}_{\text{obs}}$ ). The value of  $\beta\text{NTI}$  for pairwise comparison between samples characterizes the magnitude of the deviation from the zero distribution of  $\beta\text{MNTD}_{\text{obs}}$  from the randomly generated phylogenetic flip ( $\beta\text{MNTD}_{\text{null}}$ ). The greater the degree of deviation ( $|\beta\text{NTI}| \geq 2$ ), the more is the influence of the deterministic factors on the community, and the smaller the degree of deviation ( $|\beta\text{NTI}| < 2$ ), the more is the influence of stochastic factors on the community (Edwards et al., 2018; Shi et al., 2018).  $\beta\text{NTI} > 2$  was identified as heterogeneous selection and  $\beta\text{NTI} < -2$  was interpreted as homogeneous selection. When ( $|\beta\text{NTI}| < 2$ ), Raup-Crick ( $\text{RC}_{\text{bray}}$ ) is estimated in the second step,  $\text{RC}_{\text{bray}} > +0.95$  indicates diffusion limitation,  $\text{RC}_{\text{bray}} < -0.95$  indicates homogenized diffusion, and  $-0.95 < \text{RC}_{\text{bray}} < +0.95$  indicates undominated processes (Stegen et al., 2015) (Table 1).

## 3. Results

### 3.1 Water column physicochemical characteristics

The eight physicochemical factors of water from July to November are summarized in Fig. S1. There were significant differences among the months with respect to most physical and chemical factors, except for Chl *a* and SD (Wilcox test,  $P < 0.05$ ). From July ( $18.1 \pm 0.3$  °C) to November ( $9.5 \pm 1.2$  °C), water temperature decreased significantly ( $P < 0.001$ ).  $\text{NH}_4^+\text{-N}$ , TP, and PH in July were significantly higher than those in November (Fig. S2).

### 3.2 Diversity of bacterial community structure of epiphytic biofilm

A total of 2,764,102 high-quality filter sequences and 1169454002(bp) effective base numbers were obtained by Illumina sequencing, with a sequence average length of 423. The total OTU richness of all samples was 3996, and the similarity was 97 %. All the samples were screened and classified at the phylum level. Samples whose abundance ratio was less than 0.01 were classified as others.

Regarding the EBC, the top phyla/classes in P (*Potamogeton lucens*) were Proteobacteria (53.42 % on sum), Firmicutes (26.46 %), Cyanobacteria (8.59 %), Bacteroidetes (5.17 %), and Actinobacteria (3.47 %), whereas Proteobacteria (54.92 %), Firmicutes (21.28 %), Cyanobacteria (9.03 %), Bacteroidetes (6.74 %), and Actinobacteria (4.23 %) were found in N (*Najas marina*) (Fig. 1). Significant differences in community richness (Chao1 index) and community diversity (i.e., phylogenetic diversity and Shannon diversity) (Wilcoxon test,  $P < 0.05$ ) were observed for the different months (Fig. 2). Community richness and diversity in November were significantly higher than that in July ( $P < 0.05$ ). Nonmetric multidimensional scaling (NMDS) is based on Bray-Curtis distance ranking and is used to characterize the overall change in the composition of the EBC from month to month (Fig. 2). EBC showed significant seasonal variation, as evidenced by the ANOSIM statistical test ( $R=0.2754$ ,  $P < 0.05$ ). The RDA results showed that 19.72 % of the monthly variance in bacterial composition was explained by the first two major components (RDA1 and RDA2) (Fig. 2). Among the physicochemical factors evaluated, WT, COD, and TP had the greatest influence on the seasonal changes in the EBC. Mantel test correlation analysis showed that temperature was significantly correlated with the community structure of epiphytic bacteria ( $P=0.001$ ,  $R=0.35146$ ).

### 3.3 Co-occurrence patterns of epiphytic bacterial community

The network diagram was constructed according to the EBC (Fig. 3A-E). All networks had co-occurrence characteristics because the proportion of positive connections was more than 70 %. Table 2 summarizes the network topology characteristics for the different months. The average degree and graph density of the five subnetworks gradually increased from July to November, indicating that the complexity of the EBC network gradually increased. The EBC network mainly contained Proteobacteria, Actinobacteria, Cyanobacteria, Chloroflexi, Firmicutes, Verrucomicrobiota, Desulfobacterota, Patescibacteria, and Bacteroidetes. Fig. S4 shows the identification of putative keystone types according to within-module connectivity ( $Z_i$ ) and among-module connectivity ( $P_i$ ) of OTUs in all months in the network. The number of peripherals, module hubs, connectors, and network hubs varied for the different months. Module hubs and connectors may be similar to the key species of microorganisms in the community, as predicted by network theory. EBC network keystone phylum mainly contained Proteobacteria, Chloroflexi, Firmicutes, Actinobacteria, Cyanobacteria, and Verrucomicrobiota. In August, only one module of the hubs (belonging to Proteobacteria) was identified. More key system types were found in October (11 connectors and six module hubs) than in November (three module hubs). Cyanobacteria is the phylum with the most key system types in October. One keystone phylum, Actinobacteria, was detected in both October and November. Table 3 summarizes these key groups in more detail. We incorporated environmental factors into the species networks to interpret the

environmental predictors of co-occurrence networks. The results showed that the co-occurrence network of the bacterial groups was mainly limited by water temperature, pH, and transparency (Fig. 4).

