



Phosphorus-mediated succession of microbial nitrogen, carbon, and sulfur functions in rice-driven saline-alkali soil remediation

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ABSTRACT

Although rice cultivation holds potential for restoring unproductive saline-alkali soils and increasing food production, the mechanisms underlying the relationship between microbial functions and soil element turnover remain unclear. To clarify this relationship, this study investigated the soil physicochemical properties and microbial functions during remediation in saline-alkali soil by rice cultivation over 2, 4, 6, 8, 11, 12, 20, and 23 years. The results indicated rice cultivation markedly improved soil nutrients, soil nutrient stoichiometry, and soil aggregate stability. Additionally, rice cultivation significantly increased the microbial functions involved in nutrient cycling, such as nitrogen fixation, carbon fixation, methanogenesis, dissimilatory sulfate reduction, and thiosulfate oxidation. However, these nitrogen (N), carbon (C), and sulfur (S) cycle-related functions exhibited a similar “increase-peak-decrease” successional pattern with the years of remediation, reaching optimal levels when rice was continuously grown for 11–16 years. Furthermore, correlation analysis demonstrated that the succession of soil microbial N, C, and S functions during saline-alkali soil restoration closely related to changes in soil properties, particularly the availability of phosphorus (P). Therefore, we propose to prioritize the management of P during saline-alkali soil remediation. In conclusion, this study provides a comprehensive understanding of the microbial N, C, and S functions and soil P in the remediation of saline-alkali soils mediated by rice crop.

1. Introduction

Saline-alkali stress affects around 954 million hectares of soil worldwide, resulting in compromised fertility and ecological vulnerability (An et al., 2020), rendering it unsuitable for agriculture. The Songnen Plain in western Jilin Province, China, hosts one of the largest saline-alkali soil areas globally, comprising 3.73 million hectares (Qu et al., 2022; Ren et al., 2023). In order to address the escalating demand for arable land for agricultural production, it is critical to devise strategies that facilitate ecological restoration and simultaneously enhance soil quality for agricultural purposes (Wang et al., 2020; Cao et al., 2021). An extensive body of research has focused on restoring saline-alkali soil (Cui et al., 2021; Liang et al., 2021). One approach is to change the physicochemical properties of soil by applying soil

conditioners. For example, Zhao et al. (2020) applied corn straw biochar to amend saline-alkali soil and observed improved soil properties and functionality. Instead of organic amendment, Wang et al. (2017) used flue gas desulfurization gypsum to ameliorate saline-alkali soil.

Compared with organic and inorganic amendments, restoring saline-alkali soil by cultivating rice (*Oryza sativa* L.) may allow for more prompt adjustment to environmental changes and be more cost-effective (Du et al., 2021; Qu et al., 2022; Wang et al., 2022). The cycles of irrigation and drainage during rice cultivation can progressively remove salinity and alkalinity from the surface soil, thereby potentially reducing the negative effects of degraded soil structure (Huang et al., 2015). For example, Wang et al. (2022) found that rice cultivation for 3, 8, 13, and 18 years increased the content of soil macroaggregates with diameters of 0.25–2 mm and >2 mm in saline-alkali soil, thus significantly improving

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its aggregate structure. More importantly, several studies have shown that rice cultivation in saline soils can significantly improve soil biological activity (Cui et al., 2018; Qu et al., 2020). Xu et al. (2020) showed that after 1 and 3 years of rice restoration, there was a reduction in soil salinity and an increase in nutrient content and microbial diversity. Paddy soils experience periodic changes in redox conditions, which can affect the types and activity of microbial communities present (Qu et al., 2022). For instance, flooding of soil activates diverse anaerobic metabolic pathways involving various functional microbial communities like denitrifiers, methanogens, and sulfate reducers (Xue et al., 2022). Conversely, when the soil is drained, aerobic conditions take over, resulting in a shift towards aerobic microorganisms like nitrifiers and ammonia-oxidizing bacteria (Farooq et al., 2022). Although it is known that soil microbes are crucial for ecosystem sustainability (Dubey et al., 2019; Kong et al., 2020) and drive the nutrient cycling of specific elements such as C, N, and S through microbially catalyzed redox reactions (Delgado-Baquerizo et al., 2016; Crowther et al., 2019), it is still unclear how soil microbial functions contribute to saline-alkali soil remediation by rice cultivation.

