Nitrogen-cycling microbial community functional potential and enzyme activities in cultured biofilms with response to inorganic nitrogen availability

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ABSTRACT

Biofilms mediate crucial biochemical processes in aquatic ecosystems. It was hypothesized that eutrophication may promote the growth of biofilms, resulting in larger numbers of functional genes. However, the metabolic activity and the roles of biofilms in N cycling will be affected by ambient inorganic nitrogen availability, not by the abundance of functional genes. Biofilms were cultured either with replete inorganic nitrogen (N-rep) or without exogenous inorganic nitrogen supply (N-def) in a flow incubator, and the N-cycling gene abundances (nifH, N₂ fixation; amoA, ammonia oxidation, archaea and bacteria; nirS and nirK, denitrification) and enzyme activities (nitrogenase and nitrate reductase) were analyzed. The results showed that, comparing the N-def and N-rep biofilms, the former contained lower nifH gene abundance, but higher nitrogenase activity (NA), while the latter contained higher nifH gene abundance, but lower NA. Different patterns of NA diel variations corresponded to the dynamic microbial community composition and different stages of biofilm colonization. Ammonia oxidizing bacteria (AOB), detected only in N-def biofilms, were responsible for nitrification in biofilms. N-rep biofilms contained high nirS and nirK gene abundance and high denitrification enzyme activity, but N-def biofilms contained significantly lower denitrification gene abundance and activity. In general, the strong N₂ fixation in N-def biofilms and strong denitrification in N-rep biofilms assured the balance of aquatic ecosystems. The results suggested that evaluation of the functional processes of N cycling should not only focus on genetic potential, but also on the physiological activity of biofilms.

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Introduction

Aquatic biofilms are composed of a diverse assemblage of autotrophs and heterotrophs, mainly including algae, bacteria, archaea, fungi and protozoa (Dí et al., 2009; Lock et al., 1984). Biofilms usually form highly structured assemblages of surface-associated microbial cells and secrete an extracellular polymeric substance matrix that holds the biofilm together (Wimpenny et al., 2000; Sutherland, 2001). As one of the most widely distributed microecosystems, biofilms are the main spots for biogeochemical cycling of biogenic elements, such as nitrogen (N), carbon (C), phosphorus (P), sulfur (S) and oxygen (O), and also contribute significantly to the immobilization and transformation of contaminant molecules in aquatic ecosystems (Ancion et al., 2013; Headley et al., 1998; van Hullebusch et al., 2003). In general, highly diverse and abundant microorganisms living in biofilm communities are thought to contribute crucially to the ecological processes of water bodies (Battin et al., 2003).

There is plenty of N in the Earth’s atmosphere, hydrosphere and biosphere, but the vast majority of N is not directly usable by most living organisms (Galloway et al., 2003). As the element N is necessary to all living organisms (Francis et al., 2007), the N biogeochemical cycle, mediated by microorganisms, is of great importance. From an ecological point of view, biofilms play an important role in global N cycling. From a biological perspective, the cycle and transformation of N by biofilms, including N fixation, nitrification, denitrification, ammonification, assimilation and anammox (Revsbech et al., 2006; Zehr and Kudela, 2011), are regulated on multiple levels, such as DNA (deoxyribonucleic acid), RNA and proteins. Therefore, it is important to understand the changes of N forms during the biogeochemical cycle.

N fixation is usually the main source of bioavailable N for ecosystems. Since the nitrogenase enzyme is very sensitive to O₂, heterocystous cyanobacteria could show high nitrogenase activity (NA) in daytime, and conduct photosynthesis and N₂ fixation contemporaneously. Some nonheterocystous cyanobacteria and bacteria usually show NA in darkness (Charpy et al., 2007), but exceptions also exist (Yu, 2011). Different groups of diazotrophs have different strategies for N₂ fixation, and the separation of photosynthesis and N₂ fixation in time and space has been described (Yu, 2011). In eutrophic lakes, the excess N can be transformed into different forms by coupled microbial processes: ammonia is converted to nitrite and nitrate through nitrification, which can then be released to the atmosphere as N₂ gas through denitrification or anammox (Mosier and Francis, 2008). Ammonia oxidation is the first and rate-limiting step of nitrification, which is catalyzed by two specific microbial groups: the ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) (Konneke et al., 2005). One of the important pathways to eliminate N loading in ecosystems is to oxidizing bacteria (AOB) (Konneke et al., 2005). One of the groups: the ammonia-oxidizing archaea (AOA) and ammonia-nitrification, which is catalyzed by two specific microbial pathways to eliminate N loading in ecosystems is to oxidizing bacteria (AOB) (Konneke et al., 2005). One of the groups: the ammonia-oxidizing archaea (AOA) and ammonia-nitrification, which is catalyzed by two specific microbial