### 3.4 Ecological process

$\beta$ NTI was calculated based on a null model analysis to infer the ecological processes. The results showed that the overall EBC assembly was controlled by heterogeneous selection ( $\beta$ NTI > +2.57 %) and it was influenced by dispersal limitation ( $-2 < \beta$ NTI < 2) (22 %) and homogeneous selection ( $\beta$ NTI < -2) (21 %). In addition, undominated processes ( $-2 < \beta$ NTI < 2 &  $-0.95 < RC_{\text{bray}} < +0.95$ ) (21 %) also play a minor role in the process. The overall EBC to an assembly was driven by a deterministic assembly process ( $|\beta$ NTI|  $\geq$  2) (78 %). Furthermore, we observed from the relative proportions diagram of selection and dispersal that the relative importance of deterministic processes increases over time from August to November (Fig. 5B).

## 4. Discussion

Microbial community distribution patterns, environmental drivers, microbial community species interactions, and community building mechanisms are the focus of microbial ecology research. In this study, we analyzed the dynamics of EBC and their environmental impact factors from July to November, and revealed the structural changes, co-occurrence pattern characteristics, and community assembly processes of epiphytic biofilm bacterial communities.

The EBC diversity of the Caohai submerged plants in November was higher than that in July (Xia et al., 2020). The change in the bacterial community at the phylum level on the surface of macroalgae is driven by the season (Lachnit et al., 2011). In this study, the diversity of EBC showed an increasing trend from July to November when analyzing its characteristics. The differences in epiphyte community composition between growing seasons were mainly due to changes in host growth status and surrounding environmental factors (Xia et al., 2020). Diatom species diversity and composition exhibit large seasonal or spatial variation, which may be related to the life history, leaf morphology, and characteristics of the host plant (He et al., 2020). This difference is closely related to the development of epiphytic biofilms. Planktonic bacteria increase the diversity and abundance of EBC through adsorption, adhesion, and biofilm formation (Kimkes and Heinemann, 2020).

Biological interactions are considered the main drivers of microbial assembly processes (Ju and Zhang, 2015). Identifying microbial interactions with keystone species is essential for a better understanding of microbial community diversity and function (Ren et al., 2015). In freshwater ecosystems, the interactions between epiphytic bacterial taxa are largely unknown. Network analysis is an effective tool for describing microbial interactions at different taxonomic levels (Manoeli et al., 2014). Construction of correlation-based species–species co-occurrence networks that can reflect important details of ecological processes such as community cooperation, habitat filtering, and historical effects, as well as reflecting

mathematical associations between different bacterial populations (Zhao et al., 2016). From summer to winter, the planktonic bacterial co-occurrence network evolved from complex to simple, and the benthic bacterial community had strong seasonal stability (Jiao et al., 2020). Our study shows that the number of ecological connections (links) between EBC nodes and network connectivity and complexity gradually increased from July to November.

The differences in networks between the time periods may be because the bacterial community composition differs between seasons; bacterial diversity is higher in November, and higher biodiversity promotes interactions between bacterial communities (Sun et al., 2021a), thus increasing their co-occurrence patterns (Widder et al., 2014). Simultaneously, bacterial diversity and abundance increase, and bacteria utilize quorum sensing (Qs) to further develop a complex and well-structured community lifestyle by activating or repressing target genes (Haque et al., 2019). Intercellular communication promotes the development of biofilms and enhances interactions between bacterial communities (Guzmán-Soto et al., 2021). Finally, EBC forms a dense community with stronger interactions between bacterial members (e.g., metabolic linkage and horizontal gene transfer) (Broszat and Grohmann, 2014). The dynamics of EBC are influenced by the host plant, the period of plant growth (Li et al., 2021), differentiated morphology or metabolic profile (Hempel et al., 2009), plant leaf size and certain traits of leaves (e.g., enzyme activity and secondary metabolites) (Buchan et al., 2014), and plant characteristics (He et al., 2020). Host plants influence the bacterial community and thus, the co-occurrence pattern of the network. Furthermore, temperature is often considered to be one of the key factors influencing seasonal variation in bacterial symbiosis patterns (Ren et al., 2019). Water temperature simultaneously influences various other environmental factors (e.g., DO, Chl *a*, and metabolic rate), thus exerting a strong control on the biodiversity patterns in aquatic ecosystems (Wang et al., 2019).