Additionally, it is thought that the interdependence of plant and soil microbes has enabled them to survive in saline habitats (Mbodj et al., 2018; Pan et al., 2020; Mahmud et al., 2021). These interactions are crucial for organic matter decomposition, and nutrient mobilization (Mohanram and Kumar 2019; Gupta et al., 2021), providing prospects to enhance plant nutrition, biomass, and stress tolerance (Edwards et al., 2018; Babalola et al., 2021; Meena et al., 2022). Therefore, exploring changes in microbial functions in saline-alkali soils remediated by rice could shed light on their key roles in regulating nutrient cycling in these soils. However, most experimental studies about the remediation of saline-alkali soil have used rice cultivation as a secondary treatment and focused on the effects of different rice agronomic management practices and the application of organic or inorganic amendments on soil properties (Li et al., 2021; Huang et al., 2022). Moreover, in recent years, study on paddy soils has primarily focused on microbial diversity, soil properties, and greenhouse gas emissions (Jiang et al., 2021; Tian et al., 2022). However, there is still limited understanding of nutrient cycling in paddy soils and its relationship with soil physicochemical properties, particularly in the context of long-term restoration of saline-alkali soil.

In this study, we hypothesized that the restoration of saline-alkali soil through rice cropping leads to changes in soil properties and microbial functions at different successional stages. Additionally, we proposed that soil factors drive the functional succession of soil microbes under saline-alkali conditions. To test this hypothesis, a 23-year remediation succession experiment was carried out in saline-alkali soils with rice cropping. Soil physicochemical characteristics were determined, and the succession of soil microbial functions was identified by shotgun metagenomics.

2. Materials and methods

2.1. Field description and soil sampling

This long-term experiment was initiated in 1998 and was conducted in typical saline-alkali soil in the Songnen Plain in Qianguo County, Songyuan City, Jilin Province, China (44°55'22"N, 124°38'14"E, and 155 m a.s.l.). This region has a typical temperate continental monsoon climate with an annual precipitation of 400–500 mm and an average annual temperature of 4.5 °C. According to the World Reference Base for Soil Resources, the main soil type is classified as Solonetz, with a pH above 8.5. The rice cropping density was 1.2 million plants ha⁻¹. Chemical fertilizers were broadcast over the soil annually at rates of 165 kg ha⁻¹ N (as urea, CH₄N₂O), 90 kg ha⁻¹ P (as calcium superphosphate, CaH₄P₂O₈), and 90 kg ha⁻¹ K (as potassium sulfate, K₂SO₄); the fertilizer was mixed into the subsoil by plowing.

Soil samples were collected on 15 October 2021 (during the harvest period) from eight planted paddy fields that had been cultivated for 2, 4,

6, 8, 11, 12, 20, or 23 years and from bare land as a control. Seven samples were collected from each of the fields as replicates. We targeted non-rhizosphere soil at a depth of 0–20 cm to better reduce the potential impact of rice cultivars at field level. All samples were stored on ice and transported to the laboratory. Aliquots of 10 g of soil were stored at –80 °C and used for DNA extraction. The remaining soil was stored at 4 °C and used for the analysis of soil properties.

