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# Metagenomic data highlight shifted nitrogen regime induced by wetland reclamation

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## Abstract

Natural wetlands are mostly nitrogen-limited ecosystems, while reclamation stimulates the loss of nitrogen (N) in soils by shifting the N regime. To investigate the microbial mechanisms of the N regime shift, we first conducted a global meta-analysis to quantify the wetland reclamation impacts on soil mineral N pools and then a field campaign to sample 24 soil cores up to 100 cm depth in a natural wetland and a 23-year cultivated soybean field from the Sanjiang Plain in northeastern China. After wetland reclamation, the N regime was shifted to cause a potential risk of massive N loss in soils; their microbial mechanisms were revealed through metagenomic data. In cropland, the relative abundance of genes involved in nitrification and assimilatory nitrate reduction to ammonia (ANRA) were enriched while those in N fixation, mineralization, denitrification, and dissimilatory nitrate reduction to ammonia (DNRA) were diminished. Wetland reclamation substantially enhanced the relative abundance of genes involved in nitrification (except for genes for ammonia oxidation to $NH_2OH$) and denitrification in surface (0–30 cm) soils but decreased them in subsurface (30–100 cm) soils. After wetland reclamation, the relative abundance of genes involved in denitrification and DNRA significantly reduced in spring and summer, but such patterns were not found in autumn and winter. This change enhanced potential microbial-driven N loss in spring and summer. The metagenomic data serve as surrogate data sources for quantifying soil roles on soil N cycles under land use change.

**Keywords** Nitrogen cycling · Metagenomics · Functional gene · Wetland reclamation

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## Introduction

Nitrogen (N) is an essential element for all living organisms (Laine et al. 2018; Singh 2021; Zhang et al. 2019) and the N cycles through various components on the Earth (Dong et al. 2020; Galloway et al. 2004). However, climate change and human activities have dramatically altered the N cycle, which caused substantial environmental problems in the past century (Dong et al. 2020; Galloway et al. 2008; Wu et al. 2022). For example, drought stimulates N mineralization but inhibits nitrification (Stark and Firestone 1995), reducing the nitrous oxide ($N_2O$) emissions (Hartmann et al. 2013); N loss exacerbated cropland yield, and $N_2O$ emissions caused climate warming (Bahram et al. 2022); meanwhile, the N loading to water bodies has caused eutrophication in aquatic ecosystems (Choudhury et al. 2018; Song et al. 2022). Land use change can cause a massive N loss (Sun et al. 2015), significantly affecting the ecosystem functioning

(Arora-Williams et al. 2018; Nelson et al. 2015), such as ecosystem productivity (Singh and Gupta 2018).

Wetlands play a disproportional role in the N cycle (Fang et al. 2019), removing at least 11% of natural reactive N inputs worldwide (Finlay 2020; Jordan et al. 2011). However, due to the enormous demand for food (Reis et al. 2017), more than half of the natural wetlands have been artificially converted to other ecosystems (Davidson 2014; Pang et al. 2020). For instance, 90% of natural wetlands have been converted to agricultural lands in Brazil, and 50% have been converted to agricultural lands in Europe and North America (Borzée et al. 2018). While wetland reclamation yields economic benefits (Stagnari et al. 2017), it poses serious environmental problems (Li et al. 2014a). The biogeochemical cycling processes were shifted after wetland conversion to cropland, accelerating carbon (C) and N losses (Liu et al. 2020; Post and Kwon 2000; Yang et al. 2019). A global synthesis shows that wetland conversion to upland substantially shifted the N regime, halving $NH_4^+$-N content while doubling $NO_3^-$-N content (Fig. 1 and Sect. "Meta-analysis of reclamation effects on soil inorganic N"). However, the mechanisms determining the soil N dynamics during land use change remain elusive.

Land conversion shifts the N cycle by altering a suite of soil microbial processes (Hua et al. 2017; Ma et al. 2020; Wan et al. 2021). Studies have reported the substantial impacts of wetland conversion to croplands on the abundances of functional genes encoding N fixation (Bannert et al. 2011), nitrification (Jiang et al. 2013), denitrification (Senbayram et al. 2022; Zhang et al. 2019), and mineralization (Jiang et al. 2013). However, most studies have focused on a single process of the N cycle, risking a potentially biased understanding of the overall quantification of the N cycle. Soil microbes for N cycles are highly sensitive to abiotic and biotic properties in soils that vary over seasons (Bolaños et al. 2021; Dickens et al. 2015). The absolute and relative abundance of nitrifying genes are more resistant to seasonal variation than denitrifying genes (Chen et al. 2017; Nelson et al. 2020). Generally, studies have focused exclusively on the topsoil of 0–30 cm, where the microbial biomass, activity, and diversity are the greatest compared to other depths (Jiao et al. 2018; Xu et al. 2013). Subsurface soils (i.e., deeper soils than 30 cm) may contain greater microbial biomass and harbor more diverse microbes than top soils (Bu et al. 2020). Castellano-Hinojosa et al. (2018) found that the absolute and relative abundance of the nitrifiers shrunk along soil profiles, but denitrifier abundance enriched with depths. Thus, a comprehensive understanding of the N-cycling microbes along soil profile is urgently needed to provide a scientific basis for better managing soil N under land use change.

Changes in the N regime and losses of soil N level upon cultivation have been widely observed (Coskun et al. 2017; McIntosh et al. 1997; Raiesi 2006). However, in which form, how much, and the microbial mechanisms the N released are still unclear (Yin et al. 2022). In this study, we aim to develop a complete understanding of wetland reclamation-induced N cycle shift and its underlying microbial mechanisms. By combining metagenomic data and statistical tools, we analyzed the effects of wetland reclamation on abundance of 59 KOs involved in 7 N-cycling pathways and examined their seasonal variations along 100 cm soil profiles. We hypothesized that: (1) wetland reclamation stimulates N loss, which would be companied with shifting

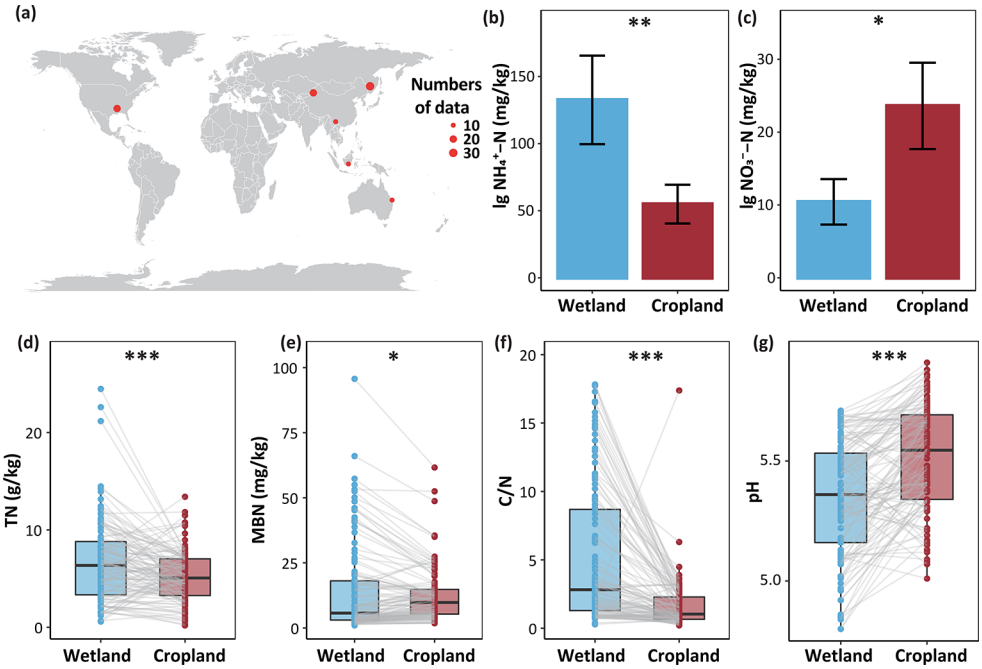


**Fig. 1** Effects of wetland reclamation on soil N and other environmental factors. (a) location of studies used in meta-analysis. Changes of soil $NH_4^+$-N (b) and $NO_3^-$-N (c) contents during wetland reclamation in meta-analysis. Changes of total soil N (TN) content (d), microbial biomass N (MBN) content (e), the ratio of C to N (C/N) (f), and pH (g) during wetland reclamation from our field experiments. Significance levels are denoted with *: $p < 0.05$; **: $p < 0.01$; and ***: $p < 0.001$

in the relative abundance of corresponding functional gene encoding N cycling; (2) the mechanism of microbial-driven N loss varied over seasons; and (3) land conversion leads to a risk of N loss by N-cycling genes along all soil depth, while different between surface and subsurface soils due to stronger disturbance in topsoil.

