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MICROBIOLOGY OF AQUATIC SYSTEMS

Microbial Succession and Nitrogen Cycling in Cultured Biofilms as Affected by the Inorganic Nitrogen Availability

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Abstract Biofilms play important roles in nutrients and energy cycling in aquatic ecosystems. We hypothesized that as eutrophication could change phytoplankton community and decrease phytoplankton diversity, ambient inorganic nitrogen level will affect the microbial community and diversity of biofilms and the roles of biofilms in nutrient cycling. Biofilms were cultured using a flow incubator either with replete inorganic nitrogen (N-rep) or without exogenous inorganic nitrogen supply (N-def). The results showed that the biomass and nitrogen and phosphorous accumulation of biofilms were limited by N deficiency; however, as expected, the N-def biofilms had significantly higher microbial diversity than that of N-rep biofilms. The microbial community of biofilms shifted in composition and abundance in response to ambient inorganic nitrogen level. For example, as compared between the N-def and the N-rep biofilms, the former consisted of more diazotrophs, while the latter consisted of more denitrifying bacteria. As a result of the shift of the functional microbial community, the N concentration of N-rep medium kept decreasing, while that of N-def medium showed an increasing trend in the late stage. This indicates that biofilms can serve as the source or the sink of nitrogen in aquatic ecosystems, and it depends on the inorganic nitrogen availability.

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Introduction

Freshwater biofilms are widely distributed in any submerged solid surface, mainly composed of a consortium of algae and bacteria [1, 2]. Biofilms play a crucial role in natural aquatic ecosystems through their influence on energy flow, nutrient recycling, and biogeochemical processes and patterns [3]. In addition, biofilms have high potential for biotechnological applications, for example, they were used for wastewater treatment in aquaculture, agriculture, constructed wetlands, and bioremediation [4–8], and for energy production [6], used as antifouling agents [9, 10] and used for gas biofiltration [11].

Nitrogen is an essential component of all living organisms and is crucial to the structures and biochemical processes of life [12, 13]. However, the majority of organisms cannot directly utilize N_2 in the atmosphere [14]. N_2 fixation is the only biological process that made N_2 accessible for use by organisms [15]. Nevertheless, N is also undersupplied in forms that can be absorbed by plants in both aquatic and terrestrial ecosystems [12]. Therefore, N fixation is particularly important.

As one of the most widespread microecosystems, biofilms are major sites of N cycling in freshwater habitats. Previous studies have shown that N plays an important role in determining primary production of ecosystems [12, 16]. N cycle of ecosystems is driven by complex biogeochemical transformations, including nitrogen fixation, nitrification, denitrification, ammonification, assimilation, and anammox [14, 17]. Microorganisms in biofilms are important contributors in regulating N cycle process in freshwater habitats. On one hand, microbial N fixation in biofilms seems to be an indispensable source of N for many

oligotrophic lakes [18]. The lack of N in ecosystem may be compensated by biological N_2 fixation. Adding N to the lake promotes primary production, but can also drive eutrophication [16]. It appears to be an environmental friendly method to obtain N through N fixation. On the other hand, in some eutrophic lakes, the autotrophs in biofilms can assimilate N in ambient water for their growth [19], and extracellular polymeric substances (EPS) in the matrix also enable absorption of N. Anammox and denitrification, mediated by microorganisms, were major biological recycling pathways of nitrogen to the atmosphere [13, 14]. Biofilms have various properties that may contribute to the uptake, storage, and transformation of nutrients [20]. In brief, biofilms play crucial roles in regulating N content of aquatic ecosystems.

Microbial community composition and succession are crucial determinants of ecosystem functions [21–23]. When subjected to a certain kind of extreme habitats, microbial community composition would change: the number of those weakly adaptable taxa may decline sharply, but those highly adaptable species would increase obviously so as to maintain the functional stability of community, and such compensatory increases not only stabilized total community biomass but also made the microbial community adapt to the changing environment [24]. N cycle of ecosystems is mainly mediated by microorganisms [14]. N availability plays important roles in determining microbial community composition or abundance; diazotrophic microorganisms are often the dominant taxa under low N level [25, 26]. It is necessary to investigate microbial community composition so as to better clarify the function of biofilms. High-throughput sequencing has a strong ability to illuminate the complicated microbial community composition of biofilms [27].

Biofilms play crucial roles in nutrient cycling of natural aquatic ecosystems. Therefore, we hypothesized that as eutrophication could change phytoplankton community and decrease phytoplankton diversity, ambient inorganic nitrogen level will affect the microbial community and diversity of biofilms and the roles of biofilms in nutrient cycling. Specifically, biofilms may serve as the source of N when it is deficient in the surrounding water, while biofilms may serve as the sink of N when it is replete in the surrounding water. This function is likely obtained by regulating microbial community composition of biofilms, i.e., diazotrophs prefer N deficient condition, while denitrifiers prefer N replete condition. The hypothesis was tested with experiments using field epilithic biofilm samples cultured under inorganic N deficient and replete conditions with a flow incubator.

Material and Methods

Biofilm Inocula Collection

Phototrophic biofilm inocula were sampled from a site which was near a sewage draining exit of Lake Nanhu in November 2014. Lake Nanhu (30° 30′ N, 114° 21′ E) is a seriously eutrophic freshwater lake in Wuhan City, China. The environmental variables at the site are pH 7.5–8.1, total phosphorus (TP) 0.2–0.6 mg/L, total nitrogen (TN) 8.7–13.0 mg/L, NH_4^+-N 4.1–4.5 mg/L, and NO_3^--N 2.3–2.6 mg/L. Submerged biofilms on flat stones in the littoral zone were collected and kept on ice until arrival in the laboratory within 2 h. Before being used as inocula, the samples were pretreated to eliminate grazers according to Guzzon et al. [28].

Biofilm Incubator and Experiment Design

The biofilm incubator was designed mainly according to Zippel et al. [29], and the detailed description was shown in Li et al. [30]. It consisted of four separate flow lanes, which were named as A, B, C, and D, respectively. The illumination system was made up of 16 strips of LED white light lamps. Ground glass slides were used as artificial substrata for biofilm growth.

Biofilm samples were used as inocula after being processed. A 200 mL of inoculum was added to 7.8-L medium and mixed in the aquarium. The inorganic nitrogen (N) concentration of the medium used in four flow lanes was different. The medium in flow lane A and flow lane C was modified BG11 without inorganic nitrogen (N-def) added, and the medium in flow lane B and flow lane D was modified BG11 with replete inorganic nitrogen ($NaNO_3$: 247.058 mg/L, N-rep) added. The modification of BG11 medium was according to Beakes et al. [31] and Guzzon et al. [4], including addition of vitamins (40 μg/L) and silicates (57 mg/L). The inoculated medium was then pumped through the incubator at 75 L/h and 25 °C. The ground glass slides substrata were subjected to a photoperiod of 16:8 light/dark with an intensity of 35 μmol photons/(sec•m^2) photosynthetically active radiation provided by LED white light lamps. This incubation phase continued for 5 days. Subsequently, the flow rate was reduced to 25 L/h; other conditions were the same as mentioned above. Samplings were conducted on days 5, 9, 13, 17, 21, 26, 31, 40, and 52 for all analyses, and the experiments were conducted for 52 days in total. During this period, an appropriate amount of distilled water was often added to the four aquariums to offset water loss due to evaporation, and the whole amount of medium always maintained at 8 L.

Biofilm Biomass and Nutrient Content Measurement

Biofilm samples were detached from the slides with a sterile scalpel blade to quantify the biomass and nutrient content on each sampling day. Phototrophic biomass production was estimated by chlorophyll *a* (Chl *a*) content. This was measured after extraction in 90 % acetone for 24 h in the dark at 4 °C and then quantified spectrophotometrically according to Arar [32]. Dry weight (DW) was analyzed by drying at 105 °C for 24 h.

