Periphytic biofilms function as a double-edged sword influencing nitrogen cycling in paddy fields

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Summary

It remains unclear whether periphytic biofilms are beneficial to N cycling in paddy fields. Here, based on a national-scale field investigation covering 220 rice fields in China, the N accumulation potential of periphytic biofilms was found to decrease from 8.8 ± 2.4 to 4.5 ± 0.7 g/kg and 3.1 ± 0.6 g/kg with increasing habitat latitude and longitude, respectively. The difference in abundant and rare subcommunities likely accounts for their geo-difference in N accumulation potential. The N cycling pathways involved in periphytic biofilms inferred that soil N and N_2 were two potential sources for N accumulation in periphytic biofilms. Meanwhile, some of the accumulated N may be lost via N_2, N_2O, NO, or NH_3 outputs. Superficially, periphytic biofilms are double-edged swords to N cycling by increasing soil N through biological N fixation but accelerating greenhouse gas emissions. Essentially, augmented periphytic biofilms increased change of TN (ΔTN) content in paddy soil from −231.9 to 31.9 mg/kg, indicating that periphytic biofilms overall benefit N content enhancement in paddy fields. This study highlights the contribution of periphytic biofilms to N cycling in rice fields, thus, drawing attention to their effect on rice production and environmental security.

Keywords: Nitrogen accumulation; Geo-distribution imprint; N source; N fate; Microbial effect; N cycling pathway.

Originality-Significance Statement

As prevalent microbial aggregates in paddy fields, it is uncertain whether periphytic biofilms are beneficial to N cycling in paddy fields. This may lead to a misunderstanding of whether the indigenous microecosystem of paddy fields should exist during rice production. Herein, by revealing the sources, fates, and transformation pathways of the accumulated N in periphytic biofilms and then substantiating their overall effect on N cycling in paddy fields, we found that although periphytic biofilms seem to have two-sided effects, they overall benefit N cycling in paddy fields. Our findings extend our understanding of the effect periphytic biofilms have on N cycling in paddy fields. Focusing on this effect may influence global policy-making toward N fertilizer use and management for sustainable rice production with minimum greenhouse gas emissions and non-point source pollution.

Introduction

In China, the high input but low utilization efficiency of N fertilizers has resulted in approximately 47% of the input N to be lost to the environment, negatively affecting the aquatic environment and even the quality of human life (Chen et al., 2014; Sun et al., 2018; Vitousek et al., 2009). Essentially, any effect on the N cycle would affect its utilization and loss. Microbes play key roles in the biogeochemistry of N in the environment (Mushinski et al., 2021; Wang et al., 2020); thus, their potential to intercept N loss and enhance N utilization efficiency is attracting increasing attention (Antonopoulos, 2010; Mooshammer et al., 2014). In nature, microorganisms usually grow in the form of microbial aggregates (Guilhen et al., 2017; Kim & Lee, 2016); for instance, periphytic biofilms are native and ubiquitous microbial aggregates in paddy fields (Sun et al., 2021b).

Bioaccumulation is an important mechanism by which periphytic biofilms affect element cycling in paddy fields (Sun et al., 2022); therefore, periphytic biofilms act as temporary pools of some elements in paddy fields (Liu et al., 2019; Liu et al., 2021; Sun et al., 2021a). However, further evidence is required to understand the impact of biofilms on the N cycle in rice fields. For instance, the potential sources and fates for accumulated N in periphytic biofilms remain unclear. Determining the sources of inputs and fates of outputs of the accumulated N in periphytic biofilms will clarify whether these biofilms are beneficial to N cycling, rice production, and even environmental security.

Currently, our understanding of the underlying mechanisms through which

periphytic biofilms affect the N cycle is insufficient. It is known that the abiotic components of these biofilms, such as extracellular polymeric substances, play key roles in the accumulation of cationic elements, such as manganese (Sun et al., 2021a; Xu et al., 2020). In contrast to the effect of cationic elements, the effect of periphytic biofilms on N cycling may be driven mainly by the biotic components of various microorganisms which, in nature, can be classified as abundant, moderate, and rare (Chen et al., 2019; Jiao et al., 2020). Accumulating evidence has confirmed that rare bacteria drive bacterial community structure changes (Hausmann & Knorr, 2016; Hua et al., 2015), while abundant bacteria contribute to nutrient cycling, carbon flow, and tolerance to environmental stresses (Delgado-Baquerizo et al., 2018; Ji & Kong, 2020). However, there are few related studies on periphytic biofilms grown in paddy fields, resulting in a lack of clarity regarding how the abundant, moderate, and rare microorganisms in these biofilms relate to N accumulation.