## Materials and methods

### Study site, soil sampling, and physicochemical analysis

This study was carried out at the Sanjiang Mire Wetland Experimental Station of the Chinese Academy of Sciences, Heilongjiang Province, China (47°35′ N,133°31′ E). Sanjiang Plain was dominated by natural wetlands before the 1950s (Xu and Tian 2012); however, more than 75% of the natural wetlands have been converted to cropland to meet the growing food demand (Liu et al. 2014). In our study area, the wetland mainly comprises *Deyeuxia angustifolia*, *Carex meyeriana*, and *Carex lasiocarpa*. The adjacent farmland was reclaimed in 1996 and was planted with soybean [*Glycine max* (L.) Merr.], since then, no fertilizers have ever been applied. The region's mean annual temperature (MAT) is 2.5℃, and the mean annual precipitation (MAP) varies from 500 to 600 mm. According to the USDA Soil Taxonomy system (Kyebogola et al. 2020), the soil texture is classified as silty loam.

The experiment was established across four seasons: October 2019 (Autumn), January 2020 (Winter), May 2020 (Spring), and July 2020 (Summer). In each season, three sampling sites were randomly selected within both a soybean field (47°35′ N,133°31′ E) and a natural wetland (47°35′ N,133°31′ E), and three soil cores were extracted from each site. Every soil core was 100 cm depth and divided into ten soil samples (i.e., 0–10, 10–20, 20–30, 30–40, 40–50, 50–60, 60–70, 70–80, 80–90, and 90–100 cm). After removing the stone and rhizome, three soil samples from the same layer were evenly mixed and then put into polyethylene bags Finally, a total of 240 soil samples were collected. A portion of soil samples was immediately stored at -80 °C for DNA extraction in less than 7 days. The remaining samples were divided into two groups: one was held at 4 °C to determine soil microbial biomass and the other was dried naturally for measuring the soil physicochemical properties.

All soil profile temperatures were collected in an automatic weather station (AWS) with a CR1000 data logger (Campbell Scientific, Inc.) at Sanjiang Mire Wetland Experimental Station (Yu et al. 2013). Soil water content (SWC) was determined by the gravimetric method (105 °C, 24 h) using fresh soil samples (Mo et al. 2020). Air-dried soil samples were used to measure soil pH and total N (TN). Soil pH was measured using pHs-25 (Shanghai INESA Scientific Instrument CO. Ltd, Shanghai, China) at a ratio of 1:10 for soil to water (An et al. 2022b). The soil TN was measured using a heating digestion method at 1100 °C with concentrated sulfuric acid (Black et al. 1992). The soil microbial biomass N (MBN) was estimated using the chloroform-fumigation extraction method (Witt et al. 2000).

### Soil DNA extraction, metagenomic sequencing, and bioinformatic analysis

DNA was extracted from fresh soil samples using the fastDNA® Spin Kit (MP Biomedicals, Inc., CA, USA). The concentration and purity of the extracted DNA were determined using the TBS-380 (Turner BioSystems, Inc., CA, USA) and NanoDrop2000 (Thermo Fisher Scientific, Inc. MA, US), respectively. Then, the integrity of the DNA was verified by 1% agarose gel electrophoresis. DNA extract was fragmented to an average size of about 400 bp using Covaris M220 (Gene Company Limited, China) for paired-end library construction. A Paired-end library was constructed using NEXTFLEX Rapid DNA-Seq (Bioo Scientific, Austin, TX, USA). Adapters containing the full complement of sequencing primer hybridization sites were ligated to the blunt end of fragments. Paired-end sequencing was performed on Illumina NovaSeq 6000 (Illumina Inc., San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Sequence data associated with this project have been deposited in the NCBI Sequence Read Archive (SRA) repository (NCBI—PRJNA853804).

The quality of the raw sequence data was assessed using the fastp (https://github.com/OpenGene/fastp, version 0.20.0) (Chen et al. 2018), and high-quality reads were extracted by filtering low-quality reads with N bases, read quality < Q20, and length < 50 bp. Then, we mapped the sequence data to host reads using burrows-wheeler alignment tool (BWA; http://bio-bwa.sourceforge.net, version 0.7.9a), and contaminated reads with high similarity were then identified and removed (Li and Durbin 2009). These high-quality reads were then assembled into contigs using MEGAHIT (https://github.com/voutcn/megahit, version 1.1.2) (Li et al. 2015). Open reading frames (ORFs) in each contig (over 300 bp) were predicted using MetaGene (http://metagene.cb.k.u-tokyo.ac.jp/) (Noguchi et al. 2006). A nonredundant gene catalog was constructed with predicted ORFs (≥ 100 bp) using CD-HIT (http://www.bioinformatics.org/cd-hit/, version 4.6.1) (Fu et al. 2012) at 90% identity and 90% coverage. All reads after quality control were aligned (95% identity) against the nonredundant gene catalog via SOAPaligner (http://soap.genomics.org.cn/, version 2.21) (Li et al. 2008), and gene abundance in each sample

was obtained. For statistical analysis, the relative abundance of genes was calculated in transcripts per million (TPM) (Wagner et al. 2012), with corrections for variations in gene length and mapped reads per sample. TPM is calculated as

$$\mathrm{TPM} = \frac{(\mathrm{R_i}/\mathrm{L_i}) * 10^6}{\sum_1^n \left(\mathrm{R_j}/\mathrm{L_j}\right)}$$

Where $R_i$ represents the abundance of Gene i in a given sample, i.e., the number of reads mapped to Gene i; $L_i$ represents the gene length, i.e., the number of nucleotides in the Gene i; $\sum_1^n \left(\mathrm{R_j}/\mathrm{L_j}\right)$ represents the total abundance of all genes after normalization by gene length (Xie et al. 2021). The non-redundant gene catalog was functionally annotated against the Kyoto Encyclopedia of Genes and Genomes database (KEGG; http://www.genome.jp/keeg/, ver. 94.2). The targeted N-cycle associated genes were filtered out based on the KEGG Orthology (KO) number (Kanehisa et al. 2015; Wang et al. 2021). Information on 59 KOs of the selected functional genes was provided in Table S1. All the functional genes involved in the N cycle were classified into 7 pathways, including N fixation, mineralization, nitrification, denitrification, assimilatory nitrate reduction to ammonium (ANRA), dissimilatory nitrate reduction to ammonium (DNRA), and ammonium assimilation (Table S1). Changes of functional genes were used to represent the potential changes of corresponding processes in this study. The taxonomic annotation for the subset of selected genes were processed based on the NCBI NR database using the blastp with an e-value cutoff of $1e^{-5}$ using Diamond (http://www.diamondsearch.org/index.php, ver. 0.8.35) (Buchfink et al. 2015). To analyze the N cycling genes, the sequences that belong to N metabolism catalog against KEGG were used for analysis of subsequent taxonomic annotations (Wang et al. 2021, 2022).

## Meta-analysis

Global data of $NH_4^+$-N and $NO_3^-$-N contents from wetland and cropland were obtained from peer-reviewed journal articles published between 1990 and June 2022. The publication databases, Web of Science, Google Scholar, and the Chinese National Knowledge Infrastructure (CNKI) were searched using the keywords (wetland* OR peatland* OR Swamp* OR Marsh* OR soybean* OR legume*) AND (nitrogen* OR N OR Dinitrogen OR N2 OR NO3* OR nitrate OR NH4* OR ammoni* OR NO2* OR nitrite OR N2O* OR "Nitrous Oxide" OR "nitric oxide") AND (reclamation OR reclaim* OR farm* OR tillage* OR cultivate* OR land-use* OR conversion* OR transition* OR abandon* OR restore*) AND (soil* OR plant* OR vegetation* OR leaf* OR leave* OR root* OR nodule*). Publications were screened if: (1) the study included both natural wetland and adjacent cropland. (2) $NH_4^+$-N or $NO_3^-$-N contents were reported for wetland and cropland ecosystems. Finally, a total of 150 observations of data from 17 publications were included in the present study (Fig. 1 and Table S5).

## Statistical analysis

Statistical analysis was performed based on the normalized abundance of each gene in R (version 4.1.1). The differences in $NH_4^+$-N, $NO_3^-$-N, TN, MBN, C/N, and pH between the wetland and cropland were evaluated with a paired *t-test*. The $NH_4^+$-N and $NO_3^-$-N contents were calculated as the common logarithm with base 10. The functional group of N-cycling genes was calculated by the sum of each gene involved in the associated process. The differences in the relative abundance of N-cycle-associated genes between natural wetlands and cultivated cropland were implemented with nonparametric statistics.

According to Hedges et al. (1999), response ratios of microbial lineages and relative abundance of genes were calculated as follows:

$$lnRR_i = ln\left(\frac{X_{ic}}{X_{iw}}\right)$$

For each group i, the $X_{ic}$ represents the average proportion of microbial lineages or N-cycling gene abundance in the cropland, and the $X_{iw}$ represents the average proportion of microbial lineages or N-cycling gene abundance in the wetland. The variance ($V_i$) was estimated by equation (Hedges et al. 1999):

$$V_i = \frac{SD_{ic}^2}{n_{ic}X_{ic}^2} + \frac{SD_{iw}^2}{n_{iw}X_{iw}^2}$$

Where $n_{ic}$ and $n_{iw}$ are the sample sizes of the cropland and wetland, respectively, and $SD_{ic}$ and $SD_{iw}$ are the standard deviations in the cropland and wetland, respectively (Borenstein et al. 2021).