TP was digested with potassium persulfate ($K_2S_2O_8$) and then determined using the phosphomolybdenum blue colorimetry method of Murphy and Riley [33]. TN was determined by digestion with $K_2S_2O_8$ and NaOH [34]. All analyses of biomass and nutrient content in each flow lane were performed in triplicates.

The thicknesses of biofilms were measured by a nondestructive method with vernier caliper: four random points were measured per slide on a total of four slides in each lane. The slides were then put back in the same position of the lane.

DNA Extractions and Illumina Miseq Sequencing

In each flow lane, biofilm samples with three replicates were collected using sterile scalpel blades and then thoroughly mixed into one sample (in one tube) for DNA extractions. Total DNA was extracted using a Power Biofilm™ DNA Isolation Kit (MOBIO, USA) according to the manufacturer's protocols. For Illumina MiSeq sequencing, theV4-V5 hypervariable region of the bacteria 16S rRNA gene was amplified by PCR with universal primers 515F and 909R [35]. The PCR mixture (50 mL) contained 1 × PCR buffer, 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at 0.4 mM, each primer at 1.0 mM and 1 U of Taq DNA polymerase (New England BioLabs, Ipswitch, MA), and 10-ng biofilm genomic DNA. The PCR amplification program included initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 40 s, 56 °C for 60 s, and 72 °C for 60 s, and a final extension at 72 °C for 10 min. Each sample was conducted two PCR reactions and combined together after PCR amplification. The band with a correct size was excised and purified using SanPrep DNA Gel Extraction Kit (Sangon Biotech, China, Cat# SK8132) and quantified with Nanodrop. All samples were pooled together with equal molar amount from each sample. The sequencing samples were prepared using TruSeq DNA kit according to manufacturer's instruction. The purified library was diluted, denatured, re-diluted, mixed with PhiX (equal to 30 % of final DNA amount) as described in the Illumina library preparation protocols, and then applied to an Illumina Miseq system for sequencing with the Reagent Kit v2 2 × 250 bp as described in the manufacturer's manual.

Statistical Analysis

In order to better clarify the results, the data were processed as follows: data gained from flow lane A and flow lane C were combined together as group N-def, and data gained from flow lane B and flow lane D were combined together as group N-rep.

The influence of nitrogen concentration on biomass and nutrient content throughout the process of biofilm colonization were analyzed by repeated measure analysis of variance (RM-ANOVA) with treatments as a fixed factor and colonization time as a random factor.

For MiSeq sequencing, the sequence data were processed using QIIME Pipeline Version 1.7.0. All sequence reads were trimmed and assigned to each sample based on their bar codes. The sequences with high quality (length >300 bp, without ambiguous base "N," and average base quality score > 30) were used for downstream analysis. The aligned ITS gene sequences were used for chimera check using the Uchime algorithm, and the chimera sequences were removed before further analysis. Sequences that removed the chimera sequences were clustered into operational taxonomic units (OTUs) at a 97 % identity threshold. Randomly resampling was conducted by QIIME Pipeline Version 1.7.0. Representative sequence of each operational taxonomic unit was used for taxonomic assignment referencing Ribosomal Database Project (RDP) classifier and Greengenes database. The community alpha-diversity indices were calculated to compare the bacterial communities in each sample. The overall structural change of microbial communities was evaluated by principal coordinates analysis (PCoA) based on the UniFrac distance metric. The cluster analysis was performed by the weight UniFrac UPGMA. The statistically significant similarities of microbial community composition were analyzed with Adonis and ANOSIM. Differences in relative abundances of taxa and alpha-diversity index between samples were evaluated using independent sample *t* test. All the statistical analyses were performed with R package vegan (R Foundation for Statistical Computing, Vienna, Austria), QIIME software pipeline (Version 1.7.0), and the software SPSS 13.0 for Windows (SPSS Inc., Chicago, USA).

Results

Biomass and Nutrient Contents

Biomass of biofilms cultured under inorganic N replete conditions was much higher than that of cultures cultivated without exogenous inorganic N supply ($p < 0.01$). The final Chl *a* content of the N-rep biofilms was 55.181 ± 2.471 μg/cm^2. However, in N-def treatment, the Chl *a* contents maintained a steady and low level in the first 30 days, and then started to increase to the final value of 3.066 ± 0.601 μg/cm^2 (Fig. 1). The DW of the N-rep biofilms kept increasing throughout the whole period of the experiment, while that of the N-def biofilms only showed a slight increase at the end of the experiment. The final value of DW in N-rep biofilms was 8.080 ± 0.484 mg/cm^2, which was about four times of the DW in N-def biofilms (2.088 ± 0.230 mg/cm^2) (Fig. 1). The thicknesses of biofilms in both treatments were gradually increasing (Fig. 2), and the values of N-rep biofilms were significantly higher than those of N-def biofilms ($p < 0.01$).

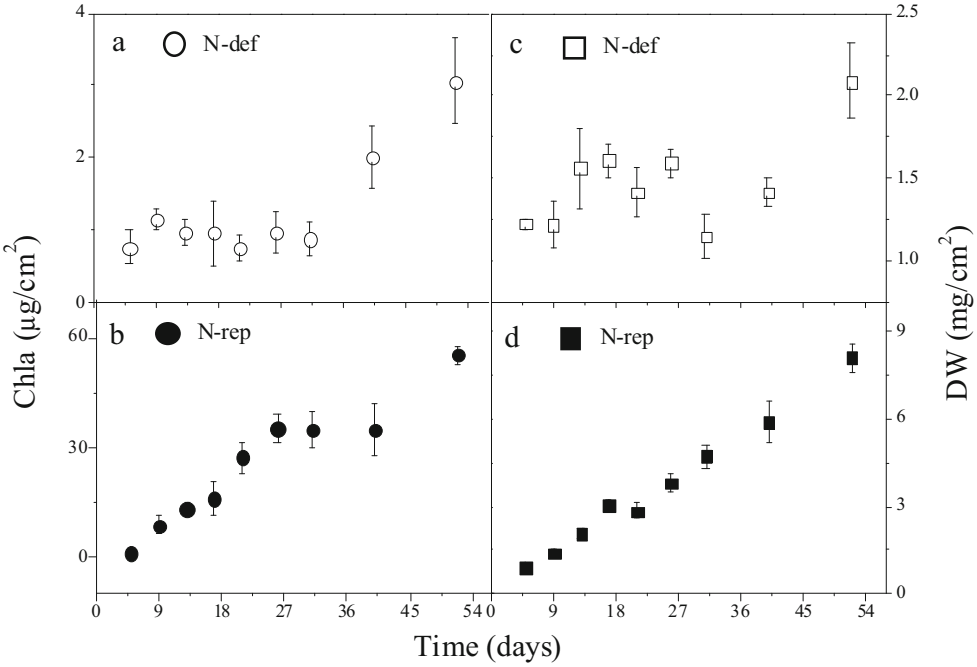


Fig. 1 Change in biomass of biofilms during the colonization period under N deficient and N replete conditions (n = 6). **a**, **b** Areal Chl *a* content of biofilms. **c**, **d** Areal dry weights of biofilms

In the N-def treatment, biofilms were cultivated without exogenous inorganic N added except that from the inocula. At the beginning of the experiment, 200-mL inoculum containing about 33 mg N was added to 7.8-L medium, so the initial concentration of N in N-def treatment was about 4.1 mg/L. However, these N mainly stored in the cells of microorganisms rather than dissolved inorganic nitrogen in the medium. In contrast, N-rep biofilms were cultured with modified BG11, which had an inorganic N concentration of about 247 mg/L. In this study, biofilms were cultured under two special conditions: deficient or replete of inorganic N.