Increased complexity can also lead to increased ecological community stability (Herren and McMahon, 2018; Mougi and Kondoh, 2012). The community structure may be more stable in November than in other months. We also determined the topological role of each OTU in a microbial network composed of all biofilm samples using network analysis. Prediction of Keystone OTUs in the EBC network is based on network scores ( $P_i$  and  $Z_i$ ), and these keystone species play an essential role in maintaining the complexity and stability of the EBC network (Banerjee et al., 2018; Herren and McMahon, 2018). More keystone species can lead to more stable and organized microbial networks (Banerjee et al., 2018; Qi et al., 2019). The influence of key taxa on community structure in co-occurrence networks is not governed by their relative abundance, but rather by their strong ecological associations (Banerjee et al., 2018; Shi et al., 2016). October and November had the most complex co-occurrence pattern and had more keystone species compared to other months, indicating a gradual stabilization of the EBC structure. The change from relatively scattered bacterial communities to the formation of mature and stable bacterial communities from July to November may be a developmental process of biofilms from formation to maturity.

Ecological niche theory and neutrality theory are used to explain the process of microbial community construction (Bahram et al., 2016; Sloan et al., 2006). The ecological niche theory (deterministic process)

emphasizes the importance of determinism and proposes that ecological selection is a unique ecological process that affects species homogeneity and abundance (Chave, 2004). Neutral theory (stochastic processes) emphasizes that neutral, often stochastic processes can largely regulate the assembly and dynamics of community structure (Vanwonterghem et al., 2014). Several studies suggest that deterministic processes play a major role in community construction (Chen et al., 2021; Gad et al., 2020; Jiao et al., 2020; Zhou et al., 2021), while others have reported the dominant role of stochastic processes (Chen et al., 2019; Sun et al., 2021b; Wang et al., 2020). In the present study, the EBC was dominated by deterministic processes. Compared to planktonic and benthic bacterial communities, epiphytic biofilms have many specific physicochemical properties, such as vegetative properties that can shape bacterial communities to exhibit "host specificity" (Hempel et al., 2009), which may lead to more complex assembly mechanisms.

After the formation of microcolonies of epiphytic bacteria, cells continue to proliferate and aggregate (Haagensen et al., 2015). The synthesis of extracellular polymers (EPS), which form a "closed microenvironment," can reduce the diffusion rate of bacteria (stochastic process) (Seymour et al., 2017). Simultaneously, complex interactions between algae and bacteria attached to the biofilm may increase biological selection (deterministic process) (Foster et al., 2011).

Our results show that the deterministic processes dominate the construction of the EBC, and the ratio of deterministic to stochastic processes varies among months, with the relative proportion of deterministic processes gradually increasing and the relative proportion of stochastic processes decreasing. Deterministic processes include selection imposed by environmental filtering and biological interactions, and stochastic processes include diffusion, random birth/death, and drift (Burns et al., 2016; Xue et al., 2018). From July to November, the complexity of the bacterial co-occurrence network increased, indicating that the interaction between species was enhanced (Wu et al., 2019), and that the deterministic process may be gradually enhanced, with the alpha diversity of bacteria also showing an increasing trend. The November EBC has higher biodiversity, higher network complexity, and more stable communities which are less susceptible to random processes (diffusion and drift). These results suggest that deterministic processes may play an important role in maintaining the diversity and function of epiphytic bacteria in aquatic ecosystems. Concurrently, the increase in temperature enhanced the metabolic dynamics and molecular irregular movement of the bacterial community, increasing the randomness of the colonization and extinction of bacterial community members (Ren et al., 2017). The bacterial community may be more affected by randomness in summer, which is consistent with our results. However, (He et al., 2020) showed that random processes played a dominant role in community construction and were significantly correlated with alpha diversity, and random processes may be beneficial for the maintenance of epiphytic bacterial diversity. Therefore, in different lakes, the process of biofilm bacterial community construction of submerged plants may not be consistent and may be related to lake nutrient status, temperature, and even host plants. Subsequently, it is necessary to carry out studies on biofilm bacterial communities in different lakes to clarify the construction mechanism and influencing factors of biofilm bacterial communities.

Co-occurrence networks allow for a more in-depth analysis of ecological processes of microbial community construction, such as neutral processes and species selection (Faust and Raes, 2012; Layeghifard et al., 2017). In this study, random and deterministic processes jointly regulated the dynamic changes in EBC. With the gradual strengthening of the dominant role of deterministic processes, the co-occurrence model had a larger network scale, and the relationship between species became closer. Deterministic processes may be beneficial in shaping the complex co-occurrence patterns of EBC.