Microbe-driven N cycling involves various N cycling pathways, which can be used to predict the N transformation processes and even the fate of the accumulated N in the microbes (Rose et al., 2021; Zhang et al., 2021; Zhou & Xing, 2021). The known pathways in single microorganisms have been well documented (Chen et al., 2013; Zhao et al., 2009); however, little is known about how many N cycling pathways are involved in periphytic biofilms and how these pathways are related to N accumulation, transformation, and the fate of the accumulated N. As periphytic biofilms are rich in microbial components, such as bacteria, microalgae, and fungi (Sun et al., 2021a; Xu et al., 2020), we speculate that they may have complicated N cycling pathways,

promoting the accumulation and transformation of N. Moreover, the N cycling pathways may potentially influence the fate of outputs of the N accumulated in them.

To further our understanding of the influence of periphytic biofilms on the N cycle in rice fields, in this study, we collected 220 periphytic biofilms nationwide in China and analyzed their N content. Thus, the geo-distribution patterns of their N accumulation potential across China were revealed, and their driving factors from three subcommunities of the abundant, moderate, and rare taxa (including prokaryotes and eukaryotes) were explored. Furthermore, the potential sources and fates of accumulated N in periphytic biofilms were analyzed by constructing the involved N cycling pathways. Finally, the synthetic effect of periphytic biofilms on the N biogeochemistry in paddy soil was verified by on-farm experiments. This study addressed the following questions: (1) What are the potential sources of inputs and fates of outputs of the accumulated N in periphytic biofilms? and (2) Are periphytic biofilms beneficial or not to N cycling in paddy fields?

Experimental procedures

Sample collection across China

For periphytic biofilm collection across China, paddy fields covering three temperature zones (tropical, subtropical, and temperate) and distributed across 22 cities and 12 main rice-planting provinces (Fig. S1A and Table S1) were selected. In each sampling city, 10 paddy fields were selected within a radius of 1 km, and one periphytic biofilm was collected from each paddy field. The 10 samples from each sampling city

were numbered 1–10. In total, 220 paddy fields, distributed from southern to northeastern China and western to eastern China, were selected to collect periphytic biofilms with collection based on our previous methods (Fig. S1B) (Sun et al., 2021a). All samples were collected within 7–15 d after the rice seedlings had been transplanted.

On-farm verification experiment

To verify the synthetic effect of periphytic biofilms on the biogeochemistry of N in paddy soil, field experiments were carried out in Jurong, Jiangsu, China (31.97° N, 119.35° E) in 2021. Before the experiment, artificial carriers that can augment the growth of these biofilms in paddy fields were prepared based on our previous method (Sun et al., 2021a). The carriers were applied immediately after rice transplanting with a dose of 30 kg/ha, and a parallel field without the carriers was set as the control. Both the experimental and control fields were 0.7 ha and sample collection was performed in triplicate. By artificially inducing the difference in biofilm growth in the paddy fields, the effect of periphytic biofilms on the biogeochemistry of N was compared. During the entire rice growth period, the paddy soils of experimental and control fields were collected weekly to detect N content (including total N, NO_3^--N, and NH_4^+-N) (Bačnik et al.), and the first sampling date was at the day of transplanting (June 11, 2021). The soil N content collected for the first time was set as the baseline, and the change in the N content (ΔN) of each field at any sampling date was calculated using the following equation:

$$\Delta N_i = N_i - N_0 \qquad \text{Eq. 1}$$

where i represents any soil sampling date; ΔN_i represents the change in soil N content between the sampling date and date before rice transplantation i; N_i is the soil N content at sampling date i; and N_0 is the soil N content collected before rice transplantation (June 11, 2021).