TN contents of both the biofilms (Fig. 3a, b) and medium (Fig. 4a, b) showed significant difference between N-def and N-rep treatments ($p < 0.01$). TN contents of the medium in N-def treatment were very low. It decreased in the early and mid stage and then showed an increasing trend in the late stage (Fig. 4a). However, TN contents of the biofilms in N-def treatment showed an overall increase trend (Fig. 3a). In the N-rep treatment, TN contents of the medium kept decreasing (Fig. 4b), while those of the biofilms kept increasing throughout the experiment (Fig. 3b). TP contents of the medium in both N-def and N-rep treatments showed continued decline, but the latter decreased faster ($p < 0.01$) (Fig. 4c, d). TP contents of biofilms in N-def and N-rep treatments followed similar trends respectively to those of TN in biofilms (Fig. 3c, d), and obvious difference between the two treatments was found ($p < 0.01$).

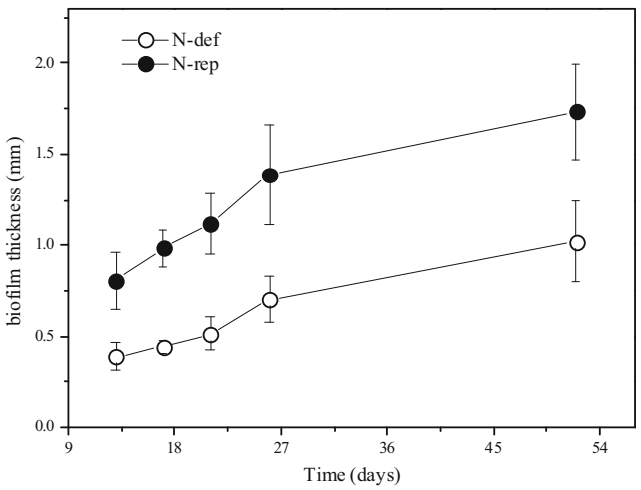


Fig. 2 Change in thickness of biofilms during the colonization period under N-def and N-rep conditions (n = 16)

Illumina™ Bacterial Community Sequencing Analysis

Alpha- and Beta- Diversity Analyses

After quality filtering and removal of chimera, a total of 1,210,791 effective sequence reads were obtained from 36 samples. All of the sequences were assigned to 108,816 OTUs at a 97 % similarity. Alpha- and beta-diversity analyses were first applied, to describe microbial communities in one specific habitat and the differentiation among habitats, respectively. The Shannon and Simpson diversity index of N-def biofilms were significantly higher than that of all N-rep biofilms ($p < 0.01$), but the species richness estimator of Chao 1 index showed no obvious difference ($p > 0.05$) (Fig. 5).

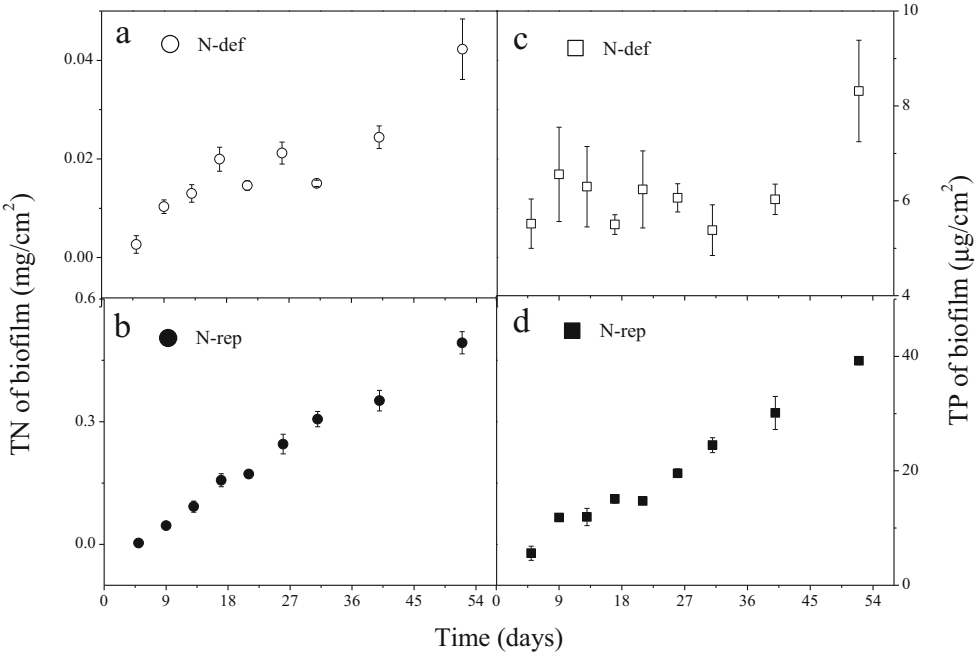


Fig. 3 Change in TN (**a**, **b**) and TP (**c**, **d**) content of biofilms during the colonization period under N-def and N-rep conditions ($n = 6$)

To understand the effect of inorganic N concentration on the overall microbial community composition, principal coordinate analysis (PCoA) was calculated based on the weighted UniFrac distance metric. As shown in Fig. 6, principal components (PC) 1 and 2 explained 29.21 and 19.05 % of the total community variations, respectively. The samples from N-rep biofilms were obviously separated from those of N-def biofilms except for the samples cultured for 5 days, and PC 1 explained the influence of nitrogen availability on microbial community of biofilms.

Concordant with PCoA, N-rep, and N-def biofilms were also well separated from each other in the UPGMA clusters (Fig. 7), suggesting clear distinctions of microbial community structure between N-def and N-rep biofilms. Furthermore, microbial communities of the N-def group were clustered into three phases: early (day 5), middle (days 9–31), and late (days 40–52). However, microbial communities of the N-rep group showed no obvious successional change.

To statistically assess differences in bacterial community structure between N-def and N-rep treatments, the OTUs of

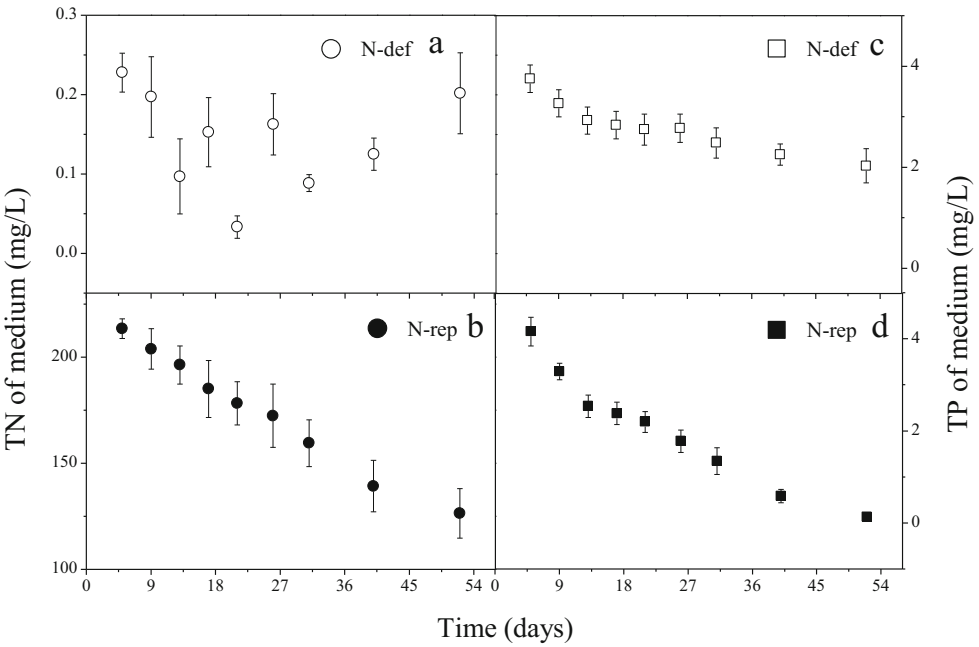


Fig. 4 Change in TN (**a**, **b**) and TP (**c**, **d**) concentration of medium during the colonization period under N-def and N-rep conditions ($n = 6$)