A weighted random-effect model was used to determine the overall effect of the altered relative abundance of genes involved in soil N cycling after wetland reclamation (Wu et al. 2022; Yue et al. 2019). The weighted mean effected size ($lnRR_{++}$) was calculated as follows (Kuang et al. 2021; Wang et al. 2019):

$$lnRR_{++} = \frac{\sum_{i=1}^n w_i lnRR_i}{\sum_{i=1}^n w_i}$$

where n is the number of groups (season and depth), w is the weighting factor of each observation; and $w_i$ was calculated by taking the inverse of $V_i$. The weighted mean effected size ($lnRR_{++}$) was transformed as a percentage to express the magnitude of variations of gene abundance after reclamation by the following equation (Kuang et al. 2021):

$$1 - \frac{1}{e^{|lnR_{++}|}} \times 100\%$$

The difference in the relative abundance of microbial lineages and genes between wetland and cropland was analyzed through the Wilcox test. The seasonal and vertical difference in the response ratios of the relative abundance of genes was analyzed through the Kruskal-Wallis test. The Bonferroni correction for *p*-values adjustment for multiple testing was adopted to test differences between seasons. Nonmetric multidimensional scaling (NMDS) based on Bray-Curtis distance was performed using the "vegan" package to display the community patterns. All histograms, heatmap, and boxplot graphs were created using the "ggplot2" package. A structural equation modeling (SEM) analysis was performed in Amos software (version 22). A principal component analysis (PCA) was carried out to identify the major genes contributing to the variations in N processes.

## Results

### Meta-analysis of reclamation effects on soil inorganic N

A global meta-analysis was conducted to quantify the response of inorganic N ($NH_4^+$-N and $NO_3^-$-N) in wetlands and croplands (Fig. 1a–c, Tables S3, and S5). This global meta-analysis showed that wetland reclamation significantly altered the soil inorganic N pools (Fig. 1a–c). Wetland reclamation causes a significant decline in $NH_4^+$-N (-58.38%; Fig. 1b and Table S3), but a substantial increase in $NO_3^-$-N (135.22%; Fig. 1c and Table S3).

### Reclamation effects on microbial community composition

Wetland reclamation dramatically altered soil microbes involving N-cycling at taxonomic levels from kingdom to order (Fig. 2). Bacteria were the dominant community in wetlands and cropland, followed by archaea and fungi. However, the relative abundance of bacteria significantly decreased after wetland reclamation ($p < 0.001$; Fig. 2a). At the phylum level, *Proteobacteria* was the most dominant phylum (32.34%), followed by *Actinobacteria* (21.40%), *Chloroflexi* (9.68%), and *Acidobacteria* (8.63%) in the wetland. Similarly, *Proteobacteria* was the most dominant phylum (28.82%), followed by *Actinobacteria* (26.87%), *Chloroflexi* (10.01%), and *Acidobacteria* (9.42%) in cropland (Fig. 2b and Table S2).

### Changes in the relative abundance of N-related genes in response to wetland reclamation

The relative abundance of N-related genes differed dramatically between wetland and cropland (Fig. 4a). Specifically, 11 genes enriched while 21 genes decreased among 53 N-related genes after wetland reclamation (Fig. 3b–d). Similarly, wetland reclamation significantly influences the weighted effect size of the relative abundance of genes involved in each N-cycling process. For example, wetland reclamation significantly augmented the relative abundance of functional genes involved in nitrification and ANRA by 17.03% and 12.47%, respectively (Fig. 8 and Table S4). On the contrary, those in N fixation, denitrification, mineralization, and DNRA significantly decreased by 77.39%, 16.94%, 10.47%, and 28.59%, respectively (Fig. 8 and Table S4).

Wetland conversion to cropland significantly decreased the relative abundance of genes involved in N fixation ($p < 0.001$; Fig. 3a). Specifically, the relative abundances of *nifH*, *nifK*, and *nifD* were significantly reduced ($p < 0.001$; Fig. 3b). Notably, *anfG*, encoding the catalytic component of nitrogenases that have iron in the active center, was not detected in cropland. In addition, wetland reclamation significantly decreased the relative abundance of genes involved in the mineralization process (Fig. 3a). For example, the relative abundances of *cynS* encoding cyanate lyase, *E3.5.1.49* encoding formamidase, and *ureAB*, *ureA*, *ureB*, and *ureC* encoding urease subunit were significantly decreased ($p < 0.001$; Fig. 3d). However, no significant change was observed in the relative abundance of genes involved in $CO_2$ to $HCO_3^-$ (Fig. 3d).

The functional group of relative abundance of genes involved in the nitrification process was not significantly changed after wetland reclamation (Fig. 3a). However, wetland reclamation increased the relative abundance of genes involved in hydroxylamine ($NH_2OH$) oxidation to nitrite and further to nitrate (Fig. 6a). Ammonia oxidation to $NH_2OH$ is the first step of nitrification by using ammonia monooxygenase (AMO). In this step, the relative abundances of *amoB* and *amoC* have no change (Fig. 6a), but *amoA* significantly increased after wetland reclamation ($p < 0.001$; Fig. 3b).

The relative abundance of genes involved in denitrification generally decreased by 16.94% after wetland