16S and 18S high throughput sequencing and data analysis

For the 10 periphytic biofilms collected from each sampling city, 1 g was sampled, mixed well, and then divided into three samples, which were used for high-throughput sequencing, and the results were used to represent the prokaryotic and eukaryotic communities in the periphytic biofilms from each sampling city. To determine the prokaryotic and eukaryotic composition of periphytic biofilms, the primers 16S V4: 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′); and 18SV4: 528F (5′-GCGGTAATTCCAGCTCCAA-3′) and 706R (5′-AATCCRAGAATTTCACCTCT-3′) were used with the HiSeq 2500 sequencing platform. After sequencing, the raw data were processed as follows: (1) paired-end raw reads data filtering using Trimmatic software (v0.33) was used to filter the quality of the double-ended raw reads data and to filter the reads containing N, those with a quality value less than 20, and those with a filtered sequence length less than 100 bp. Then, the primers at both ends of the v1.barcode and motire1.barcode were allocated to the corresponding primer sequence in the sample. (2) Paired-end clean reads splicing was conducted on the double-ended sequencing data, according to the overlapping relationship between paired-end reads.

Flash v1.2.11 software was used to splice each pair of paired-end reads into a sequence, and the minimum overlap length was set to 10 bp, the maximum mismatch ratio allowed in the overlap area of the splicing sequence was 0.1, and unqualified tags were filtered and original raw tags were obtained. (3) For raw tag sequence quality filtering, mothur V1.35.1 was used to control and filter the quality of spliced sequences to obtain effective spliced fragments (clean tags). Sequence analysis was performed using UPARSE and sequences with ≥97% similarity were assigned to the same operational taxonomic units (OTUs). For each representative sequence, the SILVA (https://www.arb-silva.de/) database was used to annotate taxonomic information. Microbial sequences were deposited in the NCBI database with the accession number PRJNA854289. The OTUs were classified as follows: abundant taxa were defined as OTUs with a relative abundance of ≥1%; rare taxa, <0.01%; and moderate taxa, within 0.01–1% (Chen et al., 2019).

High throughput qPCR gene chip

For the 10 periphytic biofilms collected from each sampling city, 1 g was sampled, mixed well, and then divided into three samples which were used for high-throughput qPCR on a Wafergen Smart Chip Real-Time qPCR platform, which can detect a total of 22 N cycling genes (e.g., *gdh*, *ureC*, *hao*, etc.). Each DNA sample was amplified with a threshold cycle value of 31 as the detection limit. Three replicates were set for each prepared sample and nine samples were taken from each sampling city. Total DNA was extracted using DNA extraction kits (MOBIO 12888-100, Carlsbad, CA, USA),

192 then purified and analyzed. Qualified samples and the reagent used for qPCR were
193 added to a 384-well plate as the sample source plate, and the primers and reagents used
194 for qPCR were added to another 384-well plate as the assay source plate. The reagents
195 of the sample and assay source plates were added into the nanopores of the chip of the
196 high-throughput qPCR using high-throughput automatic micro-sampling equipment,
197 followed by qPCR and fluorescence signal detection in the SmartChip Real-Time PCR
198 System. Amplification and dissolution curves were automatically generated. The
199 detection status and amplification cycle numbers (C_t) of each gene in each sample were
200 obtained using Canco software, and the data were standardized with 16S rRNA as an
201 internal reference to obtain the relative quantification of these. The absolute
202 quantitative information of the 16SrRNA gene was obtained using a Roche instrument,
203 and after conversion, the absolute quantitative information of other genes was obtained.
204 The quality control of raw data was conducted based on the C_t value obtained with the
205 SmartChip real-time PCR System and Canco software, and the quality control basis
206 was as follows: (1) the gene was discarded when the amplification efficiency was <1.8
207 or >2.2; (2) the gene was discarded when the negative control was amplified; and (3)
208 when the C_t value was >31, it was considered that there was no amplification, and the
209 C_t value of the gene in the corresponding sample was discarded.
210
211 *N content detection and statistical analysis*
212 Before N content detection, each periphytic biofilm (dry weight, 0.5 g) was
213 digested with HNO_3-H_2O_2 in a digestion oven (JKXZ06-8B, China), and the TN, NH_4^+-