2.2. Measurement of soil properties

Undisturbed soil samples were physically fractionated into four size classes using the wet-sieving method described by Bansal et al. (2021) and Luo et al. (2018): >2, 0.25–2, 0.053–0.25, and <0.053 mm. All fractions were dried at 70 °C for 24 h and then weighed to calculate the proportions of aggregates with different particle sizes in each soil sample. The mean weight diameter (MWD) was selected as an indicator of aggregate formation and stability (Li et al., 2020). Before soil pH and electrical conductivity (EC) measurements, the soil samples were air-dried at room temperature and sieved with a 2-mm mesh. Soil pH and EC were measured with a pH meter (PHSJ-4F, Leici, China) and conductivity meter (DDSJ-308F, Leici, China), respectively, in a soil-water suspension (1:5 w/v) after 30 min of shaking at 25 °C (Chu and Grogan 2010; Han et al., 2021). To measure soil organic matter (SOM), total nitrogen (TN), total phosphorus (TP), nitrate-N (NO₃-N), ammonium-N (NH₄⁺-N), and available phosphorus (AP), air-dried soil was passed through a 0.15-mm sieve (Luo et al., 2017). SOM and TN were determined by combustion using an automatic elemental analyzer (Elemental Analyzer System Vario Macro Cube, Germany). Soil TP was digested with HF–HClO₄, AP was extracted with 0.5 M sodium bicarbonate (Chen et al., 2023), and nitrate-N (NO₃-N) and ammonium-N (NH₄⁺-N) were extracted with 2 M potassium chloride (Xu et al., 2019). AP:TP is the ratio of AP to TP. C:N is the ratio of SOM to TN, and C:N:P, is the ratio of SOM to TN divided by TP.

2.3. DNA extraction and shotgun metagenomic sequencing

Total DNA was extracted from 0.5 g of soil by using a Fast PowerSoil DNA Isolation Kit (MP Biomedicals, USA), and the DNA quality and quantity were assessed in a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and Qubit 3.0 Fluorometer (Thermo Fisher Scientific, USA). A DNA library of 250-bp fragments was constructed with a Nextera DNA Library preparation kit (Illumina Inc., USA) and sequenced on an Illumina HiSeq 2500 platform (Illumina Inc., USA) by Biomarker Technologies Company (Beijing, China). Clean reads were obtained from the raw reads by removing adaptor sequences and trimming and removing low-quality reads (reads with N bases and a minimum quality threshold of 20). The trimmed reads were then mapped to the human and rice genomes, and Bowtie2 software was used to identify and remove human reads (human release 40, GRCh38.p13, downloaded from GENCODE). The pooled metagenomic reads from each sample were de novo assembled by Megahit (v 1.1.2). The assembly quality was evaluated by QUAST (v 2.3), and contigs with a size of at least 200 bp were assembled. The metagenes were predicted by Prodigal, and nonredundant metagenes were obtained by CD-HIT at an identity cutoff of 95%. For the genes predicted by Prodigal functional annotation was processed by eggNOG-mapper (v 2) with DIAMOND (v 0.8.22.84) against the associated reference database eggNOG 5.0 (Buchfink et al., 2015; Cantalapiedra et al., 2021). The filtered sequencing reads were aligned against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database using Diamond with default settings for the functional assignment of predicted genes. The abundance of key functional genes (in transcripts per kilobase million, TPM) involved in the first-category maps of the KEGG pathways was compared between the bare saline-alkali soil and rice-remediated soils, revealing significant changes. Subsequently, further analysis on the second-category of KEGG pathways corresponding to the first-category maps was carried out to

demonstrate the impact of different remediation years on microbial functions.

2.4. Statistical analyses

The soil property data were analyzed by using GraphPad Prism 9.0 software. All variables are reported as the mean \pm standard deviation (SD). The statistical significance of differences in soil properties between bare saline-alkali soil and rice-remediated soil were analyzed using independent *t*-test with false discovery rate (FDR) multiple comparisons corrections (Benjamini and Hochberg 1995). The least-squares linear regression model was used to fit the correlations between soil properties and the number of years of rice cultivation. Principal coordinate analysis (PCoA) based on the Bray-Curtis distance matrix was conducted in R v 4.1.2 (vegan package) to showcase the functional structural characteristics in different remediation years, and differences were examined using permutation multivariate analysis of variance (PERMANOVA) through the 'adonis' function in the "vegan" R package (Weiss et al., 2017). Statistical analyses of changes in microbial functional composition and abundance were performed in GraphPad Prism and Microsoft Excel. Pearson correlation analysis between microbial functions and soil properties was performed by using the OmicStudio tools (<https://www.omicstudio.cn/tool/62>); the hierarchical clustering of soil chemical and

physical properties was based on Euclidean distance.