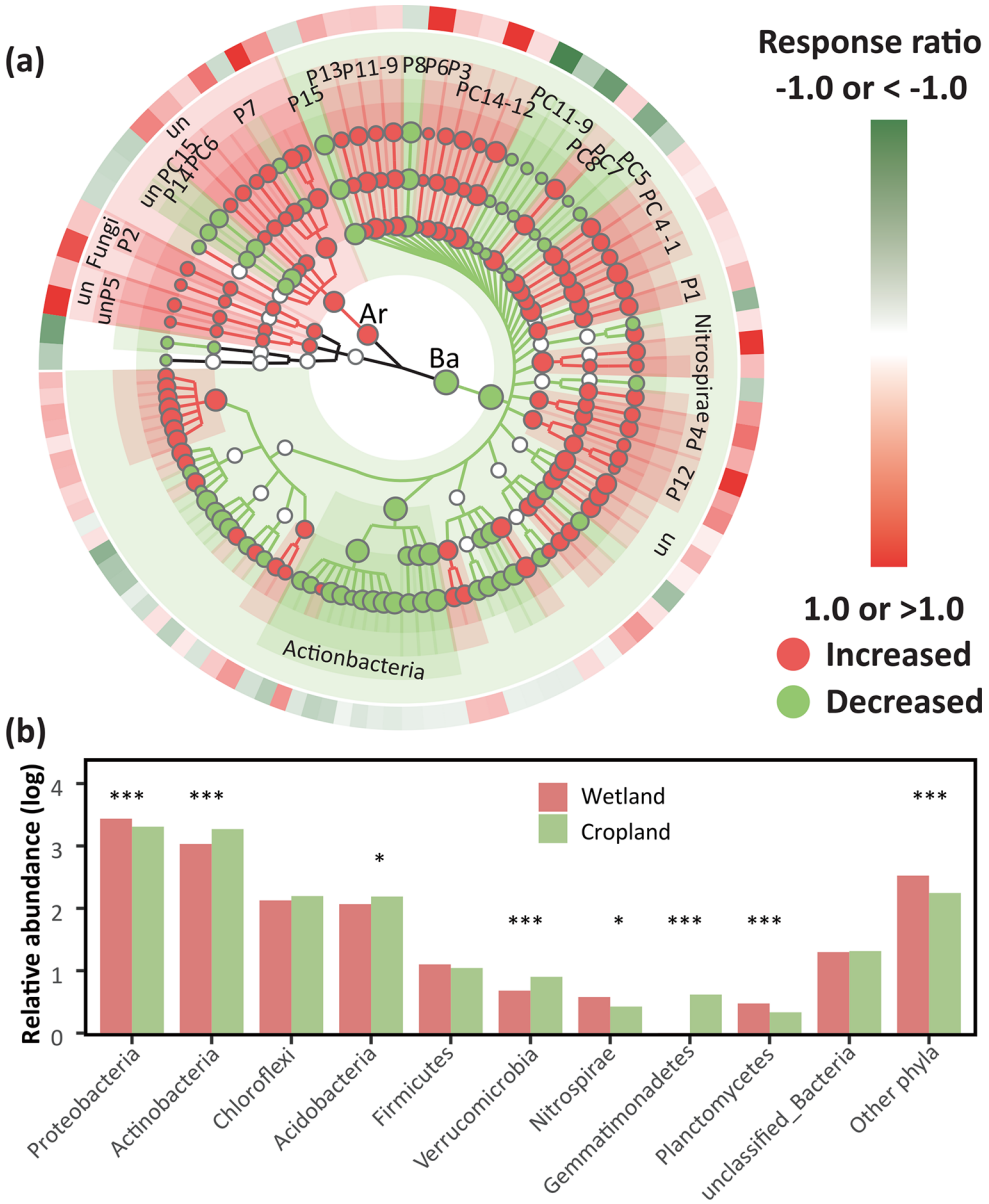


**Fig. 2** (**a**) The response of soil microbial lineages to wetland reclamation. The cladogram depicts kingdom, domain, phylum, class, and order levels from inside to outside. Taxa that significantly increased under wetland reclamation are represented as red, while taxa that significantly decreased are represented as green. The out ring represents the response ratio of each microbial order. The size of each node represents the logarithmically transformed relative abundance of each microbial lineage. Ar: Archaea; Ba: Bacteria; P1: *Aquificae*; P2: *Ascomycota*; P3: *Chlamydiae*; P4: *Chlorobi*; P5: *Chromerida*; P6: *Elusimicrobia*; P7: *Euryarchaeota*; P8: *Gemmatimonadetes*; P9: *Ignavibacteriae*; P10: *Latescibacteria*; P11: *Planctomycetes*; P12: *Spirochaetes*; P13: *Synergistetes*; P14: *Thaumarchaeota*; P15: *Verrucomicrobia*; PC1: *Candidate_division_NC10*; PC2: *Candidate_division_WOR-3*; PC3: *Candidate_division_Zixibacteria*; PC4: *Candidatus_Acetothermia*; PC5: *Candidatus_Azambacteria*; PC6: *Candidatus_Bathyarchaeota*; PC7: *Candidatus_Daviesbacteria*; PC8: *Candidatus_Omnitrophica*; PC9: *Candidatus_Parcubacteria*; PC10: *Candidatus_Peregrinibacteria*; PC11: *Candidatus_Roizmanbacteria*; PC12: *Candidatus_Rokubacteria*; PC13: *Candidatus_Saccharibacteria*; PC14: *Candidatus_Tectomicrobia*; and PC15: *Candidatus_Thorarchaeota*; un: unclassified taxa. (**b**) Soil microbial composition at phylum level in wetlands (red) and cropland (green). Only the relative abundance of top 10 phyla was shown separately, while others are represented as a sum of their proportions. The effects of the wetland reclamation were analyzed through Wilcox text. Significance levels are denoted with *: $p < 0.05$; **: $p < 0.01$; and ***: $p < 0.001$

reclamation (Figs. 3a and 8, and Table S4). However, those in nitrite reduction to nitric oxide (NO) enriched (Fig. 6a). The relative abundances of *norB* and *norC*, which encoded enzymes that catalyzed NO reduction to $N_2O$ were significantly decreased ($p < 0.001$; Fig. 3c). Relative abundance of genes involved in nitrate reduction to nitrite in denitrification was decreased significantly (Fig. 6a), especially *napA* ($p < 0.001$) and *napB*

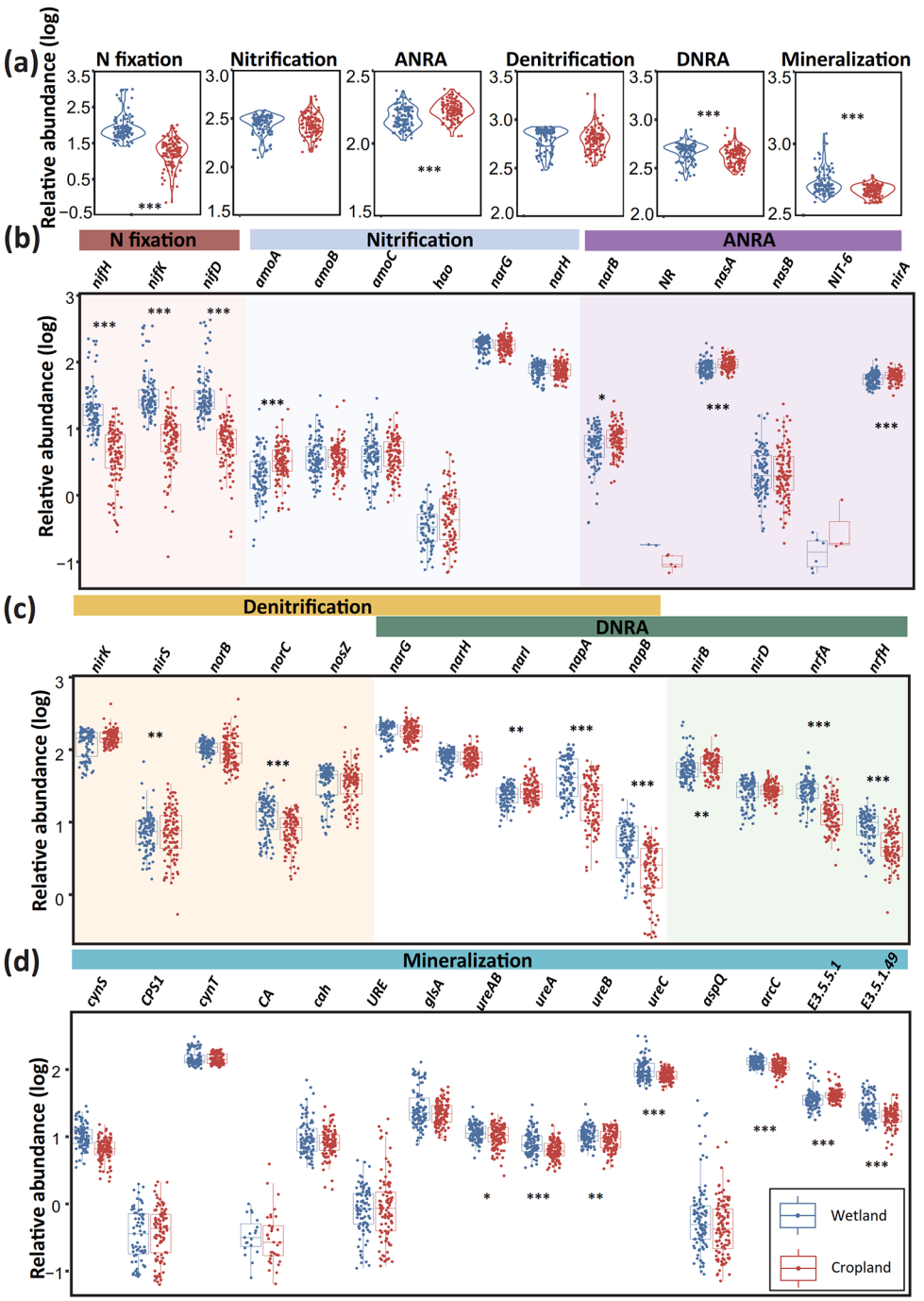


**Fig. 3** Changes of N-cycling gene abundances during wetland reclamation. (**a**) The relative abundance of genes involved in N fixation, nitrification, ANRA, denitrification, DNRA, and mineralization in wetland and cropland. (**b**) The relative abundance of each gene involved in N fixation, nitrification, and ANRA; (**c**) the relative abundance of each gene involved in denitrification and DNRA; (**d**) the relative abundance of each gene involved in mineralization. The effects of the wetland reclamation were analyzed through Wilcox text. Significance levels are denoted with *: $p < 0.05$; **: $p < 0.01$; and ***: $p < 0.001$

($p < 0.001$) that encode periplasmic dissimilatory nitrate reductases (Fig. 3c). These genes are also involved in the first step of DNRA. Consistently, the relative abundance of genes involved in the dissimilatory nitrite reduction to ammonium decreased by 28.59% significantly (Figs. 6a and 8, and Table S4), especially *nrfA* ($p < 0.001$) and *nrfH* ($p < 0.001$) that encode dissimilatory periplasmic cytochrome c nitrite reductase (Fig. 3c). Nevertheless, the relative abundance of genes involved in ANRA was increased after wetland reclamation (Fig. 3a).

There was no significant change in the relative abundance of genes involved in ammonium assimilation after wetland reclamation (Fig. S3a). Genes involved in assimilatory glutamate formation, dissimilatory glutamate formation, and urea synthesis were decreased after wetland reclamation (Fig. S3b). However, genes involved in glutamine formation and further glutamate formation (glutamine to glutamate) increased after wetland reclamation (Fig. S3b).

## Seasonal variations of N-related genes in response to wetland reclamation

Wetland reclamation significantly altered the relative abundance of N-cycling genes (Fig. 4a, $p < 0.05$) and associated microbial taxonomic composition (Fig. 4b) across seasons. In the nitrification process, the variations of relative abundance of genes in $NH_2OH$ oxidation to nitrite and further to nitrate have no significant difference between spring and summer (Fig. 5). Variations in denitrification, DNRA, and ANRA also have no discrepancy between spring and summer (Fig. 5). Instead, across all seasons, genes involved in N fixation were significantly decreased after wetland reclamation (Fig. 5). Additionally, genes involved in ammonium assimilation were significantly reduced in summer, autumn, and winter after wetland reclamation (Fig. 5).