214 N, and NO_3^--N content was detected using a flow analyzer (FS3700, OI Analytical,
215 USA). For periphytic biofilms collected at the spatial scale, the TN content was detected,
216 while for those collected from the on-farm experiments, the parameters of the TN,
217 NH_4^+-N, and NO_3^--N contents were detected. A 3D mesh-plot, prepared using
218 Sigmaplot 10.0 (Systat Software, Inc., UK), was used to exhibit the geo-distribution
219 patterns of the N accumulation potential of periphytic biofilms across China. The
220 diversities of abundant, moderate, and rare prokaryotes and eukaryotes were analyzed
221 and visualized using a circular heatmap. The overall effects of these on N accumulation
222 in periphytic biofilms were analyzed using partial least squares path modeling (PLS-
223 PM) in R version 3.5.1 with the 'psych' package. The PLS-PM model was evaluated
224 using the parameter of goodness of fit, and the estimates of path and determination
225 coefficients were validated by performing bootstrapping (br = 199). Correlation
226 analysis was further used to explore which microorganisms, at the genus level,
227 significantly affected N accumulation in periphytic biofilms (visualized using a
228 heatmap). Regression analysis was used to explore the N content geo-distribution
229 patterns and the absolute abundance of *ureC* in periphytic biofilms along the longitudes
230 and latitudes of their habitats, as well as the relationship between the absolute
231 abundance of functional genes (*nifH* and *ureC*) and the N content in periphytic biofilms
232 (visualized using R). PLS-PM was also used to analyze the potential source of
233 accumulated N in these biofilms from paddy fields. Raincloud plots were employed to
234 visualize the results of the on-farm experiments (https://www.omicshare.com/tools).
235 All statistical procedures were conducted using SPSS software (version 16.0; SPSS Inc.,

Chicago, IL, USA).

Results

Geo-imprints of N accumulation potential of periphytic biofilms across China

The N accumulation potential of periphytic biofilms showed a significant geo-distribution imprint along their habitat longitudes and latitudes (Fig. 1). On the latitudinal scale, the potential decreased from 8.8 ± 2.4 to 4.5 ± 0.7 g/kg with increasing latitude from 18°46′ N to 47°16′ N of their habitats across China ($p < 0.001$, $r = -0.35$, Fig. 1). The results indicated that the N accumulation potential of periphytic biofilms decreased along the habitats from southern to northern China. Similarly, on a longitudinal scale, the N accumulation potential decreased from 8.8 ± 2.4 to 3.1 ± 0.6 g/kg with increasing longitude from 108°88′ E to 127°17′ E of their habitats across China ($p < 0.001$, $r = -0.26$, Fig. 1). Thus, periphytic biofilms grown in western China have higher N accumulation potential than those in eastern China.

[Figure 1 here]

Difference in microbial subcommunities accounts for geo-differences in N-accumulation potential

A total of 839 genera of prokaryotes were detected in the 220 periphytic biofilm samples. Among them, 19 genera were abundant (relative abundance > 1%), 99 genera were moderate (1% > relative abundance > 0.1%), and the remaining 719 genera were

rare (relative abundance < 0.1%, Fig. 2A). Specifically, 13 genera of abundant prokaryotes (e.g., *Acinetobacter* sp., *Sphingomonas* sp., and *Massilia* sp.), 46 genera of moderate prokaryotes (e.g., *Anaeromyxobacter* sp., *Bradyrhizobium* sp., and *Roseiflexus* sp.), and 164 genera of rare prokaryotes (e.g., *Microcystis* sp., *Azospirillum* sp., and *Mycobacterium* sp.) showed significant correlations with the N accumulation potential of periphytic biofilms ($p < 0.05$). Among them, three genera of abundant prokaryotes (*Comamonas* sp., *Bacillus* sp., and *Acinetobacter* sp.), six genera of moderate prokaryotes (*Pseudoxanthomonas* sp., *Microvirga* sp., *Methanobacterium* sp., *Christensenellaceae_R-7_group*, *Brevundimonas* sp., and *Stenotrophomonas* sp.), and 42 genera of rare prokaryotes (e.g., *Aestuariibaculum* sp., *Trichormus* sp., and *Sinibacillus* sp.) showed positive relationships with the N accumulation potential of periphytic biofilms (Fig. 2C).

[Figure 2 here]

Eukaryotes in periphytic biofilms mainly consisted of microeukaryotes and meiofauna (Fig. 2B), and the biodiversity of eukaryotes was lower than that of prokaryotes. A total of 207 genera of eukaryotes were detected in the 220 periphytic biofilms, and among them, 17 genera were abundant, 45 were moderate, and the remaining 145 were rare (Fig. 2B). Correlation analysis results showed that four genera of the abundant eukaryotes (*Epitobrilus stefanskii*, *Korotnevella* sp., *Vermamoeba* sp., and uncultured *Hartmannellidae* sp.), 20 genera of the moderate eukaryotes (e.g.,

Chlamydomyxa sp., *Microascaceae* sp., and *Petalomonas* sp.), and 16 genera of the rare eukaryotes (e.g., *Cryptosporidium* sp., *Trichocomaceae* sp., and *Cercomonas* sp.) were markedly correlated with the N accumulation potential. Among these, one abundant, six moderate, and nine rare eukaryotes showed positive relationships with N accumulation potential (Fig. 2D).