1. Materials and methods

1.1. Biofilm inocula collection and culture

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 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; NH₄⁺-N, 4.1-4.5 mg/L; NO₂⁻-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 hr.

Biofilms were cultured with a flow incubator, which was designed mainly with reference to Zippel et al. (2007), and the detailed descriptions of this incubator were given in Li et al. (2016). The incubator consisted of four separate flow lanes named as A, B, C and D, respectively. The illumination system was made up of 16 strips of LED white light lamps (main wavelength peak: 460 nm, full spectrum illumination). Ground glass slides were used as artificial substrates for biofilm growth.

1.2. Experiment design

200 mL of inocula was added to 7.8 L medium and mixed in the aquarium. The inorganic nitrogen (N) concentration of the medium used in the 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 (NaNO₃: 247.058 mg/L, N-rep) added. The modified BG11 medium was prepared with reference to Beakes et al. (1988) and Guzzon et al. (2008), including addition of vitamins (40 μg/L) and silicates (57 mg/L). The inoculated medium was then pumped through the incubator at 75 L/hr and 25°C. The ground glass slide substrates were subjected to a diel cycle of 16 hr light:8 hr dark with an intensity of 35 μmol photons/(sec·m²) 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/hr, and other conditions were the same as mentioned above. Samplings were conducted on day 5, 9, 13, 17, 21, 26, 31, 40 and 52 for all analyses, including gene abundances, enzyme activity (Vila-Costa et al., 2014). The nitrite reductase genes nirS and nirK are typical marker genes for denitrifiers (Throbäck et al., 2004). Biofilms have important effects on nutrient cycling in aquatic ecosystems. Therefore, we assumed that eutrophication may promote the growth of biofilms with higher biomass accumulation, so as to obtain large numbers of functional genes, but the metabolic activity and the roles of biofilms in N cycling will be determined by ambient inorganic N availability, not by the abundance of functional genes (or biomass). The hypothesis was tested by quantifying both the N-transformation rates and gene abundances in the same aquatic system, and studies of this kind in aquatic ecosystems are rare. This experiment was performed using field epilithic biofilm samples cultured under deficient and replete inorganic N conditions in a flow incubator.
activities, biomass and nutrient contents, and the experiments lasted 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 total volume of medium was always maintained at 8 L.

1.3. DNA extraction and quantitative analysis of nitrogen-cycling genes

In each flow lane, biofilm samples with three replicates were detached from the slides using sterile scalpel blades, and then thoroughly mixed into one sample for DNA extraction. Total DNA was extracted using a Power Biofilm™ DNA Isolation Kit (MO BIO, USA) according to the manufacturer’s instruction. Quantitative PCR (qPCR) amplification was carried out for the nirK gene from nitrogen fixing cyanobacteria and bacteria as described previously (Poly et al., 2001), for amoA genes from both ammonia oxidizing archaea (AOA) and bacteria (AOB) (Francis et al., 2005; Okano et al., 2004; Rothhauwe et al., 1997; Touma et al., 2008), and for nirS and nirK genes from denitrifying bacteria as described previously (Hallin and Lindgren, 1999; Throßbäck et al., 2004). The details of primers used and qPCR conditions are shown in Table 1. The qPCR amplification was run in 96-well plates using a CFX Connect Real-time System (Bio-Rad, USA). The 20 μL volume reaction mixture contained 10 μL of SYBR Premix Ex Taq™ II (TAKARA, Japan), 5 ng of template genomic DNA, 0.2 of each primer, 7.2 μL of ddH2O. The specifics of PCR products were confirmed by melt curve analysis (60 to 95°C) and agarose gel electrophoresis. Plasmids containing the respective functional gene as an insert (nirH, amoA, nirS and nirK) were obtained by clones from environmental samples, then these known copy numbers of linearized plasmids were used as a standard for qPCR (R² > 0.99). All assays were run in triplicate, and the standard curve and negative controls were analyzed in each 96-well plate. Moreover, the detection for AOA amoA gene was also carried out in soil samples as controls. Overall, the amplification efficiencies of all detected samples ranged from 90% to 110%. Gene abundance values were normalized to surface area (copies/cm²) and dry weight (dw) content (copies/g dw). Dry weight of biofilms was analyzed by drying at 105°C for 24 hr, and then weighing.