3. Results

3.1. Rice-mediated remediation improved saline-alkali soil properties

Rice-mediated remediation markedly improved the nutrient content of saline-alkali soil. Soil TP, TN, and SOM increased significantly over time (Fig. 1a). Soil AP and $\text{NH}_4^+\text{-N}$ also increased significantly (Fig. 1b). Furthermore, the AP:TP ratio, C:N ratio, and the MWD of soil aggregates improved significantly in rice-remediated soil compared with bare saline-alkali soil (Fig. 1c and d). By contrast, the pH and EC of the saline-alkali soil decreased greatly during remediation (Fig. 1d).

As shown in Fig. 1, some of the soil variables exhibited significantly greater variation (R^2 from 0.64 to 0.94, $P < 0.05$) as the number of years of remediation increased. For instance, older fields of remediation increased their contents of TP ($R^2 = 0.64$, $P < 0.05$) and AP ($R^2 = 0.79$, $P < 0.05$) and the AP:TP ratio ($R^2 = 0.83$, $P < 0.05$) showed a quadratic response along the years of remediation. By contrast, TN content gradually decreased over time ($R^2 = 0.89$, $P < 0.05$), whereas the C:N ratio ($R^2 = 0.76$, $P < 0.05$) and C:N:P ratio ($R^2 = 0.94$, $P < 0.05$) steadily increased. In addition, soil pH and EC content showed downward trends with successive remediation mediated by rice cultivation (Slope < 0 , P