In the nitrification process, variations of genes involved in ammonia oxidation to $NH_2OH$ and further to nitrite significantly increased in summer (Fig. 5). Conversely, variations of genes involved in nitrate reduction to nitrite, NO reduction to $N_2O$, and further to $N_2$ decreased significantly in spring and summer (Fig. 5). In addition, variations of

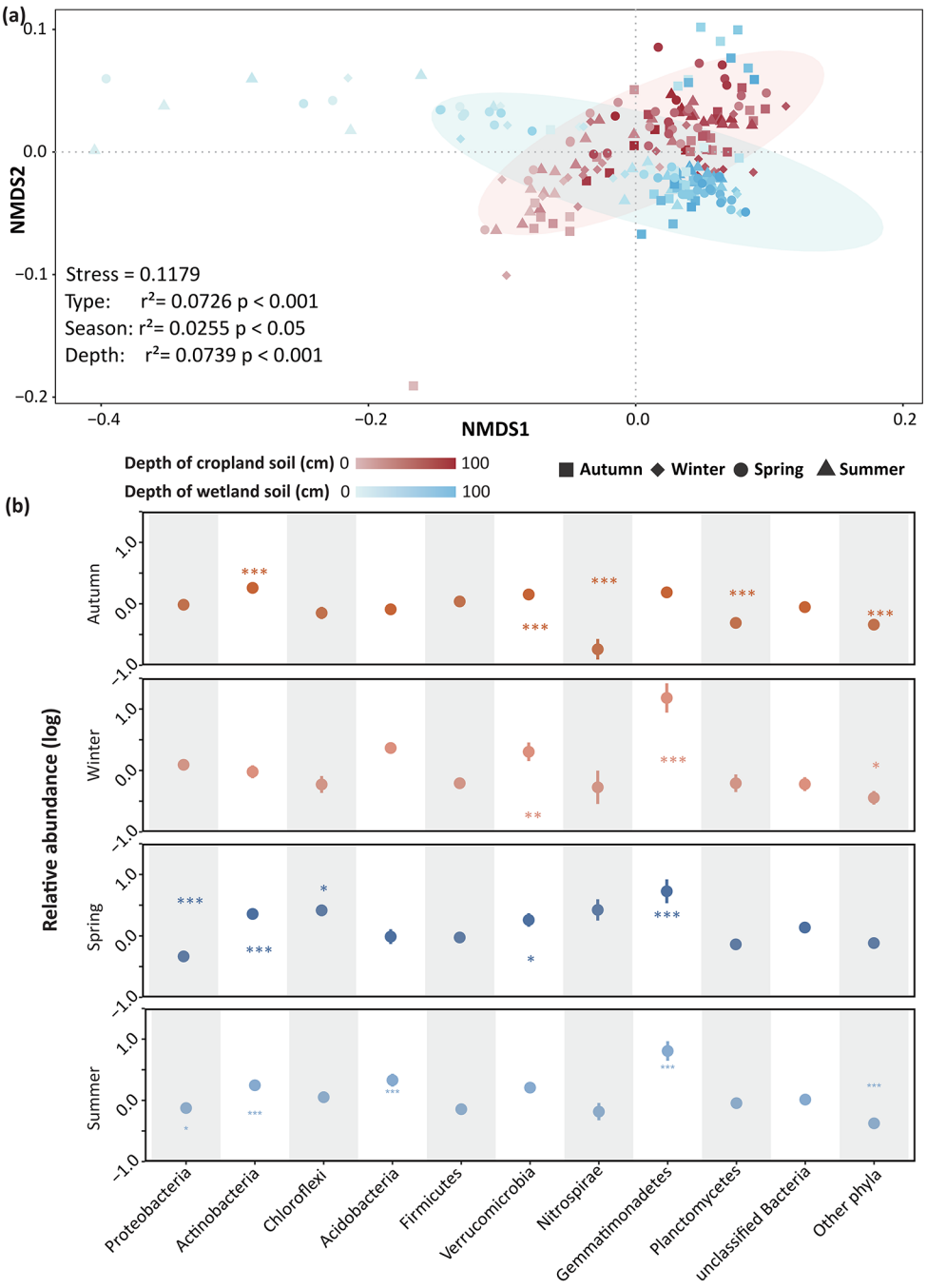


**Fig. 4** (**a**) The NMDS ordination of N-cycling genes in wetlands and cropland. The differences among four seasons and ten soil layers were quantified using the Wilcoxon rank-sum test. (**b**) The response ratio of microbial lineages to wetland reclamation over seasons at phylum level; only the relative abundance of top 10 phyla were shown separately, while others are represented as a sum of their proportions. The effects of the wetland reclamation were analyzed through Wilcox text. Significance levels are denoted with *: $p < 0.05$; **: $p < 0.01$; and ***: $p < 0.001$

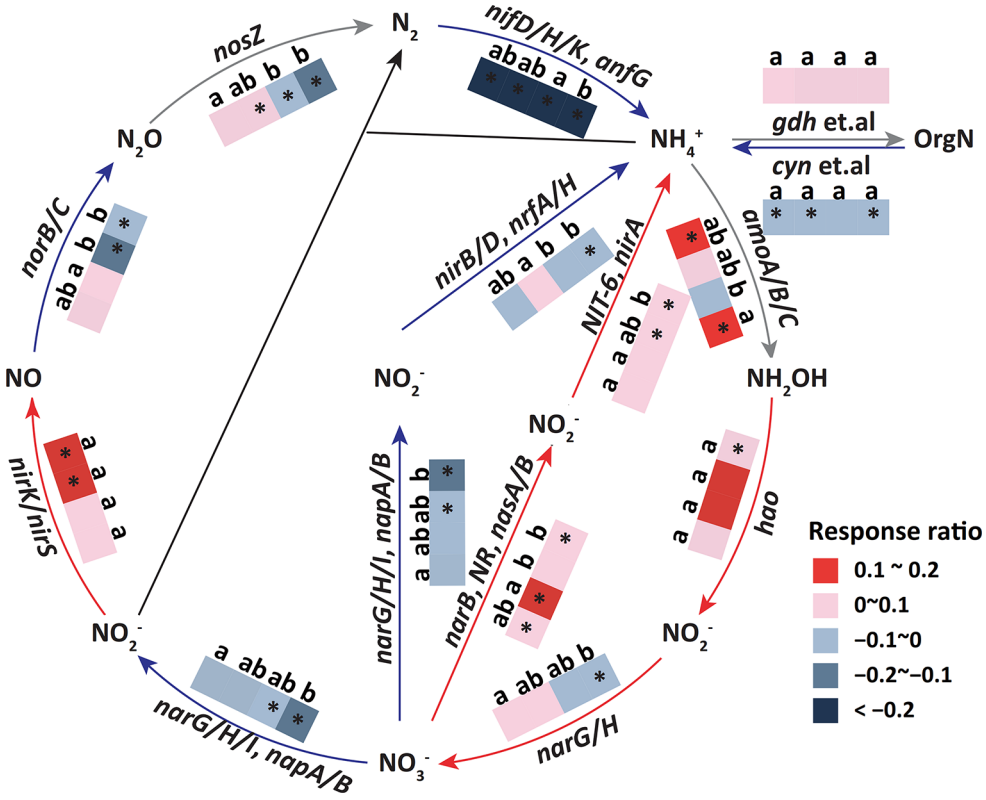


**Fig. 5** Summary of the impacts of wetland reclamation on the N-cycling gene abundance across seasons. From left to right, colorful squares are the response ratios in autumn, winter, spring, and summer; the letters above the colorful squares mean the significant difference among seasons. "*" indicates $p < 0.05$

genes involved in dissimilatory nitrite reduction to ammonium significantly reduced in spring and summer (Fig. 5). On the contrary, variations of genes involved in assimilatory nitrate reduction to nitrite in summer, and assimilatory nitrite reduction to ammonium in spring and summer, significantly increased (Fig. 5).

### Vertical patterns of the relative abundance of N-cycling genes in response to wetland reclamation

The NMDS analysis indicated that microbial functional profiles of N-cycling in cropland were significantly distinguished from wetland along soil profiles (Fig. 4a, $p < 0.001$). The relative abundance of N-cycling microbes changed substantially with soil depth in wetland and cropland (Fig. 6). Wetland reclamation caused large discrepancies between soil layers of 0–30 cm and 30–100 cm (Fig. S1). In the shallow soil (0–20 cm), wetland reclamation decreased the relative abundance of genes involved in ammonia oxidation to $NH_2OH$ while increasing those in $NH_2OH$ oxidation to nitrate and denitrification. However, the opposite tendency of these genes was found in the 30–100 cm deep soils (Fig. S1). Along all soil profiles, the relative abundance of genes involved in N fixation and ammonium assimilation reduced considerably after wetland reclamation.