## 5. Conclusion

The EBC of the Caohai submerged plants have different aggregation mechanisms during different periods. The EBC structure, co-occurrence pattern, and ecological processes of two submerged plants were explored in this study. (1) We found significant differences in the structure of submerged plant EBCs among months, with increasing diversity and abundance of bacterial communities. Temperature was the most important factor influencing the differences in microbial communities. (2) The connectivity and complexity of EBC networks differed among months, with it being significantly higher in October and November than in other months, and the complexity of co-occurrence patterns and bacterial community interactions gradually increased. Proteobacteria, Chloroflexi, Firmicutes, Actinobacteria, Cyanobacteria, and Verrucomicrobiota play key roles in the EBC network. (3) Deterministic and stochastic processes jointly influenced the aggregation of EBC; however, domination of deterministic processes overwhelmed stochastic processes, with the dominant role of deterministic processes gradually increasing. When the deterministic dominant role of EBC was stronger, the community symbiotic correlation tended to be higher. In the present study, only bacteria from July to November were analyzed, and co-occurrence networks were constructed. In the future, a broader microbial community (such as fungi, archaea, and viruses) can be considered to conduct research on the ecological process of biofilms on a longer time scale and under the influence of spatial factors.

## Declarations

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### Author Contributions Statement

**Lei Shi:** Software, Investigation, Data curation, Writing - original draft.

**Pinhua Xia:** Conceptualization, Methodology, Writing-original draft.

**Guoqing Li:** Investigation, Formal analysis, Data curation.

**Tianyou Wang:** Investigation, Formal analysis.

**Xin Du:** Investigation, Data curation.

**Tao Lin:** Resources, Writing - review & editing.

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## Tables

**Table 1** Criteria to Quantify the Importance of Ecological Processes Accounting (Stegen, Lin et al. 2015)

$\beta$ NTI	$RC_{\text{bray}}$	Processes
$< -2$		Homogeneous selection
$-2 < \beta\text{NTI} < 2$	$< -0.95$	Homogenizing dispersal
	$-0.95 < RC_{\text{bray}} < +0.95$	Undominated processes
	$> +0.95$	Dispersal limitation acting with drift
$> +2$		Heterogeneous selection

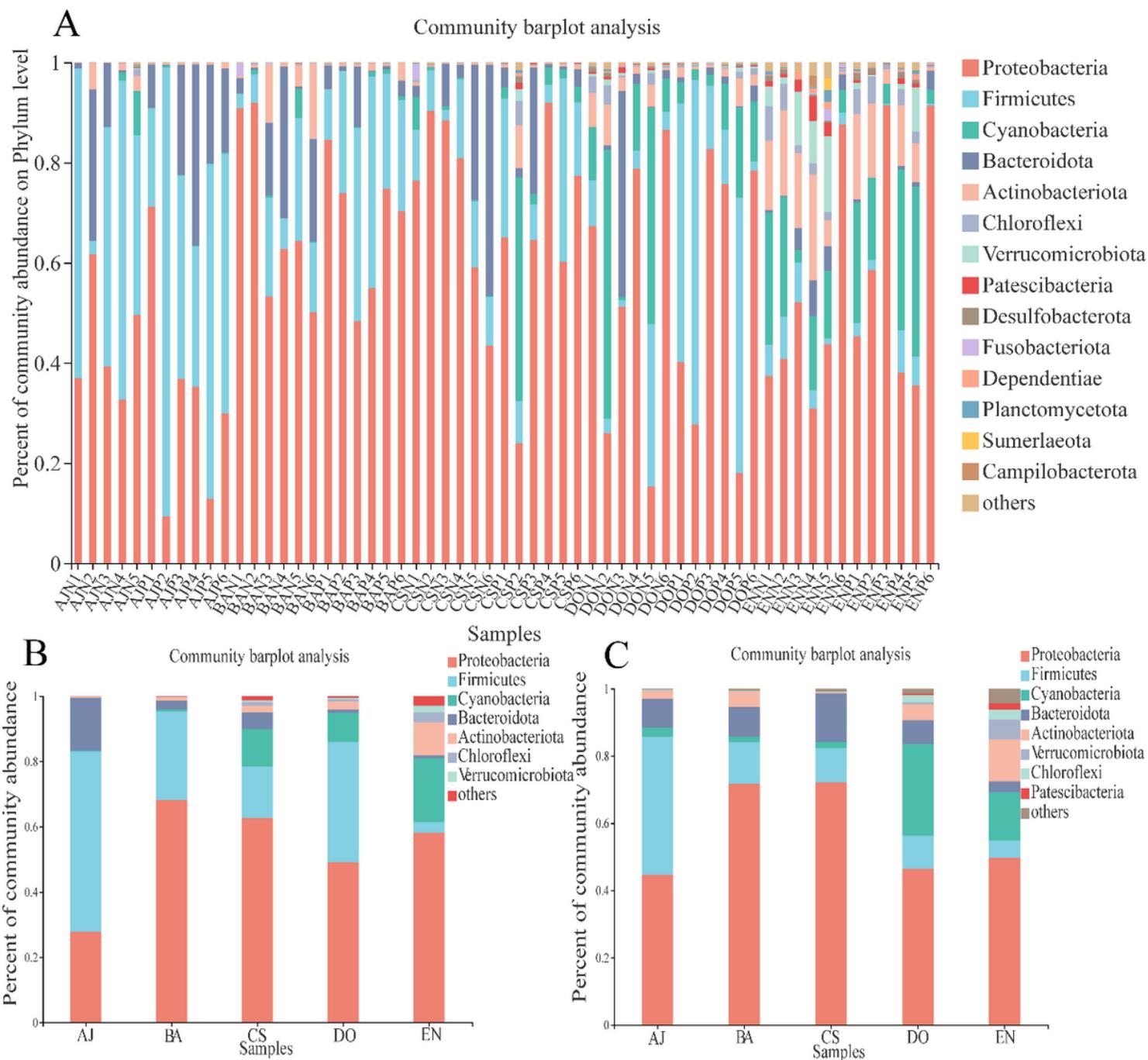
**Table 2** Topological parameters of the species-species networks of epiphytic bacterial communities.