1.4. Nitrogenase activity and nitrate reductase activity measurement

The nitrogenase activity (NA) of biofilms was measured by a modified acetylene reduction assay as previously reported (Li et al., 2010; Stewart et al., 1967; Yu, 2011), and the potential rate of N₂-fixation by nitrogenase was expressed by the amount of ethylene produced per unit dry weight of biofilms per hour (nmol C₂H₄/(hr·μg dw)). In each flow lane, 3–5 biofilm slides were chosen randomly, each biofilm sample was cut into pieces (2 cm × 2.5 cm), and each piece was placed into a separate 20 mL glass cuvette. The glass cuvette contained 2 mL of sterile modified BG11 medium without inorganic nitrogen, which could totally submerge the biofilm samples. Then the glass cuvette was sealed with a rubber stopper and 10% of the volume of air in the glass cuvette was replaced with acetylene gas. The glass cuvettes were then incubated under the same condition as biofilms cultured in the flow lane: 25°C with an illumination of 100 μmol photons m⁻² s⁻¹ for 12–16 h. All assays were run in quintuplicate.

At specified intervals, 200 μL of gas was sampled from the headspace of the glass cuvette and injected directly into a gas chromatograph equipped with a gas chromatography column (2 m in length × 3 mm in diameter) and a flame ionization detector (FID) to measure the content of ethylene (Shimadzu GC14C, Japan). The retention time of ethylene in the column was 0.7 to 0.9 min. The temperatures of the gas chromatography column, gas injection port and detector were 65, 135, and 150°C, respectively. Calibration with standard ethylene was performed at the time of observation. Glass cuvettes with sterile modified BG11 medium but without biofilm samples were chosen as controls.

After NA measurement, the samples were removed from the glass cuvettes for dry weight determination, and the NA values of biofilms were normalized to dry weight content. The biofilm samples were firstly centrifuged at 10,000 r/min for 10 min, then the pellets were rinsed with distilled water, dried at 105°C for 24 hr, and finally weighed.

The nitrate reductase activity (NRA) of biofilms was measured as previously reported with some modifications (Pigaglio et al., 1999), and the actual rate of nitrate reduction was

| Table 1 – Quantitative PCR conditions for the targeted N-cycling genes. |
|-----------------------|--------------------------|-------------------|-------------------|
| Target gene           | Primer set               | Sequence (5’–3’)  | Amplicon length (bp) | Annealing temperature (°C) | Reference               |
| nifH                  | Pol F                    | TGGAYCCSAARCGAGCATCT | 360               | 59          | Poly et al., 2001          |
| AOA amoA              | Pol R                    | ATGGCCATCATCCTGCGGA | 635               | 50–60       | Francis et al., 2005       |
| AOB amoA              | ArchamoA-1F              | STAATTGCTTGCTTACAGGC | 628               | 50–60       | Touma et al., 2008         |
|                      | ArchamoA-2R              | CCCCGGACATCTGACGTTT    | 491               | 50          | Rothhauwe et al., 1997     |
|                      | CrenamoA23f              | ATGTCCTGCTGACGTTT     | 425               | 57          | Throßbäck et al., 2004     |
|                      | CrenamoA616r             | GCCATCCATCGCTGCTT2CA | 472               | 57          | Hallin and Lindgren, 1999  |
| nirS                  | amoA-1F                  | GGGTTTCTACGGTGTTT     |                   |             |                          |
|                      | amoA-2R                  | CCCCCTGCGTTAGGCTTCT   |                   |             |                          |
|                      | Cd3s3f                   | GSSAAGTCTGAGGARCRGG   |                   |             |                          |
| nirK                  | R3cd                     | GASTCGGVTGCTGTTGTA    |                   |             |                          |
|                      | Fl1aCu                   | ATCGTGTCGTTGCTGTTT   |                   |             |                          |
|                      | R3Cu                     | GCCTCGATCAGRTTGTGTTT |                   |             |                          |