Our study found that soil N was significantly influenced by edaphic factors and N-cycling microbes both in wetlands and cropland (Fig. 7). The soil depth was particularly and negatively relevant to soil N in wetlands and cropland. In addition, the soil depth had a direct or indirect significant negative influence on the microbes of mineralization. In the wetland, the microbes of mineralization, influenced by soil depth, soil water content, and soil pH, were significantly positively correlated with soil N (Fig. 7a). In the cropland, the microbes of mineralization, are also influenced by soil depth, pH, and temperature (Fig. 7b). However, edaphic factors and N-cycling-related microbes had different impacts on soil N between wetlands and cropland. For example, soil water content influences the relative abundance of genes involved in the nitrification of wetlands but not cropland, while pH influences gene abundance in the N fixation of cropland but not wetlands (Fig. 7a, b).

## Discussion

### Wetland reclamation accelerates soil N loss by shifting microbial N cycling

As soil microbes are identified as the pivotal driver in the terrestrial N cycle at the global scale (Kuypers et al. 2018), wetland reclamation would considerably shift the N cycling by changing microbial functional profiles of N. This is supported by the results from our field experiment and a

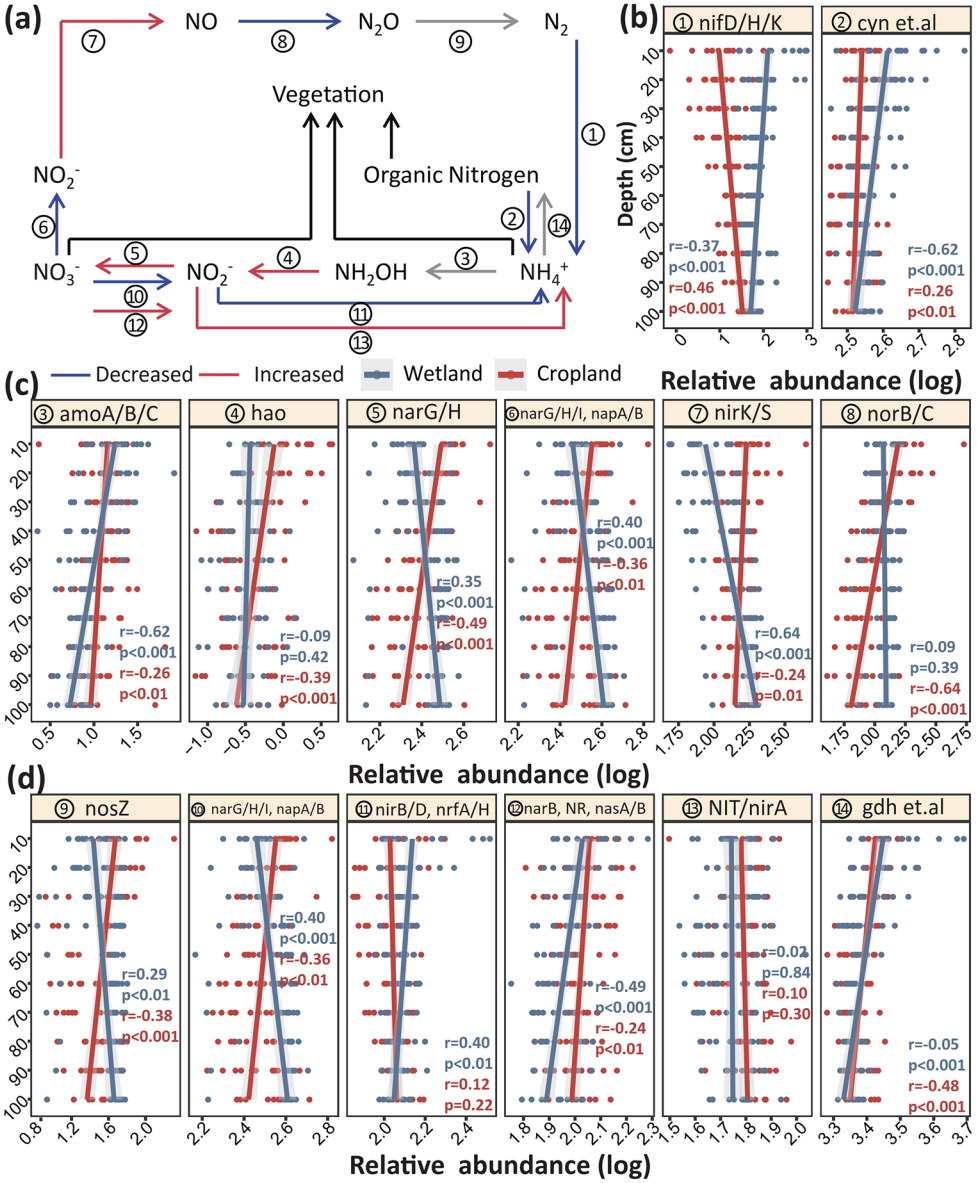


**Fig. 6** Wetland reclamation impacts on the N-cycling gene abundance among soil depths. (**a**) The variations of gene abundances in different N cycling processes. Red solid lines indicate that the gene abundances are significantly increased after wetland reclamation; solid blue lines indicate that the gene abundances are significantly decreased after wetland reclamation; gray lines indicate no significant difference between wetland and cropland, and black lines indicate no gene is detected in our experiments. The relative abundance of genes involved in N fixation and mineralization (**b**), nitrification and denitrification (except *nosZ*) (**c**), and denitrification (*nosZ*), DNRA, ANRA, and ammonium assimilation (**d**) of each layer in the wetland (blue point and line) and cropland (red point and line)

global meta-analysis, which indicates that wetland reclamation significantly increased soil $NO_3^-$-N concentrations but decreased the concentrations of soil $NH_4^+$-N (Fig. 1 and Table S3), and the relative abundance of genes involved in nitrification also improved after wetland reclamation. Compared to $NH_4^+$, $NO_3^-$ readily leaches as its overall negative charge and has a higher diffusion coefficient (Luo et al. 2011). Moreover, wetland plants generally have higher uptake capacities for $NH_4^+$ than $NO_3^-$ (Wang et al. 2020). Such a result confirmed our first hypothesis that land cultivation bears a risk of increasing the extent of N loss by enhancing the $NO_3^-$-N produced by nitrification (Fig. 7). This finding is in accordance with the findings of other studies, where N loss increased by enhancing the nitrification rates (Bakhsh and Kanwar 2007; Helfrich et al. 2020; Laine et al. 2018; Qin et al. 2016).

In addition, N loss after wetland reclamation is also supported by other N cycling processes reflected by changes in the relative abundance of corresponding genes including DNRA, N fixation, and nitrite reduction to NO (Fig. 3). Specifically, the inhibitions of the relative abundance of genes in N fixation and DNRA lead to potential N loss by decreasing $NH_4^+$ concentration (Zhang et al. 2021). Since nitrate contents in cropland were higher than in wetlands, and nitrate levels inhibited multiple stages in root nodule growth and N fixation (Nishida and Suzaki 2018), this resulted in a decrease in the relative abundance of genes involved in N fixation (Fig. 3). The increase of the relative abundance of genes involved in nitrite reduction to NO led