mouth	notes	Edges (Positive / Negative)	Average degree	Graph density
A	49	42/38/4	1.714	0.036
B	51	65/55/10	2.549	0.051
C	126	209/209/0	3.317	0.027
D	254	3711/3632/79	29.22	0.115
E	396	5697/5527/170	28.77	0.073

**Table 3** EBC network hubs and connectors in different months

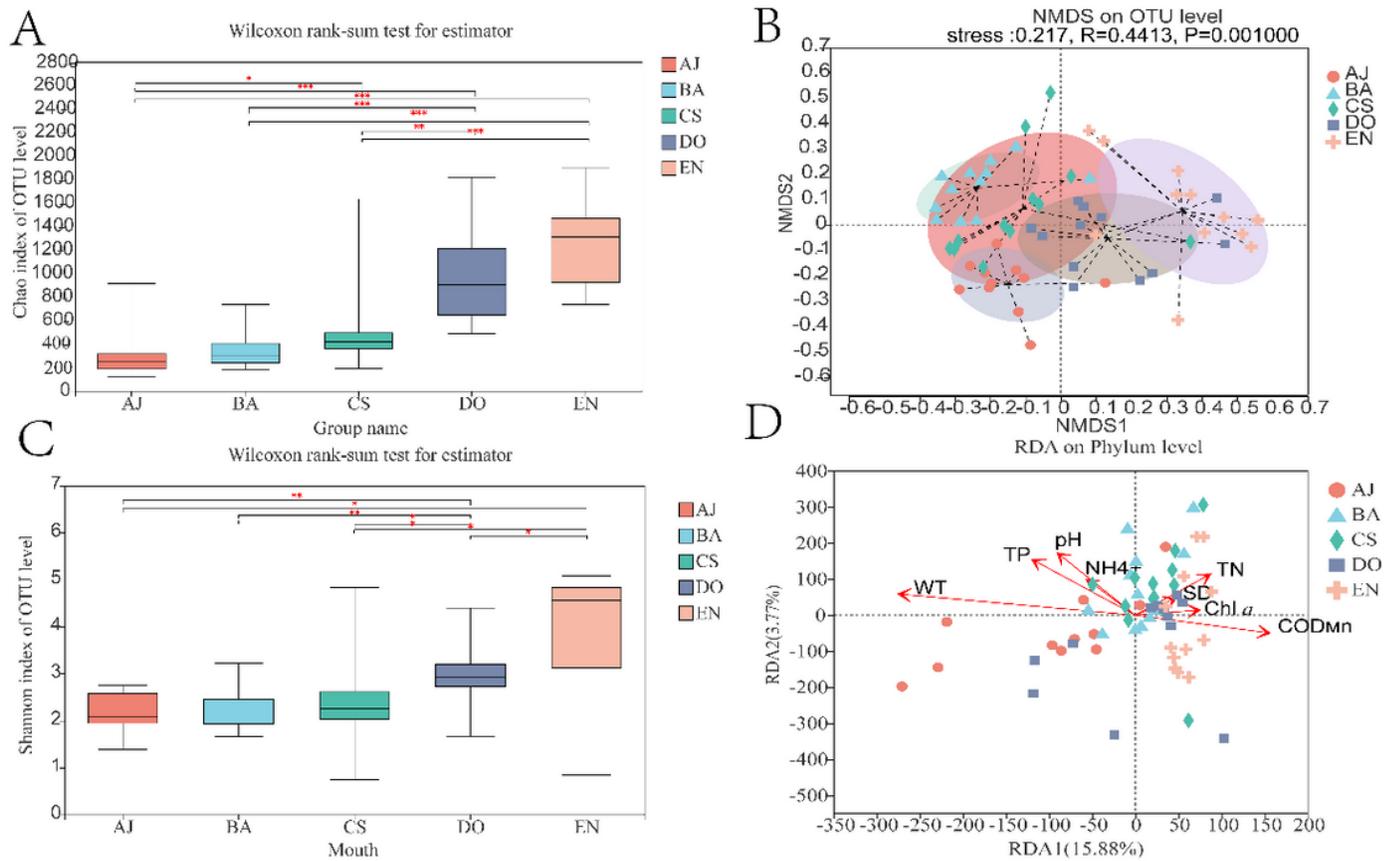
	group	z	p	taxa_roles	Phylum
OTU7328	B	3.04	0.38	Module hubs	Proteobacteria
OTU2421	D	0.74	0.63	Connectors	Chloroflexi
OTU4220	D	0.74	0.62	Connectors	Proteobacteria
OTU4255	D	0.71	0.63	Connectors	Actinobacteriota
OTU2320	D	0.51	0.64	Connectors	Cyanobacteria
OTU7752	D	0.47	0.63	Connectors	Proteobacteria
OTU7548	D	0.35	0.64	Connectors	Chloroflexi
OTU4123	D	0.02	0.66	Connectors	Actinobacteriota
OTU4622	D	-0.16	0.63	Connectors	Cyanobacteria
OTU4403	D	-0.25	0.63	Connectors	Cyanobacteria
OTU7698	D	-0.40	0.66	Connectors	Cyanobacteria
OTU630	D	-1.24	0.64	Connectors	Acidobacteriota
OTU7774	D	3.96	0.00	Module hubs	Proteobacteria
OTU7238	D	2.91	0.54	Module hubs	Actinobacteriota
OTU7739	D	2.81	0.00	Module hubs	Chloroflexi
OTU7708	D	2.59	0.55	Module hubs	Cyanobacteria
OTU7261	D	2.56	0.55	Module hubs	Cyanobacteria
OTU8074	D	2.52	0.61	Module hubs	Actinobacteriota
OTU6963	E	3.69	0.00	Module hubs	Actinobacteriota
OTU3171	E	2.79	0.06	Module hubs	Verrucomicrobiota
OTU2693	E	2.56	0.44	Module hubs	Verrucomicrobiota