* The PCR amplification program included initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 sec and 1 min, then annealing at the listed temperature for 1 min, and 72°C for 60 sec, and final extension at 72°C for 10 min. The specifics of PCR products were confirmed by melt curve analysis (60 to 95°C) and agarose gel electrophoresis.
expressed as the amount of nitrite produced per unit dry weight (or area) of biofilms per hour (\(\mu g\ NO_2-N/(cm^2\cdot hr)\) or \(\mu g\ NO_2-N/(mg\ dw\cdot hr)\)). Biofilm samples submerged in phosphate buffer were sonicated under ice bath conditions in order to extract the enzyme, and the samples were checked microscopically post-sonication to ensure that the majority of cells were lysed. NRA was then determined using a colorimetric method at 520 nm and expressed in terms of \(\mu g\ NO_2\) formed per mg dry weight and per cm\(^2\) surface area. All assays were run in triplicate.

1.5. Statistical analysis

The data were processed as follows: data obtained from flow lane A and flow lane C were combined together as group N-def, and data obtained from flow lane B and flow lane D were combined together as group N-rep.

The influence of inorganic nitrogen concentration on the abundance of nitrogen-cycling genes, nitrogenase activity and nitrate reductase activity throughout the process of biofilm colonization were analyzed by repeated measures analysis of variance (RM-ANOVA) with treatments as a fixed factor and colonization time as a random factor. On each sampling day, significant differences of NA values at different times during a diel cycle were determined using one-way ANOVA followed by a LSD post hoc test. All the statistical analyses were performed with the software SPSS 13.0 for Windows (SPSS Inc., Chicago, USA).

2. Results

2.1. Growth of biofilms

The surface color and morphology of N-def and N-rep biofilms were noticeably different during the experiment period (Fig. 1). In the initial and mid stages, the surface color of N-def biofilms was yellowish-brown, and the surface of N-def biofilms was relatively smooth with fewer small peaks. In the late stages (day 40–52), some green spots appeared on the slides of N-def biofilms. After microscopic identification, the spots were identified as \(N_2\)-fixing heterocystous cyanobacteria. However, in the N-rep treatment, the surface color of biofilms was dark green, the color deepened gradually with the development of biofilms, and the surface of biofilms was uneven with more small peaks.

The biomass (Chlorophyll \(a\), dry weight) and thickness of N-rep biofilms were significantly higher than those of N-def biofilms (\(p<0.01\), Figs. S1 and S2, our published data) (Li et al., 2017). The deficiency of N limited the growth of biofilms, while replete N promoted the growth of biofilms.

2.2. Abundance of nitrogen-cycling genes

2.2.1. Abundance change of \(nifH\) gene
As shown in Fig. 2a, the \(nifH\) gene abundance of N-def biofilms kept increasing during the experiment period, while the abundance of \(nifH\) genes in N-rep biofilms showed an increase-decline-increase tendency, which was likely to be affected by the biomass and microbial community of the biofilms. In general, the abundance of \(nifH\) genes in N-rep biofilms was significantly higher than that of N-def biofilms (RM-ANOVA, \(p<0.01\)). In Fig. 2b, the \(nifH\) gene abundance of N-def biofilms also increased gradually, but its change in N-rep biofilms was totally different. In the N-rep treatment, the \(nifH\) gene abundance increased rapidly from day 13 to day 21, and then decreased rapidly from day 21 to day 31, and the maximum value was obtained on day 21.