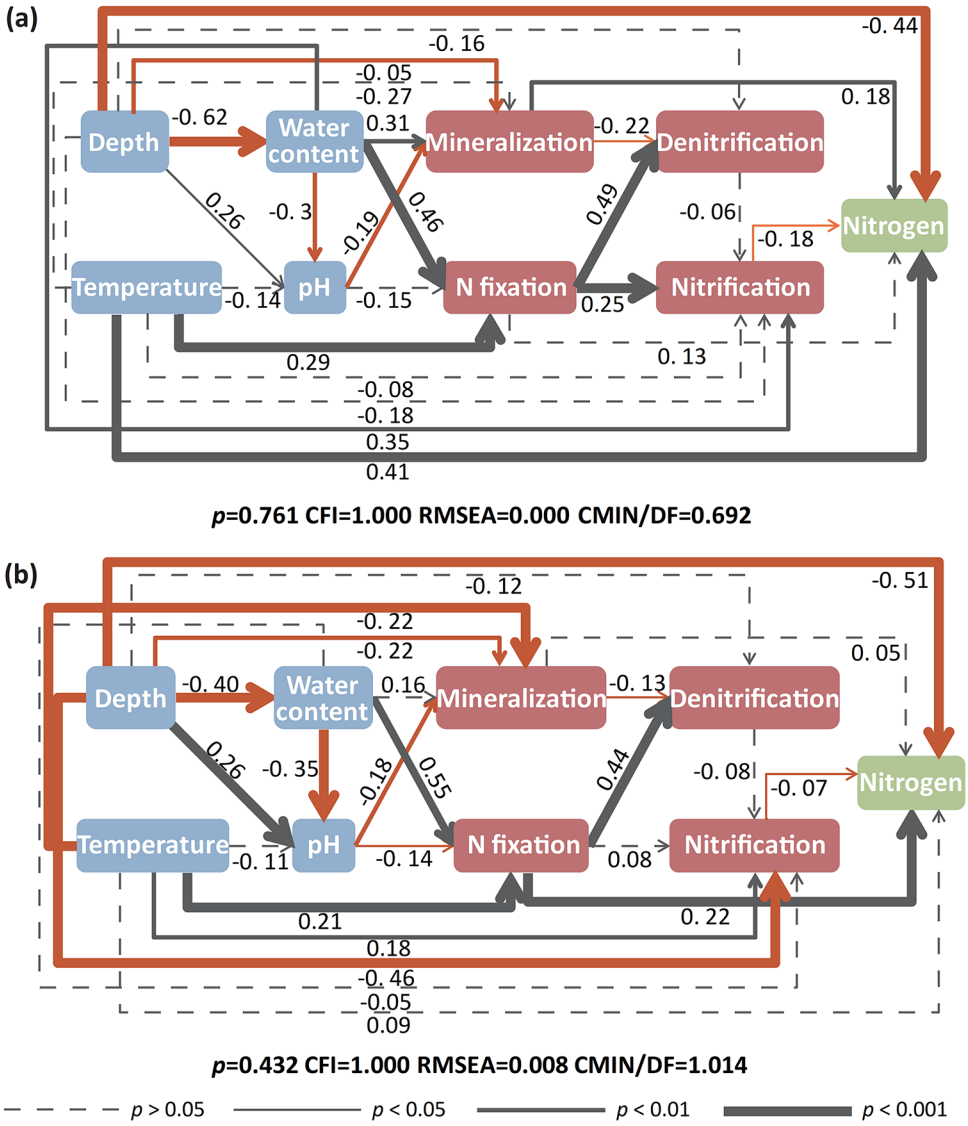


**Fig. 7** Structural equation modeling (SEM) illustrating the direct and indirect effects of physicochemical characteristics and the relative abundance of genes involved in N cycling on soil total N in (a) wetland and (b) cropland. Arrows in gray and red indicate positive and negative effects, respectively. The numbers adjacent to the arrows are standardized path coefficients proportional to the line thickness. Continuous and dashed arrows indicate significant and non-significant correlations, respectively. Arrows indicate the hypothesized direction of causation. CFI: Comparative-of-fit index; RMSEA: Root mean square error of approximation; CMIN/DF: Chi-square to degrees of freedom ratio

to an increase in NO emission. Overall, the changes in soil inorganic N pools under the wetland reclamation could shift the N regime and accelerate soil potential N loss by affecting N-cycling functional genes and microbial community composition.

The significant decrease in $NH_4^+$-N concentrations and increase in $NO_3^-$-N concentrations were attributed to the substantial increase in the relative abundance of genes involved in nitrification (Fig. 3a). Previous studies reported similar patterns in the response of N-cycling microbial communities to land-use change (Qin et al. 2016). As revealed by Che et al. (2017), an increase in nitrifier abundance is associated with greater soil N loss. Nitrifiers are mainly aerobic (Li et al. 2014b; Martens-Habbena et al. 2009), and dry croplands provide the aerobic conditions for them. As the phylogenetic marker to detect aerobic ammonia-oxidizing microbes (Jiang et al. 2010; Mohamed et al. 2010), the *amoA/B/C* gene significantly increased after the wetland converted to cropland (Fig. 3b). Most of the nitrifier communities belong to *Proteobacteria* (Zhang et al. 2014; Zheng et al. 2013), which is also supported by the decreased *Proteobacteria* after wetland reclamation (Fig. 2b). Nitrifiers were induced with a low C/N (Tsujino et al. 2021; Wang et al. 2023), and most soil nitrification process is accomplished by autotrophic nitrifiers (Islam et al. 2007; Pedersen et al. 1999; Tortoso and Hutchinson 1990). In this study, the C-to-N ratio (C/N) was lower than 25 in wetlands and lower than 10 in cropland (Fig. 1f). Under lower C/N in cropland, lower N demand of other heterotrophs allows the low competition with nitrifiers (Adair and Schwartz 2008; Lee et al. 2018). Recent studies also suggested the extremely high heterotrophic nitrifiers and nitrifying activities in croplands (Gao et al. 2023b), explaining the increase of relative gene

abundance involved in nitrification (17.03%) shifting N regime after wetland reclamation (Fig. 3).

The relative abundance of genes involved in nitrite reduction to NO significantly increased after wetland reclamation (Fig. 3a), which contributes to high N loss as $N_2O$ emissions (Gao et al. 2023a; Highton et al. 2023). As the first step in soil gas emission in the N cycle, NO diffusion is limited by water conditions of the surface soil (Friedl et al. 2022; Peirce and Aneja 2000; Pilegaard 2013). Therefore, NO emissions from dry well-aerated soils are much more than flooded wetlands (Smith 2005). Alternatively, a recent study demonstrated that mineral N addition can enhance the production of $N_2O$ (Parajuli et al. 2022), which was supported by the increasing $NO_3^-$-N under wetland reclamation in our meta-analysis (Fig. 1). Although *nirS* and *nirK* genes are functionally similar in nitrite reduction to NO, they mostly belong to different bacterial strains and owe unrelated evolutionary relationships (Sun and Jiang 2022), showing varying responses in different habitats. Previous studies have reported that *nirK* was more abundant than *nirS* in soil but lower than *nirS* in aquatic habitats (Mao et al. 2023; Palacin-Lizarbe et al. 2019). Similarly, we found evidence that the *nirK* was more abundant than the *nirS* in wetlands and cropland (Fig. 3b). Additionally, the habitat selectivity of *nirK* was greater than *nirS* (Jones and Hallin 2010), confirming the significantly increased *nirK* under wetland reclamation in our study (Fig. 3b). This pattern was similar to that found in an earlier study of tidal wetlands in which the *nirK* gene increased with rice cultivation time and dominated its functionally redundant counterpart *nirS* (Bannert et al. 2011).

Consistent with the previous result (Bedard-Haughn et al. 2006), the significant decrease in the relative abundance of microbes involved in (dissimilatory) nitrate reduction to nitrite also contributed to the substantial increase in $NO_3^-$-N concentrations (Fig. 3a). The significant decrease in *napA* (periplasmic nitrate reductase) and *napB* (cytochrome c-type protein) can be due to aerobic conditions (Fig. 3a) (Li et al. 2020b; Zaki et al. 2019). While no obvious decreasing trend was shown in *narG/H/I* and *nirB/D*, which control the DNRA process under anaerobic conditions (Huang et al. 2020; Zaki et al. 2019). Unlike DNRA, ANRA microbes contribute to N conservation by transforming it into organic N. ANRA is carried out by bacteria to incorporate ammonia for cell growth (Feng and Li 2019), which inferred that microbes prefer to utilize N to maintain their metabolism and growth. This corresponds to our results that microbial biomass weakly increased after wetland reclamation (Fig. 1e).

Collectively, after the wetland reclamation, the increased relative gene abundance in nitrification and ANRA, and the decreased relative gene abundance in N fixation and DNRA accelerate the loss of inorganic N together. The increased relative gene abundance in nitrite reduction to NO accelerates the N loss as gas emission. Therefore, the first hypothesis was supported. The impact of wetland reclamation on the specific process of the N cycle shifted the N regime and accelerated N loss, as shown by the functional genes encoding those processes (Fig. 8).

## Different seasonal changes of N-cycling genes after wetland reclamation

Effects of reclamation on N cycling genes are different across seasons, reflected by the significant influence on N cycling genes in spring and summer rather than in autumn and winter, except for nitrite reduction to NO (Fig. 5). This is primarily due to the strong temperature dependence on microbial activities (Zhu et al. 2022). The enhancement of microbial activity by high temperatures in summer is stronger in cropland than in wetlands (Li et al. 2004). Furthermore, the difference of plant species in cropland and wetland in the growing season (spring and summer) might also enhance the effect of reclamation on N cycling. Alternatively, most plants grew in summer (Li et al. 2020a). Changes in plant species can affect the N cycle by regulating the microbial community in summer (Kuzyakov and Xu 2013). Consistently, we observed apparent distinctions in microbial community controls after wetland reclamation across seasons (Fig. 4b). Therefore, microbial responses to wetland reclamation varied in different seasons and the second hypothesis was supported.