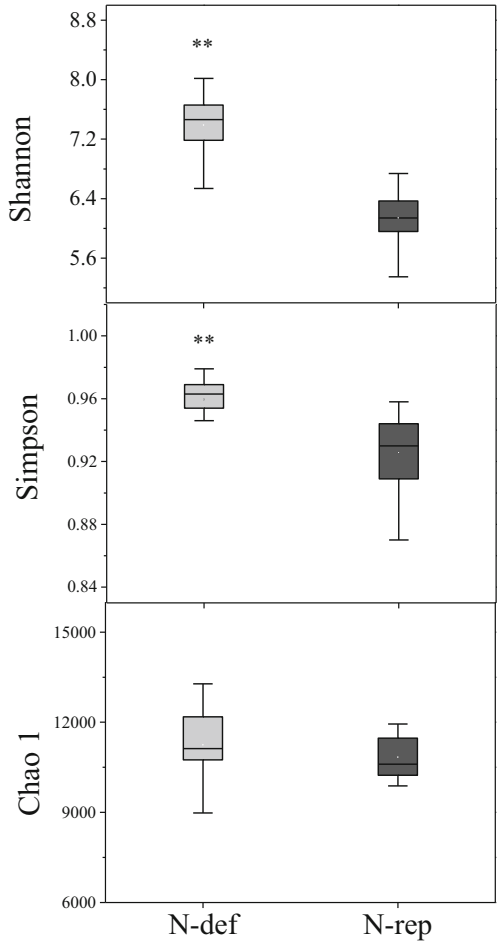


Fig. 5 Alpha-diversity index (Shannon, Simpson, Chao 1) of biofilms cultured under N-def and N-rep conditions (n = 18). Significant differences between N-def and N-rep samples are depicted with an *asterisk* (**p < 0.01)

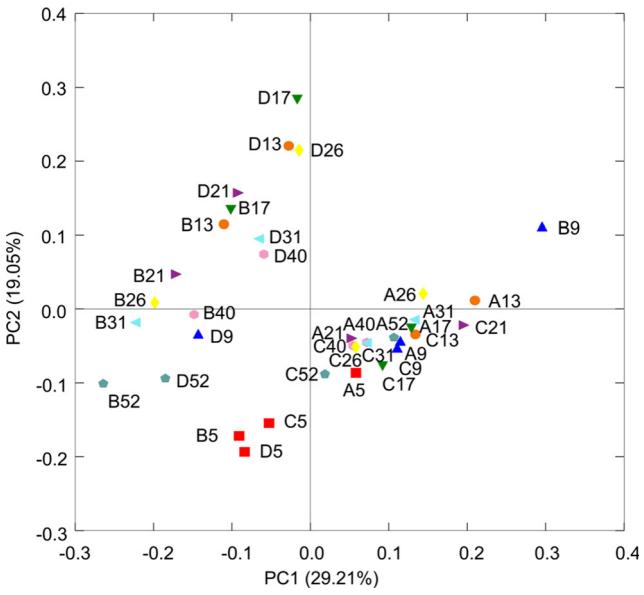


Fig. 6 Principal coordinates analysis (PCoA) of weighted Unifrac distance of 16S rRNA genes. *Capital letters* represent different concentrations of N: *A* and *C*, inorganic N deficient; *B* and *D*, inorganic N replete. *Numbers* represent the age of the biofilm in days

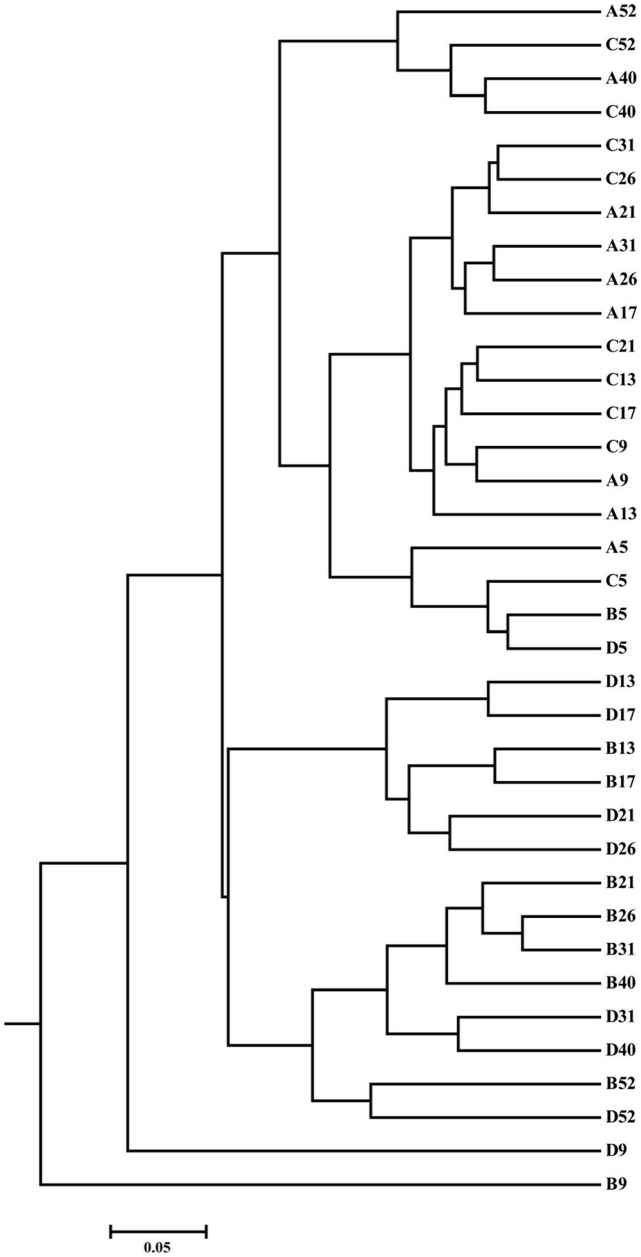


Fig. 7 UPGMA clustering of 16S rRNA gene in biofilm samples. *Capital letters* represent different concentrations of N: *A* and *C*, inorganic N deficient; B and D, inorganic N replete. *Numbers* represent the age of the biofilm in days

each sample were compared using Adonis and ANOSIM. Both of the two statistical methods showed that there was significant difference in microbial community composition between N-def and N-rep biofilms (Adonis: F = 12.603, p = 0.005; ANOSIM: R = 0.564, p = 0.001).

Microbial Community Composition

The taxonomic richness detected in this study comprised 44 phyla with Cyanophyta, Bacteroidetes, and

Proteobacteria being the most abundant in both treatments (Fig. 8). These three dominant phyla accounted respectively for 85.2 to 98.3 % of total sequences in N-def and N-rep treatments during the colonization period. The percentage of Bacteroidetes showed different trends in N-def and N-rep treatments during biofilm colonization. It firstly increased and kept a stable high level in N-def biofilms, while it decreased in late stage after it increased in early stage in N-rep biofilms. At the end of the experiment, the relative abundance of Cyanophyta in N-rep biofilms (67.4–77.1 %) was obviously higher than that of N-def biofilms (44.9–56.5 %). The Firmicutes were detected in 5-day-aged biofilms with relative abundance of 8.7 ± 0.45 %, in which more than 94.5 % was *Exiguobacterium*, but then Firmicutes decreased sharply with less than 0.1 % in 52-day-aged biofilms. Moreover, the N-rep biofilms had higher percentage of Armatimonadetes than N-def biofilms, while the N-def biofilms had higher percentage of Chloroflexi, Verrucomicrobia, Acidobacteria, and an unclassified genus of bacteria.