PLS-PM analysis was used to reveal the overall effect of the three subcommunities (abundant, moderate, and rare) of prokaryotes and eukaryotes on N accumulation potentials of periphytic biofilms across China. Both abundant prokaryotes and eukaryotes showed direct and positive effects (path coefficients of 0.31 [$p = 0.02$] and 0.16 [$p = 0.03$] for prokaryotes and eukaryotes, respectively), while both rare prokaryotes and eukaryotes showed the largest but negative direct effects (path coefficients of −0.92 [$p < 0.001$] and −0.79 [$p < 0.001$] for prokaryotes and eukaryotes, respectively). In comparison, the direct effects of moderate prokaryotes and eukaryotes on N accumulation was negative but small (path coefficients of −0.07 [$p = 0.82$] and −0.06 [$p = 0.79$] for prokaryotes and eukaryotes, respectively, Fig. 3). Thus, the difference in abundant and rare microbes in periphytic biofilms potentially accounted for their difference in N accumulation potentials across China, as well as showing contrasting effects.

[Figure 3 here]

Potential input sources of accumulated N in periphytic biofilms

Based on the results of high-throughput qPCR gene chips, 20 N cycling genes with high absolute abundance were detected in periphytic biofilms (Fig. 4A). Among them, *ureC* had the highest absolute abundance, ranging from 2065 to 2 361 923, with a mean value of 786 043 ± 617 516 copies/g biofilm (Fig. 4A). Furthermore, correlation analysis results showed that the absolute abundance of *ureC* significantly correlated with N content in periphytic biofilms ($p = 0.01$, $r = 0.21$, Fig. 4B), providing evidence that they can potentially assimilate N from hydrolyzed chemical N fertilizer (urea). Thus, N in paddy fields is one potential source of the accumulated N in periphytic biofilms. Furthermore, PLS-PM results indicated that N in soil is the first potential source for the accumulated N in periphytic biofilms (path coefficient = 0.31, $p < 0.001$, Fig. S2). More importantly, the geo-distribution pattern of *ureC* in periphytic biofilms was consistent with that of the N content in periphytic biofilms, which decreased with increasing latitude ($p = 0.02$, $r = -0.21$, Fig. 4C). This finding further provides a genetic interpretation of the regional differences in the N accumulation potential of periphytic biofilms.

[Figure 4 here]

nifH also had a high absolute abundance, varying from 121 to 1 317 297 copies/g biofilm, with a mean value of 125 240 ± 201 421 copies/g biofilm (Fig. 4A). The absolute abundance of *nifH* also showed a significantly positive correlation with N content in periphytic biofilms ($p = 0.01$, $r = 0.40$, Fig. 4D), implying that biological N

fixation may be another mode of N accumulation; thus, N_2 may be another source of the accumulated N in periphytic biofilms.

Potential output fates of the N in periphytic biofilms

Based on the results of high-throughput qPCR gene chips, we identified the potential N cycling pathways in periphytic biofilms, which included nitrification and denitrification, ammoxidation, anaerobic ammoxidation, N assimilation, nitrate dissimilation reduction, and biological N fixation (Fig. 5). Among these N cycling pathways, only two—biological N fixation and N assimilation—contribute to N accumulation. The remaining N cycling pathways promote the mutual transformation of different forms of nitrogen in periphytic biofilms, even causing some nitrogen loss.

[Figure 5 here]

Accompanied by the complicated N cycling pathways in periphytic biofilms, some gaseous intermediates or end products may be produced, causing N loss. For instance, as a product of denitrification, nitrous oxide reduction, ammoxidation, and anaerobic ammoxidation reactions, N is released into the air in the form of N_2. As an intermediate product of the denitrification reaction and nitrate reduction, N is lost via N_2O, and NO and NH_3 are produced during nitrite reduction, which would also overflow from periphytic biofilms. Thus, some of the N in periphytic biofilms may be lost in the form

of N_2, N_2O, NO, and NH_3 during the N cycling processes; however, the exact magnitude of each loss still needs to be further verified.