The potential risk of N loss under wetland reclamation greatly depends on the transition from winter to spring (Jia et al. 2022; Treusch et al. 2009). Microbial death and proteolysis during the freeze-thaw period generate the highest available N pulse of the year (Jia et al. 2022). Moreover, the wetland has a better capacity for N retention than cropland due to a large amount of inorganic N losses through $NO_3^-$ leaching in cropland (Helfrich et al. 2020; Yang et al. 2019), especially in growing seasons (Shafreen et al. 2021). Consistently, we observed the abundance of functional genes involved in nitrification significantly increased under wetland cultivation in summer (Fig. 5). When soil $NO_3^-$ supply is insufficient, the denitrification process will be restricted by the limited substrate in growing season (Davidsson et al. 2002; Liu et al. 2023). Similarly, decrease in the relative abundance of genes in DNRA and nitrate reduction to nitrite was more significant in spring and summer than in autumn and winter (Fig. 5), which also resulted in N loss being higher in spring and summer than in autumn and winter.

The above findings were consistent with our second hypothesis. On the basis of these findings, the application of nitrifying inhibitors is a potentially promising management

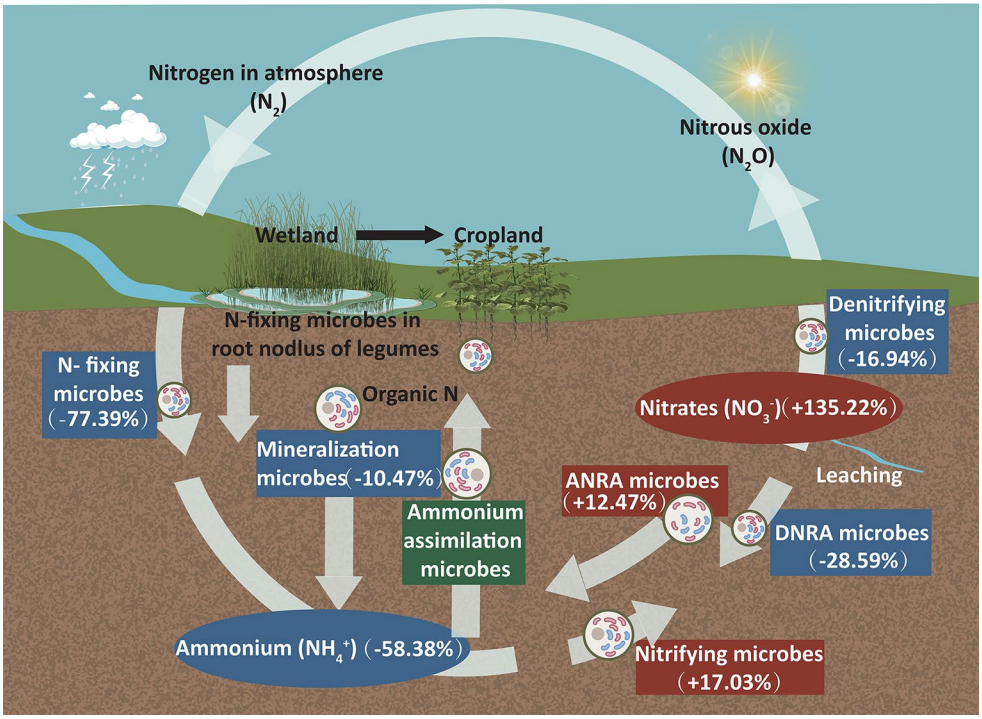


**Fig. 8** Graphic diagram showing the microbial mechanisms of wetland reclamation impacts on soil N cycling. Wetland reclamation significantly altered soil inorganic N and microbial gene abundances in the N cycle. The ellipse shape indicates soil N; the rectangle shape indicates the relative abundance of genes associated with soil N-cycling. Shape with red background color with (+) indicates the positive effect of wetland reclamation, which shows that N content or relative abundance of genes significantly increases after wetland reclamation. Shape with blue background color with (-) indicates the negative effect of wetland reclamation, which shows that N content or relative abundance of genes significantly decreases after wetland reclamation. Shape with a green background color indicates a non-significant impact of wetland reclamation

practice to alleviate the accelerated N loss during land cultivation (Subbarao et al. 2009). To obtain the best ecological benefits and crop production, synthetic nitrification inhibitors in fertilizer is one of the ongoing practices in land cultivation (Hatano et al. 2019), such as dicyandiamide (DCD) and 3,4-dimethylepyrazole phosphate (DMPP). However, synthetic nitrification inhibitors were not advocated due to their high cost and environmental pollution issues (Otaka et al. 2022). Biological nitrification inhibitors (BNI), released from plant roots (Zhang et al. 2022), are a promising strategy to control this high cost and reduce N losses (Ekwunife et al. 2022). In this study, we suggest that the use of BNI in tillage must be considered.

## Microbes accelerate N loss in surface rather than sub-surface soils after wetland reclamation

Wetland reclamation bears a risk of N loss along all soil depths (Figs. 6 and 7, and S1). Decreased relative abundance of genes involved in mineralization after wetland reclamation decreases the potential of N loss along all soil profiles (Fig. 6). However, genes involved in N fixation reduced significantly after wetland reclamation along all soil profiles (Fig. S1), which increased the potential of N loss. This can be partly attributed to the diverse microbial communities (Li et al. 2016). Moreover, the relative abundance of genes involved N fixation decreased with soil depth (Fig. 6), confirming previous studies (Bu et al. 2020).

Effects of wetland reclamation accelerates potential N loss in surface rather than sub-surface soils reflected by significantly shifting the relative abundance of genes involved in denitrification among soil depth (Fig. 6). The relative abundance of genes involved in denitrification decreased at a depth of 30–100 cm after the wetland reclamation (Fig. S1), which is related to more anaerobic conditions of wetland than cropland in deep layers (Hunter and Faulkner 2001). In wetland and cropland, soil depth influences the relative abundance of genes involved in mineralization directly and indirectly (Fig. 7). Available N produced by mineralization is an important source of nitrification (Seitzinger 1994), the decline in mineralization may cause a decrease in denitrification in deep soils (Fig. 6). In contrast, the relative abundance of genes involved in denitrification increased by more than 10% at the depth of 0–30 cm after the wetland reclamation (Fig. S1). This increase, according to our data, contradicts some previously published studies, showing that gene abundance in denitrification decreased at surface soil during land conversion (Bu et al. 2020; Emer et al. 2017). These differences may be caused by the application of mineral N fertilizers (An et al. 2022a). The increase in the relative abundance of genes involved in denitrification in surface soils resulted in the permanent removal of N as gases NO, $N_2O$, and dinitrogen ($N_2$) after wetland reclamation (Fig. S1) (Harrison et al. 2011). Therefore, N loss might be partially induced by gas emission in surface soil after wetland reclamation, which is also supported by a recent investigation (Helfrich et al. 2020). Overall, these findings were consistent with our third hypothesis.

Effects of wetland reclamation accelerates potential N loss also reflected by significantly shifting the relative

abundance of genes involved in $NH_2OH$ oxidation to nitrate (Fig. 6). At a 0–30 cm depth, the relative abundance of genes involved in soil $NH_2OH$ oxidation to nitrate increased (Fig. S1). Increasing the relative abundance of genes involved in nitrification in surface soils exerts the potential for $NO_3^-$ production. It could be explained by the regular input of fresh plant residues in the wetland, and their mineralization favors an increase in soil nitrification (Németh et al. 2014). This finding was also confirmed in our study: the relative abundance of genes involved in soil mineralization was found to significantly decrease after wetland reclamation (Fig. 3). The higher content of nitrifiers is related to the optimal environments as the more aerobic conditions on the surface than deep soil after reclamation (Pett-Ridge et al. 2013). The risk of $NO_2^-$ leaching was higher in sub-surface rather than surface soils after wetland reclamation (Wyland et al. 1996), supported by our result that the relative abundance of these genes decreased at the depth of 30–100 cm (Figs. 6 and S1).

## Conclusion

This study quantified the effects of wetland conversion to cropland on soil N-cycling genes in northeastern China by analyzing the metagenomic data. The conversion of wetlands to cropland significantly enriched the relative abundance of genes involved in nitrification and ANRA by 17.03% and 12.47%, respectively. The relative abundance of genes involved in N fixation, denitrification, mineralization, and DNRA declined by 77.39%, 16.94%, 10.47%, and 28.59%, respectively. These changes in N-cycling genes would result in higher $NO_3^-$but lower $NH_4^+$ content in soils, which would lead to a risk of N loss. It can be further advocated by the global meta-analysis showing a significant decrease in $NH_4^+$ and an increase in $NO_3^-$. The changes in N-cycling genes in growing seasons were more apparent than in non-growing seasons. In addition, land use change led to a higher risk of N loss by N-cycling genes in surface soils (0–30 cm) than in deep soils (30–100 cm). Nevertheless, we acknowledge some limitations of our study. First, we only obtained functional genes in the present study, which would only proxy for the potential of N cycling processes. Second, we only presented the abundance of N-cycling genes in a wetland and 23-year cultivated cropland. Further studies should extend the mechanistic understanding of the shifting N-cycling along the chronic sequence. Even with these limitations, our work can be useful for evaluating the N cycle under land use change in perspective of microbial metabolism, and provide mechanistic insights into the impacts of human activity on the soil N cycle.

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## Declarations

**Competing interests** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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