Successional change on composition of cyanobacteria and bacteria (excluding cyanobacteria) genus was analyzed respectively by choosing 10-first genus with the highest relative abundance in each sample (Fig. 9). The community composition of dominant species were almost similar in day 5 but then started to show difference.

The cyanobacterial genus *Leptolyngbya* was most highly enriched with average relative abundance of 32.3 ± 6.4 % in N-def biofilms, while its average relative abundance in N-rep biofilms was 22.5 ± 9.2 %, significantly lower than that in N-def biofilms ($p < 0.01$). *Microcoleus* was another dominant taxa but showed totally different trends in N-def and N-rep treatments ($p < 0.01$). The relative abundance of this genus increased continuously from day 13 in N-rep biofilms and became a prominent biofilm component with final values of 27.4–36.7 %. However, it decreased continuously and became negligible with final values of 0.1–0.5 % in N-def biofilms. *Arthronema* (Pseudanabaenaceae) and another two genera of Pseudanabaenaceae all showed higher relative abundance in N-def biofilms than in N-rep biofilms. Two genera of Oscillatoriophycideae were also found and showed similar

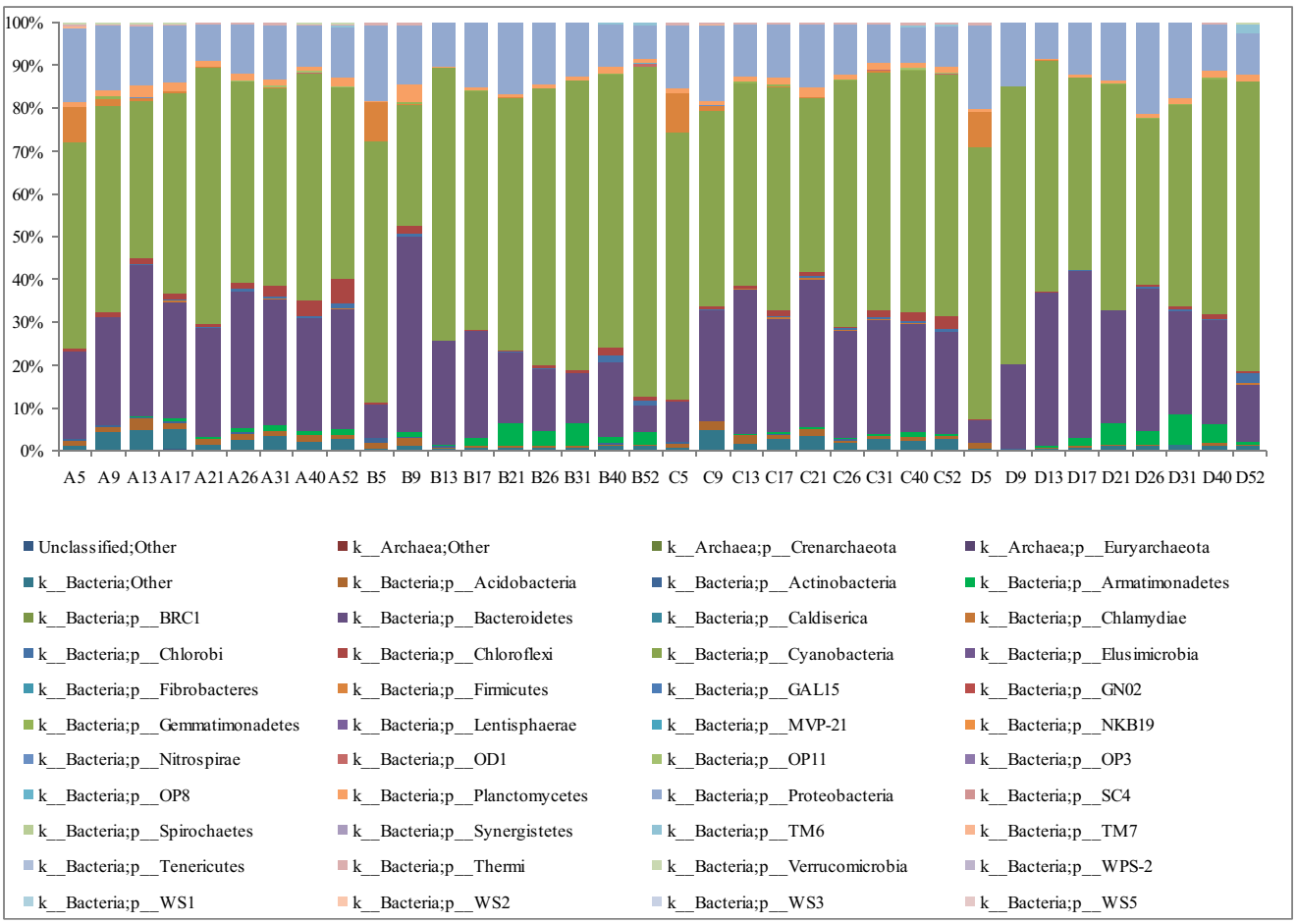


Fig. 8 Relative abundance of microbial community composition in biofilms at phylum level. *Capital letters* represent different concentrations of N: *A* and *C*, inorganic N deficient; *B* and *D*, inorganic N replete. *Numbers* represent the age of the biofilm in days

relative abundance in the initial 5–13 days, but then became obviously higher in N-rep biofilms than in N-def biofilms from day 13 to day 40. An unclassified genus of Phormidiaceae also showed higher relative abundance in N-rep biofilms than that in N-def biofilms from day 13 to day 52. However, an unclassified genus of Cyanophyta and an unclassified genus of Xenococcaceae showed similar relative abundance throughout the experiment in the two treatments. In addition, *Calothrix*, emerged from day 31 in N-def biofilms, was kept increasing with relative abundance of 0.7–9.0 % in biofilms of day 52, but almost not detected in N-rep biofilms.

Some genera of bacteria were more suitable for living under the condition without nitrogen. The bacteria genus *A4* (Sphingobacteriales, Flammeovirgaceae) began to be detected in the ninth day of biofilm colonization. It showed obvious increase trend with maximum values of 8.6–8.8 % in N-def biofilms, and its relative abundance was significantly higher than that in N-rep biofilms ($p < 0.01$). Another genus of Saprospiraceae (Sphingobacteriales) also showed higher relative abundance in N-def biofilms, lower relative abundance in N-rep biofilms from day 17. In addition, the genera *Haliscomenobacter*, *Methyloversatilis*, and an unclassified genus of Acidobacteria were found in the late stage (days 40–52) of N-def biofilms, but the relative abundance of them in N-rep biofilms was negligible. Several other genera of bacteria, such as an unidentified bacterial genus, an unclassified genus of Chloracidobacteria, an unclassified genus of Flexibacteraceae, and two genera of Sphingomonadales, were also found in N-def biofilms, but significantly lower in relative abundance in N-rep biofilms.

Some genera of bacteria grew well in the condition with replete nitrogen. *Flavobacterium* showed similar relative abundance in N-def and N-rep biofilms in days 5–9. However, it increased rapidly and became a dominant species of N-rep biofilms with maximum values of 17.0–28.3 %. The relative abundance of this species was significantly higher than that of N-def biofilms from day 13 to day 31 ($p < 0.01$). *Aquimonas* firstly appeared in 13-day-aged biofilms and increased rapidly in N-rep biofilms but almost was undetectable in N-def biofilms. Its maximum relative abundance in N-rep biofilms was 6.0–10.9 % and then obviously decreased in the late stage. In addition, an unclassified genus of Fimbriimonadaceae and an unclassified genus of Rhizobiales were also more likely to be found in the mid stage of N-rep biofilms.