Synthetic effect of periphytic biofilms on N biogeochemistry in paddy soil

Based on the results of the on-farm experiments, TN content in the experimental field soil increased by 290.4 mg/kg at the end of the rice growth period, of which the average value of ΔTN was 31.9 mg/kg throughout sampling; however, in the control field soil, TN content decreased by 329.6 mg/kg at the end of rice growth period, and the average value of ΔTN was -231.9 mg/kg throughout sampling (Fig. 6a). The results indicated that augmented periphytic biofilms in paddy fields could enhance TN content in the soil. Furthermore, the increase in NH_4^+-N, in particular, and NO_3^--N may partially account for the increase in TN. Throughout sampling, the averages of ΔNH_4^+-N in the control and experimental fields were −8.5 and −2.7 mg/kg, respectively (Fig. 6B). Similarly, the averages of ΔNO_3^--N in the control and experimental fields were −0.9 and 0.2 mg/kg, respectively (Fig. 6C). The results indicated that augmented periphytic biofilms could potentially enhance the N content in paddy soil, that is, the more they occurred in paddy fields, the more N (TN, NH_4^+-N, and NO_3^--N) content formed in the soil. Therefore, periphytic biofilms are beneficial overall to N cycling in paddy fields.

[Figure 6 here]

Discussion

Here, by determining the sources of inputs and fates of outputs of the accumulated N in periphytic biofilms, our findings emphasize that they may potentially increase the N content in rice fields by biological N fixation, while also potentially accelerating greenhouse gas emissions (Zhao et al, 2021). On-farm experiments substantiated that the overall effect of periphytic biofilms on N cycling in paddy fields is positive.

To verify whether it is common for periphytic biofilms to affect N cycling in rice fields, we investigated and revealed the geo-distribution patterns of their N accumulation potential in different paddy fields across China. Our results are of practical importance as they indicate that it is feasible to regulate N cycling by regulating the growth of periphytic biofilms in paddy fields (Zhao et al, 2021). However, rice fields are distributed zonally (Jonai & Takeuchi, 2014); therefore, we investigated whether different paddy fields manipulate these biofilms by varying measures. Our findings showed that periphytic biofilms in different paddy fields varied in their effect on N cycling, suggesting that rice producers in different rice-growing areas should implement different measures to regulate them and improve N cycling in paddy fields.

Periphytic biofilm is essentially a microbial aggregate (Liu et al., 2019; Liu et al., 2021; Sun et al., 2021a), and understanding their diversity, composition, and function (e.g., N accumulation and tansformation) is crucial for exploiting biofilms for sustainable rice production (Xiong and Lu, 2022). Every microbe needs N to grow; thus, all periphytic biofilm microbes influence their accumulated N. This study determined that N accumulation by periphytic biofilms is a microbe-driven process, as verified using 16S/18S high-throughput sequencing and high-throughput qPCR gene chips.

From a microbial community perspective, abundant prokaryotes and eukaryotes mainly contribute to N accumulation in these biofilms; however, rare taxa showed significantly negative effects. These findings are consistent with the traditional understanding that abundant bacteria usually contribute to nutrient cycling (Delgado-Baquerizo et al., 2018; Ji & Kong, 2020). By contrast, rare bacteria represent most of the Earth's biodiversity and play important roles in maintaining the stability of crop mycobiomes and ecosystem functions (Xiong et al., 2021). They have been identified as driving spatial and temporal structural changes of bacterial communities in various ecosystems (Hausmann & Knorr, 2016; Hua et al., 2015) and maintaining continuous ecosystem functioning (Caron et al., 2009). Therefore, the difference in abundant and rare microbes mainly induces the difference in the N accumulation potential of periphytic biofilms across China. In further work, the keystone taxa of these biofilms in N accumulation may be identified by multi-omics techniques (Xiong and Lu, 2022).