2.2.2. Abundance change of \(amoA\) genes
The abundance changes of AOB \(amoA\) genes in biofilms are shown in Fig. 3. When normalized to both surface area (Fig. 3a) and dry weight content (Fig. 3b), the abundance of AOB \(amoA\)
genes in N-def biofilms showed an overall decrease trend, but AOB amoA genes in N-rep biofilms were only detected in the initial 9 days, and the abundance was obviously lower than that of N-def biofilms.

In terms of AOA amoA genes, two pairs of universal primers were tested by gradient PCR, and the annealing temperature ranged from 50 to 60°C. However, AOA amoA genes were not detected in either N-def or N-rep biofilms throughout the whole period of the experiment. In addition, positive controls were carried out in soil samples to verify the results.

2.2.3. Abundance change of nirS and nirK genes
The abundance of denitrifying bacteria was detected by means of nirS and nirK genes. No matter whether normalized to the surface area or dry weight content, the nirS and nirK gene abundances of N-rep biofilms were significantly higher than those of N-def biofilms (p < 0.01, Figs. 4 and 5).

The nirS gene abundance of N-def biofilms increased slowly, with final values of \((7.15 \pm 2.98) \times 10^6\) copies/cm², and that of N-rep biofilms increased rapidly in the initial 21 days, then it remained stable, with final values of \(4.35 \times 10^7 \pm 4.77 \times 10^6\) (Fig. 4a). As shown in Fig. 4b, the nirS gene abundance of N-def biofilms also increased slowly, while the nirS gene abundance of N-rep biofilms kept increasing in the initial 21 days, then started to decline, and the maximum value was obtained on day 21 during the experiment period.

The nirK gene abundance of N-def biofilms maintained a steady and low level during the experiment period, while that of N-rep biofilms kept increasing before day 40 (Fig. 5a). As shown in Fig. 5b, the changes of nirK gene abundance in both N-def and N-rep biofilms over time were irregular. Similar to the nirS genes of N-rep biofilms, the abundance of nirK genes of N-rep biofilms also achieved maximum value in the mid stage of biofilm development.

2.3. Nitrogenase activity
The NA of biofilms during the colonization period was measured on each sampling day (Fig. 6). The NA values were measured after biofilm samples were incubated in the dark for 4 hr. In the N-def treatment (Fig. 6a), biofilms only showed notable NA on day 9 during the initial 21 days, after then NA started to increase rapidly, especially in the late stage of biofilm colonization (day 40–52) with final values of \(C_2H_4 (1448.53 \pm 287.07) \text{ nmol/(hr \cdot \mu g dw)}\). In the N-rep treatment (Fig. 6b), NA was only detected on day 5 with values of \(C_2H_4\).
No NA was detected at other times during the experiment period. In the initial 21 days, NA of N-def biofilms only occurred under dark conditions, but not under light conditions. Therefore, in order to better clarify the level and pattern of NA at different times during a diel cycle, the NA of 26, 31, 40 and 52-day aged N-def biofilms were recorded (Fig. 7). On day 26, NA increased slowly during the 8-hr dark period \((p > 0.05)\), but increased rapidly and persistently upon illumination \((p < 0.05)\,\text{Fig. 7a}\). On day 31 and 40, the level and pattern of NA were similar: (1) NA increased slowly during the 8-hr dark period \((p > 0.05)\); (2) NA continued to increase slowly when entering the light period \((p > 0.05)\); (3) NA showed an overall increase trend during the 16-hr light period except for a slight decrease at the 20th hr, and the final NA values in the light period were significantly higher than NA values in the dark period \((p < 0.05)\). Thus, this may be a turning point for the NA pattern when conversion occurred between dark and light conditions (Fig. 7b, c). On day 52, NA was mainly analyzed at the conversion point of dark and light (Fig. 7d). However, different from other dates, NA decreased when entering the light period \((p < 0.05)\).