Some genera of bacteria showed similar relative abundance in the two treatments. Two genera of Chitinophagaceae were the dominant species of both N-def and N-rep biofilms. In addition, *Acinetobacter*, *Exiguobacterium*, *Delftia*, *Luteimonas*, and an unclassified genus of Burkholderiales only appeared at the beginning (day 5) of the biofilm colonization.

Fig. 9 Successional change in composition of 10-first genus with the highest relative abundance in each sample. **a** Cyanobacteria. **b** Bacteria (excluding cyanobacteria). *Capital letters* represent different concentrations of N: *A* and *C*, inorganic N deficient; *B* and *D*, inorganic N replete. *Numbers* (both on the vertical axis and on the right side of capital letters) represent the age of the biofilm in days

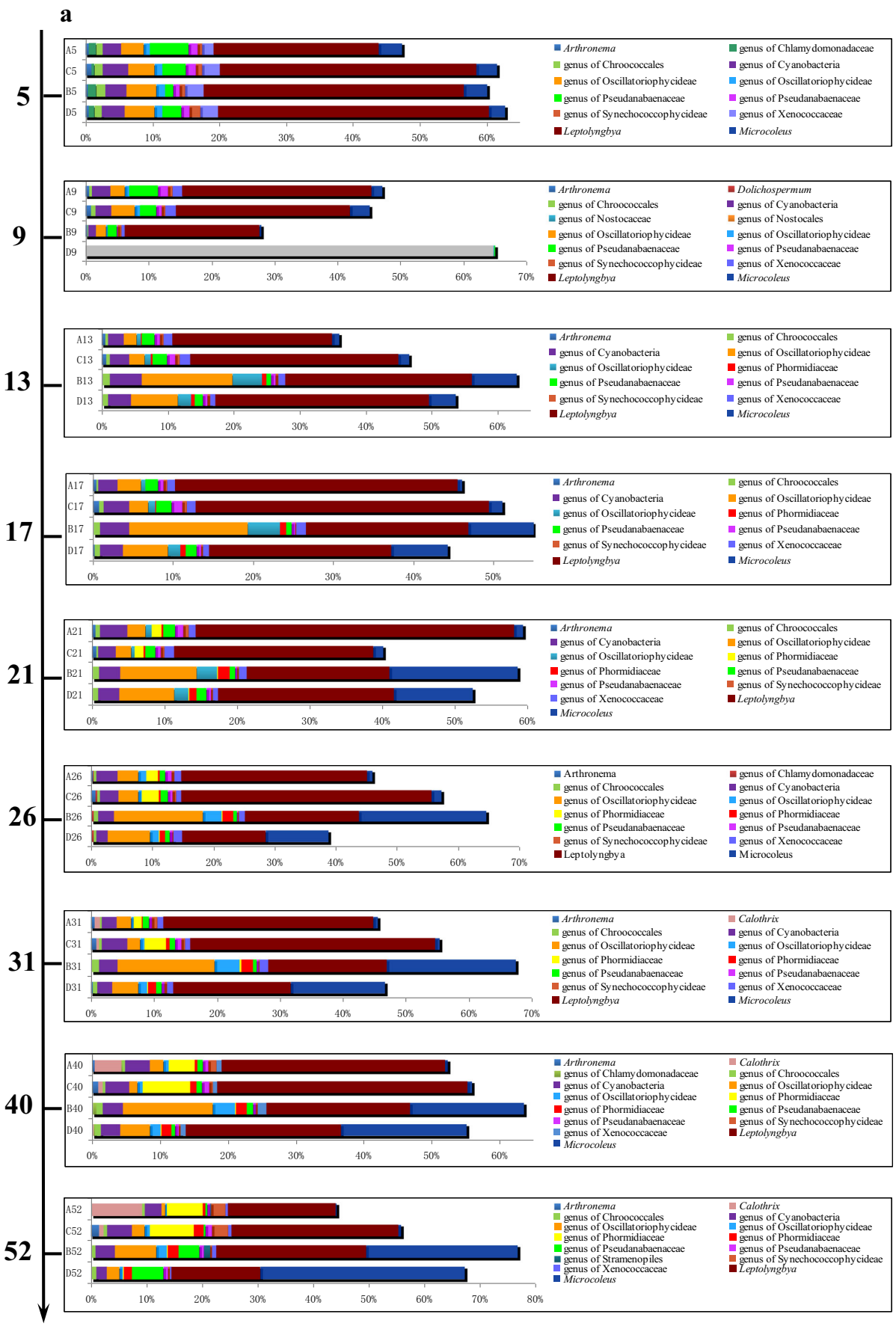
Discussion

Formation and Development of Biofilms under N-def and N-rep Conditions

In this study, two groups of biofilms were cultured under the same environment conditions except for inorganic nitrogen supply. The result showed an integrated biofilm could be formed under conditions without exogenous inorganic N supply, suggesting that inorganic N was not an absolute limiting factor for biofilm formation and development. A previous study suggested that N limitation of ecosystem could be alleviated, even eliminated, by biological N_2 fixation [14].

However, the availability of inorganic N has important effect on structure, physiology, and microbial community of biofilms. Biomass (Chl *a*, DW) was the most common indicator for N limitation evaluation in biofilms. In this study, biomass of N-def biofilms was significantly lower than that of N-rep biofilms; this indicated that inorganic N was an essential element for the growth of biofilms and nutrient enrichment could enhance the accumulation of biomass in biofilms [36]. In the present study, the thickness of biofilms was also closely related to N concentration, and N deficiency severely limited the thickness of the biofilms. Stoodley et al. [37] suggested that biomass and thickness of biofilms possibly had an effect on flow regime of ambient water, resulting in change in local shear rate. In addition, biofilm morphology also changed with N concentration. Compared with N-def biofilms, N-rep biofilms that were more compact and with more surface protrusions showed that the biofilms had changed their morphology and structure to adapt to the changing nutrient conditions [38]. Meanwhile, the study also showed that there were significant differences in microbial community between the N-rep and N-def biofilms, although the inocula of the two treatments were the same. Inorganic N availability was an important factor in shaping microbial community composition and succession.

In turn, biofilms of N-def and N-rep treatments showed different impact on the ambient environment. Under N-rep condition, biofilms absorbed much more N and P from the medium, and direct uptake of nutrients by the microorganisms in biofilms was a main way to reduce N in ambient water. In contrast, the N-def biofilms took in less P and even released a little N to the medium, so the N concentration of N-def medium showed an increase at the end of the experiment.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Leptolyngbya
Microcoleus
17
A17
C17
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Arthronema
genus of Chroococcales
genus of Cyanobacteria
genus of Oscillatoriophycideae
genus of Oscillatoriophycideae
genus of Phormidiaceae
genus of Pseudanabaenaceae
genus of Pseudanabaenaceae
genus of Synechococcophycideae
genus of Xenococcaceae
Leptolyngbya
Microcoleus
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genus of Pseudanabaenaceae
genus of Pseudanabaenaceae
genus of Synechococcophycideae
genus of Xenococcaceae
Leptolyngbya
Microcoleus
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C26
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Arthronema
genus of Chlamydomonadaceae
genus of Chroococcales
genus of Cyanobacteria
genus of Oscillatoriophycideae
genus of Oscillatoriophycideae
genus of Phormidiaceae
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genus of Pseudanabaenaceae
genus of Pseudanabaenaceae
genus of Synechococcophycideae
genus of Xenococcaceae
Leptolyngbya
Microcoleus
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Arthronema
Calothrix
genus of Chroococcales
genus of Cyanobacteria
genus of Oscillatoriophycideae
genus of Oscillatoriophycideae
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genus of Pseudanabaenaceae
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genus of Synechococcophycideae
genus of Xenococcaceae
Leptolyngbya
Microcoleus
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Arthronema
Calothrix
genus of Chlamydomonadaceae
genus of Chroococcales
genus of Cyanobacteria
genus of Oscillatoriophycideae
genus of Oscillatoriophycideae
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genus of Pseudanabaenaceae
genus of Pseudanabaenaceae
genus of Synechococcophycideae
genus of Xenococcaceae
Leptolyngbya
Microcoleus
52
A52
C52
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Arthronema
Calothrix
genus of Chroococcales
genus of Cyanobacteria
genus of Oscillatoriophycideae
genus of Oscillatoriophycideae
genus of Phormidiaceae
genus of Phormidiaceae
genus of Pseudanabaenaceae
genus of Pseudanabaenaceae
genus of Stramenopiles
genus of Synechococcophycideae
genus of Xenococcaceae
Leptolyngbya
Microcoleus