## Figures



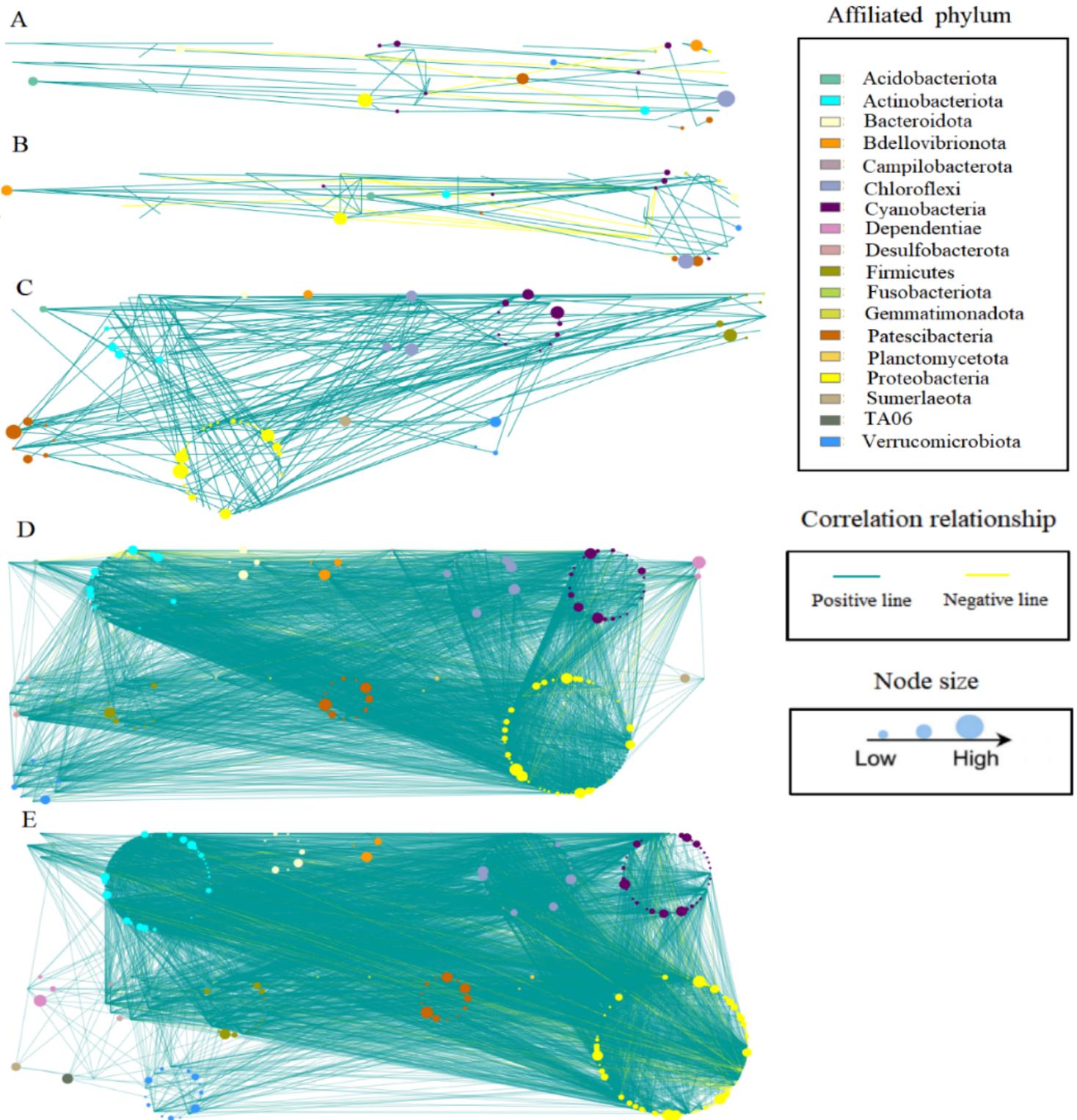
**Figure 1**

(A) percent of abundance for all samples (B) percent of abundance for *Potamogeton lucens* on phylum level (C) percent of abundance for *Najas marina* on phylum level.