### 2.4. Nitrate reductase activity

Nitrate reductase catalyzes the conversion of \(\text{NO}_3^-\) to \(\text{NO}_2^-\). When normalized to surface area (Fig. 8a), NRA occurring in N-def biofilms was always at a very low level, while it kept increasing in N-rep biofilms during the experiment period, and was significantly higher than that of N-def biofilms \((p < 0.01)\). When normalized to dry weight (Fig. 8b), NRA was also low in N-def biofilms, while NRA increased first and then decreased, reaching its maximum value on day 21 in N-rep biofilms.

### 3. Discussion

### 3.1. Nitrogen fixation in biofilms

Biological N fixation is an essential source of N for many oligotrophic waters (Howarth et al., 1988). Nitrogen fixation by microorganisms in biofilms not only supports nutrients for the biofilm itself, but also provides nutrients for the surrounding water. Therefore, it is crucial to explore the regulation mechanism for microbial N fixation in biofilms.
In the present study, compared with N-rep biofilms, the absolute abundance of \( \text{nifH} \) genes in N-def biofilms was relatively lower. However, the N-def biofilms had very high NA, while the N-rep biofilms had no detectable NA most of the time during the experiment period. Therefore, the lack of N did not necessarily lead to abundant nitrogen-fixing genes, and the presence of nitrogen-fixing genes did not mean that they would function (Steppe and Paerl, 2005; Zani et al., 2000). The reason why the absolute abundance of \( \text{nifH} \) genes was higher in N-rep biofilms might be that: firstly, the biomass of biofilms was the main factor determining the abundance of \( \text{nifH} \) genes per unit surface area of biofilms (copies/cm²). The initial microbial community and numbers in the two treatments were exactly the same, but the nutrient concentrations between the two treatments were different during the colonization period. The N-rep biofilms were growth-promoted, with more biomass, while the N-def biofilms were growth-limited, with less biomass. Therefore, obvious biomass differences were found between the two treatments (Figs. S1 and S2) (Li et al., 2017), so this was the possible reason that more numbers of microorganisms with

![Fig. 6 – Change in nitrogenase activity (NA) of biofilms during the colonization period under N-def and N-rep conditions.](image)

![Fig. 7 – Nitrogenase activities of N-def biofilms cultured for 26, 31, 40, 52 days during the colonization period. The circadian rhythms for nitrogenase activity measurement were the same as that of the biofilm incubator. The white bar above the plot shows the light period, the black bar shows the dark period. NA values with different letters are significantly different \((p < 0.05)\).](image)
more functional genes were discovered in the N-rep biofilms. This could be also verified in the nifH gene abundance per unit dry weight (copies/g dw), as the difference between the two treatments was relatively small, especially in the middle and late stage (day 31–52) of biofilm colonization. Secondly, the diazotrophs could survive under N-def condition through N fixation, but might also exist under N-rep conditions with activated or inactivated NA. This was consistent with our published results, reporting that diazotrophs were also detected in N-rep biofilms by Illumina MiSeq sequencing (Li et al., 2017). Previous studies suggested that higher abundance of nifH genes did not necessarily mean a higher rate of nitrogen fixation and higher microbial diversity (Collavino et al., 2014), and there was no correlation between the transcription of nifH genes and NA (Severin and Stal, 2010). In general, the gene abundance to some extent only represented the microbial community functional potential for N fixation and microbial community of the biofilms, and the presence, even transcription, of nifH genes did not necessarily mean that microorganisms would show activated NA and fix N₂ (Severin and Stal, 2010; Woebken et al., 2015). This was because the NA could be regulated on multiple levels such as transcription, translation and post-translational protein modification (Chen et al., 1998; Kim et al., 1999). In general, sufficient N availability could facilitate the biomass accumulation of biofilms with higher abundance of nitrogen-fixing genes in N-rep biofilms, but the deficiency of N could stimulate higher NA in N-def biofilms. Therefore, it was necessary to investigate the N fixation of biofilms on multiple levels, and the metabolic activity of nitrogenase seemed to be particularly important.