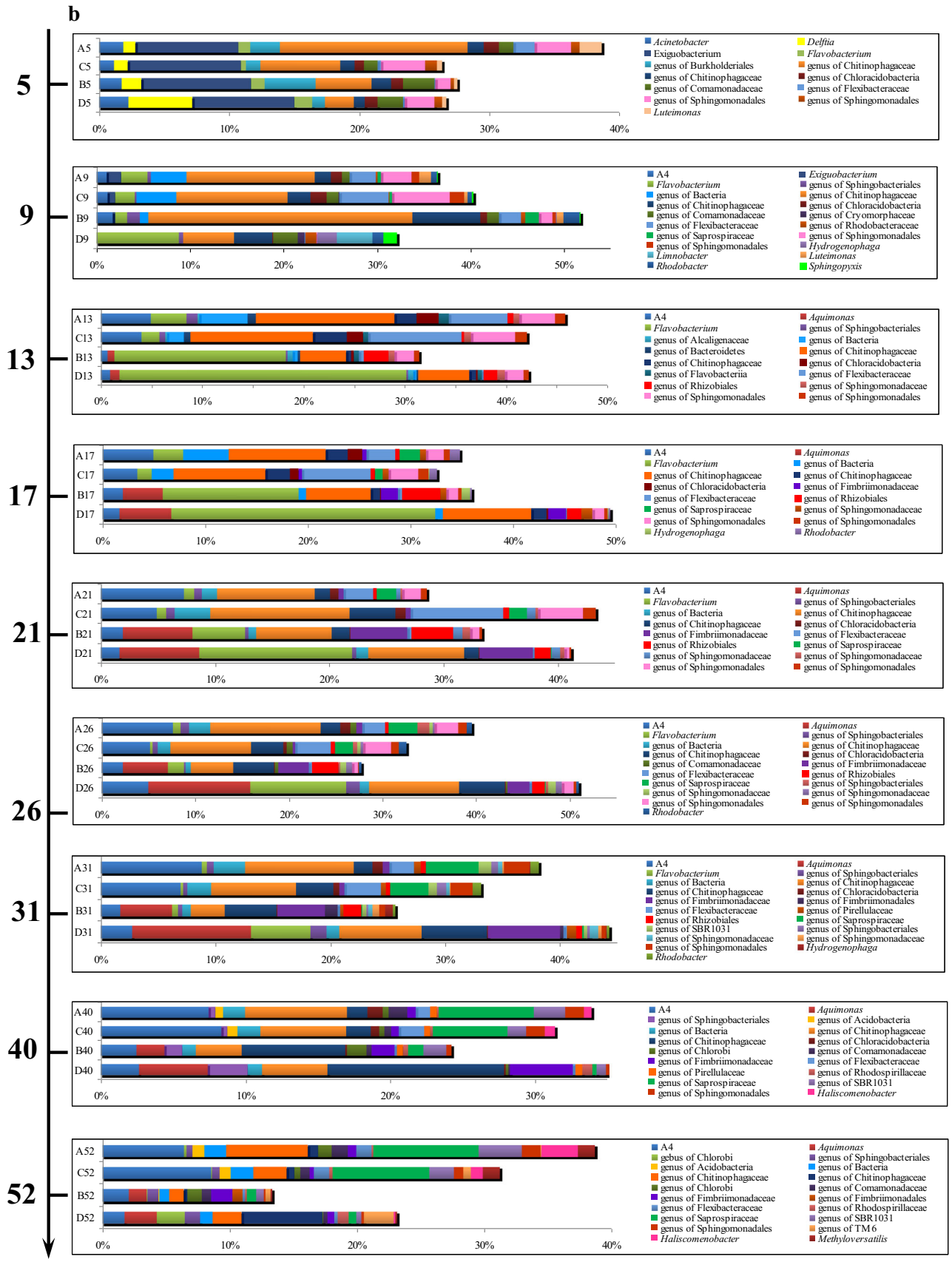

Fig. 9 (continued)

Moreover, we detected high nitrogenase activities in the N-def biofilms, but not detected in the N-rep biofilms (unpublished data). The lack of inorganic N could stimulate the nitrogenase activity (NA) in biofilms, and the lack of inorganic N in ecosystem may be compensated by biological N_2 fixation. So biofilms could serve as the source or sink of N in aquatic ecosystems.

Microbial Diversity of Biofilms

Although the growth was markedly limited, the N-def biofilms had significantly higher microbial diversity than that of N-rep biofilms. As eutrophication could decrease phytoplankton diversity and change phytoplankton community, it was probably the same situation in N-rep biofilms. Some highly adaptive microorganisms dominated the biofilms and suppressed the growth of other species, and this then decreased the microbial diversity of N-rep biofilms. Microbial diversity may be crucial for an ecosystem adapting to changing conditions and maintaining its functional stability [21, 38]. The Shannon and Simpson diversity indexes indicate not only the richness but also the evenness of species [39, 40]. Higher Shannon and Simpson diversity indexes of N-def biofilms could be considered as responses of biofilms against N limited condition. Previous studies had demonstrated that a highly diverse community contained more functional groups could offer more powerful functionality to an ecosystem, and contribute to resist the completed environmental stress [24, 40]. In addition, Yao et al. [41] also showed that a high N addition would decrease diversity of species and bacterial interaction and then weaken the stability of microbial community structure.

Microbial Community Composition and Succession

Nutrient limitation is dynamic and will continuously affect the microbial community structure and the productivity of ecosystems [14]. At the level of phylum, the dominant phyla (Cyanophyta, Bacteroidetes, and Proteobacteria) in both N-def and N-rep biofilms were the same, and they were the most common microorganisms in biofilms [42–46]. At the level of phylum, the obvious difference between N-def and N-rep biofilms was the different percentage of Verrucomicrobia, Chloroflexi, Acidobacteria, Armatimonadetes, and an unclassified genus of bacteria. The reason why Verrucomicrobia is likely to be found in N-def condition may be that it has *nifH* gene, which encodes the iron protein subunit of nitrogenase and thus has the genetic potential for N_2 fixation [47]. Moreover, a previous study also demonstrated that N addition could significantly decrease the relative abundance of Verrucomicrobia, Chloroflexi, and Acidobacteria but increase the relative abundance of Armatimonadetes, indicating its nitrophilous nature [41], and this was consistent with our study. In addition, an unclassified genus of bacteria, which could not be assigned to any taxa, showed high relative abundance in N-def treatment, may also have the ability to fix N_2.

In the present study, the development of biofilms cultured under N-def condition exhibited three obvious stages, but biofilms cultured under N-rep condition did not show such obvious successional stages. The variations in microbial community were affected by environmental conditions, and such a shift in turn would also change the environmental conditions, for instance, the N availability might be regulated. In N-def treatment, stage one (days 0–5) was the initial attachment of microorganisms. During this period, the microorganisms attached to the slides were mainly from the inoculum. Stage two (days 9–31) was biomass accumulation and biofilm maturation. Stage three (days 40–52) was probably a turning point for the development of N-def biofilms, owing to the great change in not only the microbial community composition but also the physiology of biofilms. A previous study also reported that the change of microbial community would be the leading factor to the variation of metabolites [48]. During this time, the abundance of N_2-fixing heterocystous cyanobacteria increased, meanwhile, the biomass, nutrient content, nitrogenase activity, and thickness of biofilms also increased remarkably.