The high diversity of microbes in periphytic biofilms implies that they may contain complicated N cycling pathways. Here, we detected 20 cycling genes that are highly abundant in periphytic biofilms and constructed the involved N cycling pathways. Accordingly, we can conclude that there are two modes of periphytic biofilm N accumulation: assimilating N fertilizer (supported by the high absolute abundance of *ureC*) and biological N fixation (supported by that of *nifH*). The significant correlation between the absolute abundance of *ureC* and the N content in these biofilms (Fig. 4B) provides evidence for N accumulation by assimilating chemical N fertilizer (urea) from paddy fields. This is because *ureC* encodes urease, which catalyzes the hydrolysis of

411 urea to release ammonia, promoting the accumulation of N fertilizer by periphytic
412 biofilms (Alonso-Sáez et al., 2012; Conthe et al., 2018). PLS-PM results indicated that
413 soil N is a potential source for the accumulated N in these biofilms, which seems to
414 influence N cycling in paddy fields (Chen et al., 2014; Sun et al., 2018; Vitousek et al.,
415 2009). Additionally, another mode (biological N fixation) of N accumulation by these
416 biofilms can be identified based on the high absolute abundance of *nifH* in them (Chua
417 et al., 2014; Warshan et al., 2016) and the significant correlation between *nifH* and N
418 content (Fig. 4D). Overall, biological N fixation may enhance the N content in paddy
419 soils; however, the respective contributions of the two N accumulation modes should
420 be further quantified using ^{15}N tracing experiments.

421 Functional genes are commonly used biomarkers to forecast the potential process
422 and reactions involved in element cycling (Tang et al., 2022); therefore, the N cycling
423 pathways in periphytic biofilms are expected to be involved as there are many reactions
424 driving the transformation between different N forms. For instance, the prevalence of
425 anaerobic ammoxidation can be demonstrated by the high abundance of *hzsB* (Dietl et
426 al., 2015; Yang et al., 2015). *hao*, *amoA1*, *amoA2*, and *amoB* are marker genes of
427 ammonia-oxidizing bacteria that contribute to the ammoxidation pathway (Soler-Jofra
428 et al., 2020; Su et al., 2021; Vajrala et al., 2013). *nxrA* in nitrite-oxidizing bacteria
429 encodes a key enzyme responsible for the oxidation of NO_2^- to NO_3^- (Franck et al.,
430 2008). Nitrite reductases, such as *nirK1*, *nirK2*, *nirS1*, *nirS2*, and *nirS3*, are functional
431 markers for investigating denitrification reactions (Rose et al., 2021; Zhang et al., 2021;
432 Zhou & Xing, 2021). *narG*, *nasA*, and *napA* are key genes representing nitrogen

433 dissimilation reactions (Asamoto et al., 2021; Pisarenko et al., 2013), and *nosZ1* and
434 *nosZ2* are responsible for reducing nitrous oxide to nitrogen (Conthe et al., 2018). Four
435 forms of gaseous nitrogen (N_2, N_2O, NO, and NH_3) may be produced by the
436 abovementioned reactions, leading to N loss in periphytic biofilms. We have previously
437 substantiated and quantified this N loss via NH_3 (Zhao et al, 2021); however, further
438 studies are needed to quantify the amount of N loss via the three other forms of gaseous
439 nitrogen.

440 Overall, the sources of inputs and fates of outputs of the N accumulated by
441 periphytic biofilms in paddy fields were confirmed in this study. Furthermore, based on
442 on-farm experiments, we found that the synthetic effect of these biofilms on the
443 biogeochemistry of N in paddy fields is exerted by enhancing TN, NH_4^+-N, and NO_3^--
444 N content in the soil. Thus, although these biofilms functions as a double-edged sword
445 through their N cycling influence in paddy fields, the advantages (increase in N content
446 by biological N fixation) may outweigh the disadvantages (acceleration of greenhouse
447 gas emissions), and their overall effect on the N cycle is therefore beneficial.

448 This study also points to a need for additional work to be done. First, direct
449 evidence on how much N accumulates in the biofilm from N_2 and soil N should be
450 quantified. By doing this it may be possible to discover a new N fate in paddy fields as
451 4–22% of the input N fates are currently unclear (Chen et al., 2013; Dong et al., 2012;
452 Ghoneim et al., 2008; Ju et al., 2009; Koyama, 1981; Tan et al., 2015; Zhao et al., 2009;
453 Zhu & Chen, 2002). Second, further attention should be given to how much N in
454 periphytic biofilms would be lost via gaseous N, such as N_2, NO, NH_3, and N_2O.