Biofilms cultured without exogenous inorganic N showed higher NA, and the biological fixation of N was the most important source of N for N-def biofilms, which maintained the development of N-def biofilms. The N-def biofilms showed high NA at the initial stage (day 9) of biofilm colonization, which was probably a response to the N deficiency. Compared with the initial stage, the NA of N-def biofilms increased gradually at the middle and late stages of biofilm colonization. The nitrogen availability had an effect on not only the N-cycling related microbial community composition, but also their metabolic activity (Lyautey et al., 2013). In our published data, the inorganic N availability was an important factor in shaping the microbial community composition and succession (Li et al., 2017), meanwhile the microbial community structure was a leading factor affecting the metabolic activity of biofilms (Gao et al., 2014). The N₂-fixing heterocystous and non-heterocystous cyanobacteria, such as Calothrix sp., Hapalosiphon sp., Fischerella sp., Aphanizomenon sp. and Leptolyngbya sp., were the dominant taxa of N-def biofilms at the late stage of biofilm colonization (Li et al., 2017); at the same time, the NA of biofilms reached a very high value. This probably meant that these cyanobacteria made a great contribution to the NA.

Cyanobacteria are likely to be regarded as the main contributors to N₂ fixation in biofilms, as they were often morphologically dominant and could survive under N-deficient conditions (Paerl et al., 1991). Actually, the biofilms were composed of a rich assemblage of microorganisms; in addition to cyanobacteria, many bacteria and archaean taxa also contained nitrogenase genes and were responsible for N₂ fixation (Zehr et al., 1995). In the initial 21 days, N-def biofilms only showed NA in the dark, but not in the daytime, so it was non-heterocystous cyanobacteria and bacteria, not heterocystous cyanobacteria, that assumed the dominant N₂-fixing role in this stage. They fixed N₂ in the dark so as to avoid oxygen from photosynthesis, and respiration in the dark could also consume some oxygen to sustain a hypoxic environment (Brauer et al., 2013; Dron et al., 2012). However, ATP produced by photosynthesis was an essential energy source for N₂ fixation (Tucker et al., 2001). From day 26 on, N-def biofilms showed high NA in both light and dark periods, and this suggested that the members responsible for N₂ fixation comprised not only non-heterocystous cyanobacteria and bacteria, but also heterocystous cyanobacteria. This situation was consistent with our published data showing that the abundance of N₂-fixing heterocystous cyanobacteria in N-def biofilms increased rapidly at the late stage (Li et al., 2017). Furthermore, N₂ fixation was also affected by the microenvironments within biofilms, which were complex and dynamic. The mature biofilms would form complex three-dimensional structures, some of which contained micro-oxic or anaerobic microzones, and this would facilitate N₂ fixation. Moreover, some diazotrophs could also create
anaerobic microenvironments through cell aggregation (Belnap, 1996). The microenvironments within biofilms would have particular effects on the N2 fixation of non-heterocystous cyanobacteria and bacteria. Thus, at the late stage of biofilm colonization, it was likely that N2-fixing non-heterocystous cyanobacteria and bacteria also showed NA during the light period. The patterns of NA in biofilms were the sum contributions of all active diazotrophs, and it was hard to identify the contribution of a single species (Yu, 2011). It was thought that the different patterns of NA in the biofilm colonization stage and diel cycle were synthetically influenced by environmental conditions, diazotroph assemblages, the photosynthetic community and biogeochemical cycling and gradients (Steppe and Paerl, 2005).

In general, the deficiency of inorganic N can promote the emergence of diazotrophs, and activate the NA, which guarantees the input of new N to oligotrophic ecosystems. N2 fixation greatly contributes to the growth and development of biofilms under N-def conditions.