At day 5, the dominant cyanobacteria and bacteria assemblages were similar in N-def and N-rep biofilms. This was consistent with the results of PCoA and UPGMA clusters, where 5-day-aged samples of both treatments also clustered together. On one hand, nitrogen level seemed to have no effect on microbial composition in a relatively short time. On the other hand, microbial composition of the initial stage was largely determined by the inocula of biofilms [49, 50]. However, not all microorganisms of the inocula could attach to the substrata and survive. Jackson et al. [51] demonstrated that the initial attachment of bacteria to substrata was random, but Atabek and Camesano [52] suggested that the lipopolysaccharide-producing bacteria were more likely to attach to the surface of substrata. The EPS, especially lipopolysaccharides, could enhance the adhesion force of cells [1, 53]. In the present study, the dominant phyla such as Cyanophyta, Proteobacteria, and Bacteroidetes were gram-negative bacteria, and the cell surface of gram-negative bacteria was rich in lipopolysaccharides [53]. In addition, the morphology of bacteria may also play roles on the initial attachment of microorganisms to a solid surface. The dominant taxa of 5-day-aged biofilms almost were rod-shaped bacteria in this study, such as *Acinetobacter*, *Delftia*, *Exiguobacterium*, *Flavobacterium*, an unclassified genus of Chitinophagaceae, and an unclassified genus of Comamonadaceae. The reason was not clear, which needs further researches.

Delftia was the richest in the 5-day-aged biofilms with the average relative abundance of 2.1 %, but then decreased sharply with less than 0.1 %. *Acinetobacter* was also dominant

in 5-day-aged biofilms, but then became negligible. It was known from early studies that *Delftia* and *Acinetobacter* were able to degrade some pollutants excellently [54, 55]. In addition, *Exiguobacterium* (Firmicutes), just prevailed in 5-day-aged biofilms, was reported to have the ability to degrade structurally diverse organic compounds [56, 57]. An unclassified genus of Burkholderiales was also dominant in 5-day-aged biofilms, and studies showed that some genus of Burkholderiales have the ability of denitrification [58, 59]. These bacteria in the initial stage were probably from biofilm inocula, which were sampled from a sewage draining exit. The water in the sampling site was seriously polluted, so this may be the reason why the pollutant-degrading bacteria were prevailed in biofilms of the initial stage.

The variation of medium N concentrations in the two treatments showed different trends: N content of N-rep treatment kept decreasing, while that of N-def treatment had an obvious increasing trend. Therefore, it could be expected that N would no longer be the limiting factor for the development of N-def biofilms. Previous studies had demonstrated that nutrient concentration was an important factor regulating the microbial community composition [26, 36]. In terms of macroscopic observation, the surface color of N-def biofilms was yellowish-brown in the early and mid stages, but some green spots appeared in the slides of N-def biofilms cultured for about 40 days, then the spots became bigger and the numbers also gradually increased. After microscope identification, these spots were identified as *Calothrix* sp., *Hapalosiphon* sp., *Fischerella* sp., and *Aphanizomenon* sp., which all were nitrogen-fixing cyanobacteria with plenty of heterocysts. Their appearance could provide more N for the system by N fixation. So, these species probably played important role in the development of N-def biofilms. Our results also showed that the appearance of these heterocystous cyanobacteria was accompanied by the rapid increase of nitrogenase activity (NA) (unpublished data) and obvious increase of N content of biofilms. Of course, the dramatic increase of NA in the late stage of colonization also includes the contribution of other diazotrophs.

In general, biofilms cultured without exogenous inorganic N would develop better with the increase in abundance and diversity of heterocystous cyanobacteria and other diazotrophs. In the present study, biofilms covered the entire surface of flow lanes and blocked the inlets and exits on day 52, so our experiment did not last for much longer time.

Dominant Taxa of Biofilms

Microbial community composition under N-def treatment differed greatly in species composition from those developed under N-rep condition.

Leptolyngbya was the most abundant genus in biofilms, especially in N-def treatment. It was speculated that *Leptolyngbya* in our experiment may have the ability of nitrogen fixation. Previous studies had demonstrated that some species of *Leptolyngbya* could grow well in medium free of inorganic nitrogen [60, 61]. Moreover, it was reported that *Leptolyngbya* had *nifH* gene [62], which encodes the iron protein of the nitrogenase enzyme complex and resulted in nitrogenase activity [63–65]. Nevertheless, *Leptolyngbya* was filamentous non-heterocystous cyanobacteria. Since nitrogen fixation occurred in anaerobic place, such as in heterocyst [66], the non-heterocystous cyanobacteria had evolved some strategies, including behavioral, metabolic, and structural characteristics, to protect nitrogenase not only from environmental oxygen but also from photosynthetically produced oxygen [67]. Some diazotrophs were found to create anaerobic microzones by cell aggregation [68]. In the present study, biofilms could form a complex three-dimensional space structure, some of which were micro-oxic or anaerobic microzones, and this would contribute to nitrogen fixation. In addition, some other species fixed N_2 mainly during the dark period in order to avoid oxygen from photosynthesis in the daytime [69, 70]. Schütz et al. [63] suggested that *Leptolyngbya* could fix N_2 under micro-oxic conditions. There were also a lot of *Leptolyngbya* in N-rep treatment, but nitrogenase activity was not detected. This was probably because there was plenty of N in N-rep treatment and no need to fix N_2. Therefore, the actual role one microorganism may play should be analyzed in combination with the environmental conditions. *Microcoleus* was also a common genus in biofilms [46], which could produce a lot of EPS to hold biofilms together. In the present study, *Microcoleus* preferred to appear in N-rep biofilms, so it was a nitrophilous taxon. The heterocystous cyanobacteria *Calothrix*, only dominated in N-def biofilms, made great contribution to the increase of N in biofilm and medium through N fixation at late stage of the experiment, and promoted the sustainable development of N-def biofilms.

It was considered that phylogenetically closely related species showed similar metabolic capabilities [71]. Two groups of bacteria, genus *A4* and an unclassified genus of Flexibacteraceae (both belong to Sphingobacteriales), *Haliscomenobacter,* and an unclassified genus of Saprospiraceae (both belong to Saprospiraceae), were phylogenetically close in the phylogenetic tree, respectively. Moreover, all of them were more likely to survive under N-def condition. It was inferred that these bacteria might have some properties such as N_2 fixation for their survival in N-def condition. The reason was not clear, and additional research was needed.

Under N-def condition, some diazotrophs prevailed. Their biological nitrogen fixation to some extent compensated for the lack of nitrogen in the environment and was beneficial to the development of biofilms. The diazotrophs then could provide combined N for other microorganisms [72], which would reshape the community structure of N-def biofilms.

The dominant taxa of N-rep biofilms were regarded as typical heterotrophic denitrifying bacteria, including *Flavobacterium* [73, 74], *Aquimonas* [75, 76], and an unclassified genus of Rhizobiales [59, 77]. The relative abundance of these species was significantly higher than that of N-def biofilms, and the biological processes of denitrification could effectively remove nitrogen from the ecosystem. The present study showed that these denitrifying bacteria mainly emerged or became dominant in the mid stage of the experiment, but not in the initial stage. This suggested that their dominance was a result of the environmental stress, owing to the high N concentration of the medium in N-rep treatment. The denitrifying bacteria made great contribution to reduce N concentration of the surrounding water. In general, biofilms could reduce N concentration of medium through denitrification and photosynthesis.

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