Gaseous N emissions, such as NO and N_2O, may intensify climate problems, such as the greenhouse effect (Chen et al., 2013; Dong et al., 2012; Shan et al., 2016; Zhao et al., 2009). Anaerobes participate in different biogeochemical processes of ecological and environmental significance (Prakash, 2022); thus, whether nitrite-dependent anaerobic methane oxidation processes contribute to N cycling in periphytic biofilms needs investigating as this may provide novel strategies to reduce N loss (Gómez-Gallego, 2022). Based on the double-edged sword effect of periphytic biofilms on N cycling in paddy fields, further focus on this is needed. The results may aid in greatly improving N cycling in rice fields for sustainable rice production via the regulation of periphytic biofilms to increase their N fixation potential and reduce gaseous N emissions.

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Data availability statement

Supplementary information is available in the online version of the paper. Correspondence and requests for materials should be addressed to Yonghong Wu (yhwu@issas.ac.cn).

Conflict of interest

The authors declare no competing interests.

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651 **Supporting information**

652 Additional Supporting Information may be found in the online version of this
653 article at the publisher's web-site:

654 **Fig. S1** Map of the 22 sampling areas located in different rice growing areas across
655 China (A), and diagram of periphytic biofilm collection (B). The 22 sampling areas
656 are TS: Taishan, RH: Renhua, QZ: Quanzhou, FZ: Fuzhou, NP: Nanping, CS:
657 Changshu, YC: Yancheng, NB: Ningbo, HZ: Hangzhou, WH: Wuhu, WHA: Wuhan;
658 CZ: Chizhou, YY: Yueyang, JZ: Jingzhou, YiC: Yichang, JJ: Jiujiang, YT: Yingtan, TL:
659 Tieling, DD: Dandong, WC: Wuchang, QQHR: Qiqihar; and LD: Ledong.

660 **Fig. S2** Potential source analysis of N accumulated in periphytic biofilms from paddy
661 fields. A) Partial least squares path modeling analysis. Soil N, Floodwater N, and
662 Biofilm N represent N in soil, floodwater, and periphytic biofilm, respectively. P values
663 in brackets represent the correlation between the two factors. Goodness of fit value of
664 the model was 0.3891.

665 **Table S1** Detailed information for the sample collections

666

667

668 **Figure Captions**

669 **Fig. 1** Geographical imprints of N accumulation potential of periphytic biofilms in
670 paddy fields (Z-axis) along with the latitudes (X-axis) and longitudes (Y-axis) of their
671 habitats in China.

672

673 **Fig. 2** Community structure of the top 10 abundant (blue), moderate (black), and rare
674 (red) subcommunities of prokaryotes (A) and eukaryotes (B) in periphytic biofilms,
675 presented at the genera level using circo heatmap; community structure of abundant
676 (blue), moderate (black), and rare (red) subcommunities of prokaryotes (C) and
677 eukaryotes (D) and their positive correlation with the N content in periphytic biofilms,
678 presented at the genera level using correlation heatmap. In panels A and B, the color
679 represents the size of species abundance; In panels C and D, * indicates $p < 0.05$, **
680 indicates $p < 0.01$, and *** indicates $p < 0.001$.

681

682 **Fig. 3** Synthesis of the overall effects of abundant (*abu.*), moderate (*mod.*), and rare
683 prokaryotes (A) and eukaryotes (B) on N content in periphytic biofilms, analyzed by
684 PLS-PM. The goodness of fit values of PLS-PM in A and B were 0.6284 and 0.4184,
685 resepctively. Blue and red arrows in the model indicate a positive and negative effect,
686 respectively.

687

688 **Fig. 4** N cycling genes in periphytic biofilms. (A) Absolute abundance of N cycling
689 genes; (B) correlation between the absolute abundance of the *ureC* gene and the N

690 content; (C) regression analysis between the absolute abundance of *ureC* and the
691 absolute latitude of the habitats of periphytic biofilms; and (D) correlation between the
692 absolute abundance of the *nifH* gene and the N content.
693
694 **Fig. 5** Postulated N cycling pathways in periphytic biofilms, based on functional gene
695 analysis. Blue lines represent the potential sources of inputs, while red dotted lines show
696 the potential fates of outputs of the accumulated N.
697
698 **Fig. 6** Synthetic effect of periphytic biofilms on TN, NH_4^+-N, and NO_3^- -N in paddy
699 soils. In the experimental fields, the growth of the biofilm was artificially increased,
700 while in the control field, it grew naturally. In the figures, the nuclear density curve
701 shown by the cloud plot was used to display the distribution status of original data. Jitter
702 scatters represent the dispersion degree of data. The gray lines connect the average
703 values in boxplots and could intuitively compare the data of experimental and control
704 soil.