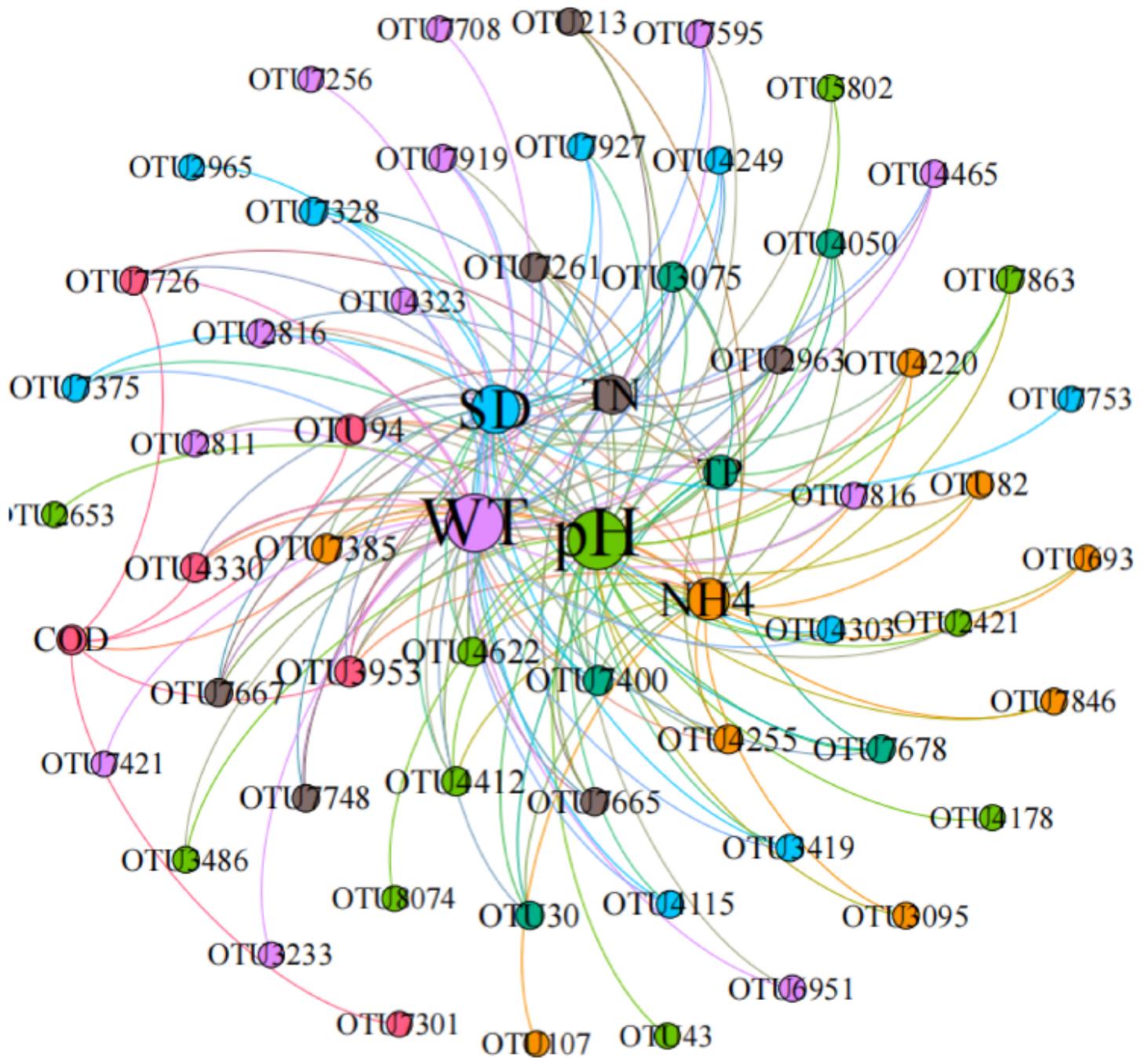
**Figure 2**

(A) Chao1 index in different months based on OTU level. (B) NMDS diagram of different months, different months are represented by different shapes and colors (C) Shannon index in different months based on OTU level (D) RDA analysis of bacterial community with environmental factors from July to November.



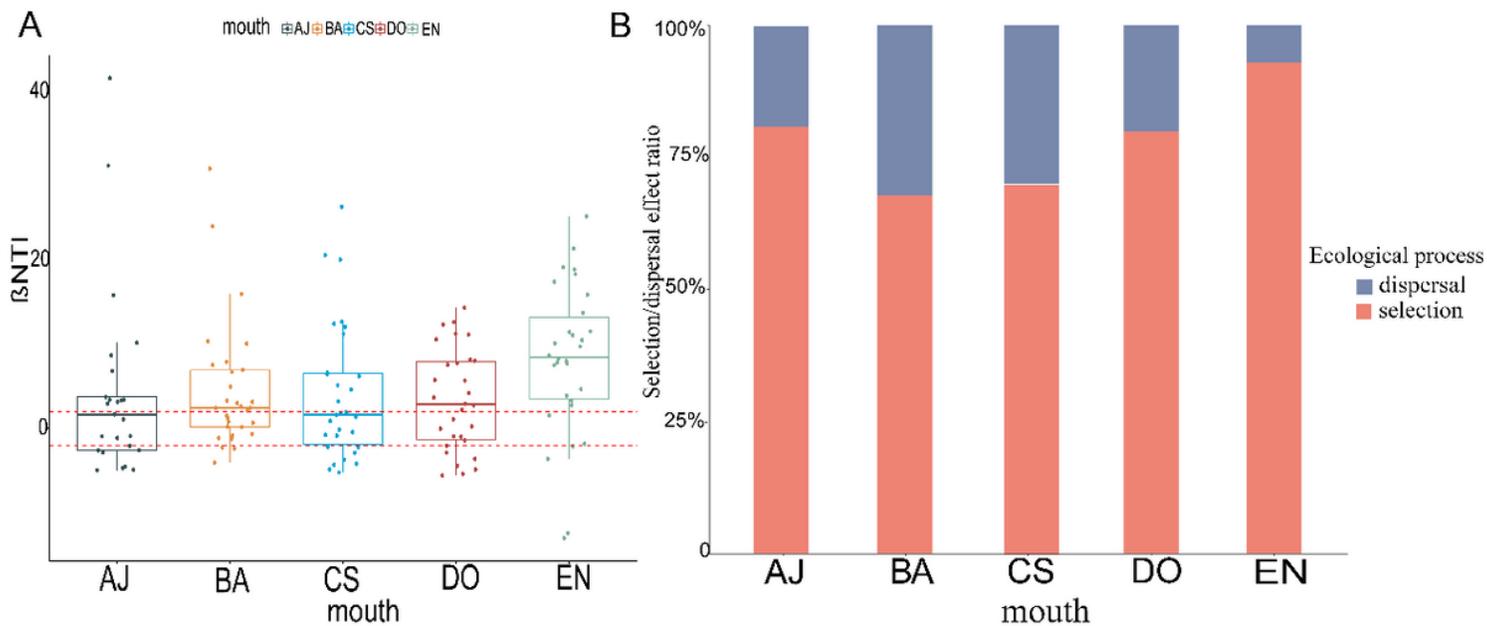
**Figure 3**

Bacterial species-species network structures in biofilm. (A) July (B) August (C) September (D) October (E) November. The nodes in the network represent OTUs, the color of the edge connecting two nodes indicates the positive or negative correlation between them.



**Figure 4**

Modular associations between bacterial OTUs and environmental factors in all samples. Different modules are shown in different colors. The size of each node (OTU or environmental factor) is proportional to the node degree (that is, the number of connections).



**Figure 5**

(A) Distribution of  $\beta$ -NTI values in different months, the horizontal dashed lines indicate the importance thresholds for up (+2) and down (-2) (B) Proportion of selection and dispersal in different months.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMaterial.docx](#)