3.2. Nitrification in biofilms

Ammonia oxidation plays a crucial role in the cycling and removal of N in aquatic ecosystems (Hou et al., 2013b). The studies of factors influencing ammonia-oxidizing microorganisms (AOA, AOB), including the abundance, diversity and activity, are very important. AOA have been recognized as an important component of the microbial community in performing ammonia oxidation (Reigstad et al., 2008), and the abundance of AOA sometimes even exceeds the abundance of AOB in soils and aquatic systems (Coo len et al., 2007; Hou et al., 2013a; Leininger et al., 2006). However, the present study showed that only AOB were responsible for ammonia oxidation in both N-def and N-rep biofilms. On one hand, the biofilm inocula were collected from a hypertrophic site, and Hou et al. (2013b) suggested that hypertrophic conditions markedly inhibited the growth of AOA, but promoted the growth of AOB as well as nitrification, so the abundance of AOA in the inocula might be very low. On the other hand, it was likely that the environmental conditions of N-def and N-rep treatments were not suitable for AOA growth, as the same environmental factors had different effects on AOA and AOB, and niche differentiation has been detected between AOA and AOB (Wessén et al., 2011).

Massive amounts of NO3− existed in the N-rep treatment, and this would inhibit the process of ammonia oxidation, so this was the possible reason why AOB genes were only detected in the initial stage of biofilm colonization, with much lower values. However, the N2 fixation of N-def biofilms produced some ammonium, which may contribute to biological ammonia oxidation, and it was also reported that the nitrate concentration was negatively correlated to AOB, while the ammonium concentration was positively correlated to AOB (Vila-Costa et al., 2014). The higher AOB gene abundance of N-def biofilms only demonstrated the microbial community functional potential of ammonia oxidation.

3.3. Denitrification in biofilms

Inorganic N availability was an important factor affecting denitrification in biofilms. In the present study, there were high nirS and nirK gene abundances concomitant with high denitrification enzyme activity in N-rep biofilms. This was also consistent with our published data showing that abundant denitrifying bacteria dominated in N-rep biofilms (Li et al., 2017). The situations between N-rep and N-def biofilms were totally opposite: the latter contained significantly lower denitrification gene abundances and activity. This indicated that the replete inorganic N not only promoted the biomass accumulation of N-rep biofilms with higher abundance of denitrification genes, but also stimulated the denitrification enzyme activity.

Despite all this, both the gene abundance and enzymatic activity demonstrated the co-occurrence of denitrification and N2 fixation in N-def biofilms. Halm et al. (2009) also showed the co-occurrence of the two opposite functions in a meromictic lake. As N-def biofilms were cultured without an exogenous inorganic N supply, the NO3−/NO2−, which were the substrates of denitrification, were probably derived from nitrification. Therefore, these results along with the N content change in N-def biofilms and medium (Li et al., 2017) revealed not only the co-existence of N-fixing microorganisms, ammonia-oxidizing bacteria and denitrifiers, but also the co-existence of N2 fixation, nitrification and denitrification in N-def biofilms.

Both nirS and nirK gene abundances and NRA in N-rep biofilms reached maximum values in the mid stage of biofilm colonization, and the denitrifying bacteria analyzed by Illumina MiSeq sequencing, such as Flavobacterium, Aquimonas and an unclassified genus of Rhizobiales, also dominated N-rep biofilms in the mid stage (Li et al., 2017). Therefore, N-rep biofilms showed their strongest denitrification in the mid stage of biofilm colonization. This was probably because the TN contents of the N-rep medium kept decreasing during the experiment period (Li et al., 2017). It could be assumed that a high concentration of inorganic N could promote denitrification in biofilms, and that the denitrification would weaken when the inorganic N concentration decreased. This mode of regulation would benefit the balance of inorganic N availability in aquatic ecosystems.

4. Conclusions

The inorganic N availability of biofilms was found to be an important factor influencing N cycling in aquatic ecosystems. The results indicated that adequate nutrients guaranteed the growth of biofilms, with higher biomass accumulation, offering higher microbial community functional potential. However, the metabolic activity of biofilms was regulated by nutrient availability. The lack of inorganic N could stimulate high NA in N-def biofilms, which then exhibited strong N2 fixation, and the excess N could stimulate NRA in N-rep biofilms, which then exhibited strong denitrification. In summary, the evaluation of the functional processes of N cycling should not only focus on genetic potential, but also the physiological activity of biofilms. This study demonstrated an important fact in establishing the influence of inorganic N availability on both the N-cycling gene abundances and enzyme activities of cultured biofilms, with crucial consequences for the biogeochemical cycle of N in aquatic ecosystems.