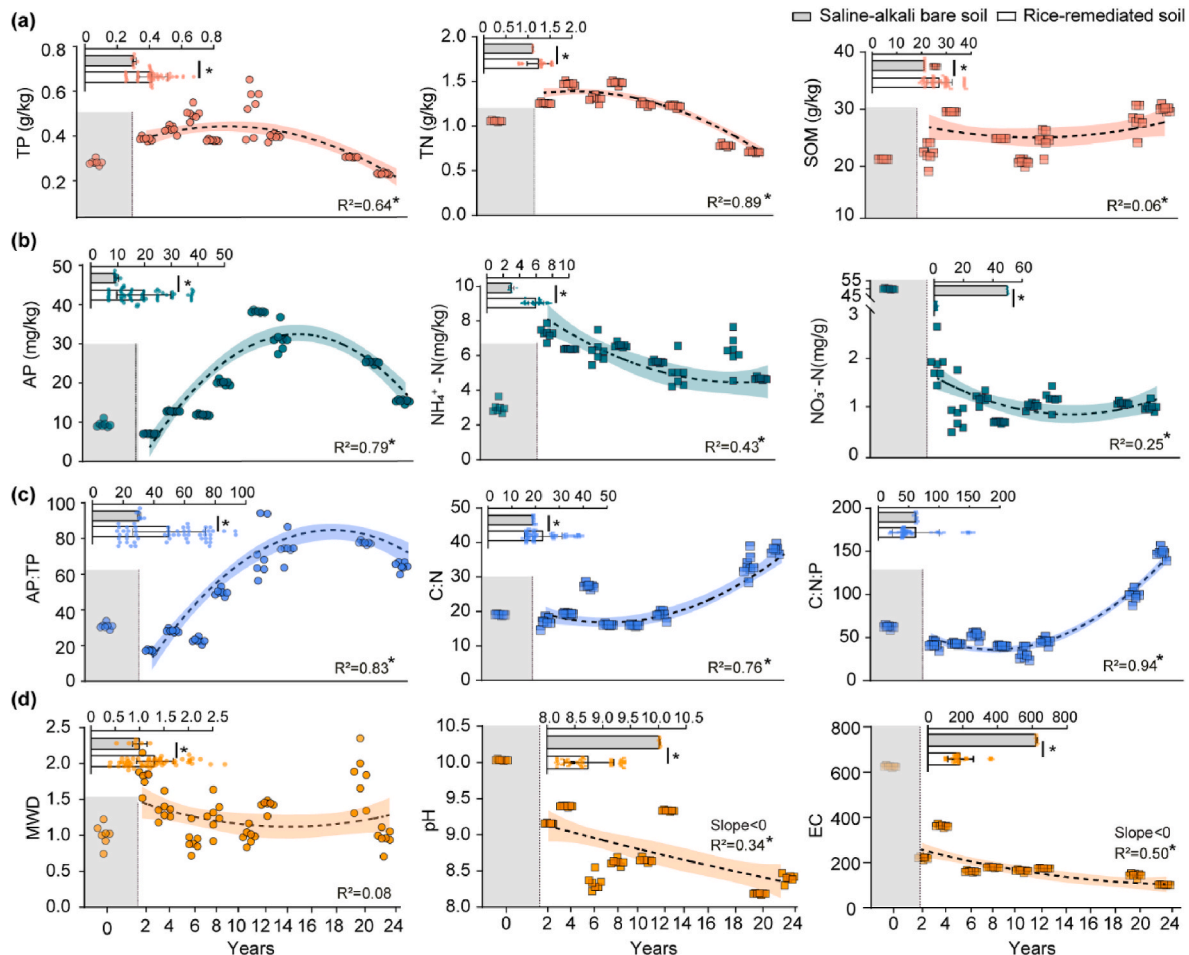


Fig. 1. Effects of rice cultivation on soil properties in saline-alkali soil (horizontal bar chart) and the trend pattern in different years (scatter plot). TP, total phosphorus; TN, total nitrogen; SOM, soil organic matter; AP, available phosphorus; $\text{NH}_4^+\text{-N}$, ammonium nitrogen; $\text{NO}_3^-\text{-N}$, nitrate nitrogen; AP:TP, available phosphorus: total phosphorus ratio; C: N, soil organic matter: total nitrogen ratio; C: N: P, soil organic matter: total phosphorus ratio; MWD, mean weight diameter; EC, electrical conductivity. Inset statistics in bar charts are the mean (saline-alkali bare soil, $n = 7$ soil samples; Rice remediation, $n = 56$ soil samples), error bars are standard deviations (SD), and asterisks indicate significance differences ($P < 0.0001$, FDR) by *t*-test. Trend lines in scatter plot display the least squares linear regression model, shaded region indicates the standard deviations ($n = 7$ soil samples per year), and the asterisks indicate the variation explained by the model is statistically significant ($P < 0.05$).

< 0.05; Fig. 1d).

3.2. Microbial function succession during saline-alkali soil remediation

The PCoA based on Bray-Curtis distance metrics demonstrated that there were significant differences in microbial functional structure during remediation, which was confirmed by PERMANOVA ($F = 28.83$, $P = 0.001$; Fig. 2a). To further identify the variations in functional composition in succession of remediation, functional differences were detected by transcripts per million (TPM) values in first-category maps of the KEGG pathways. Compared with the bare saline-alkali soil, all functions increased to varying degrees in succession stages of remediation. Surprisingly, only the increase in the energy metabolism category was significant ($FC > 2.0$, $FDR < 0.05$; Fig. 2b). Therefore, regression analysis of the Bray-Curtis similarity of energy metabolism during remediation was performed to explore the succession of energy metabolism. A significant negative correlation between the Bray-Curtis similarity of energy metabolism and the number of years of remediation was observed, indicating a shift in energy metabolism with increasing duration of remediation ($R^2 = 0.11$, $P < 0.05$, Slope < 0; Fig. 2c). Additionally, the abundance of reads associated with energy metabolism

gradually increased as the number of years of remediation increased from 2 to 12 and decreased thereafter ($R^2 = 0.66$, $P < 0.05$; Fig. 2d). The main nutrient cycling processes within energy metabolism were the N (Fig. 3), C (Fig. 4), and S (Fig. 5) cycles.

3.2.1. Effects of saline-alkali soil remediated by rice cultivation on the nitrogen cycle

The main process of the N cycle is shown in Fig. 3a. In bare saline-alkali soil, the relative abundance of nitrite reduction was highest (43.43%); however, after remediation, N fixation (33.65%) dominated the N cycle, with an increase in abundance of 50.21-fold compared with bare saline-alkali soil (Fig. 3b, pie chart). As the number of years of remediation increased, the relative abundances of nitrite oxidation and nitrate reduction gradually increased, while the relative abundances of nitrite reduction and nitric oxide reduction decreased (Fig. 3b, bar chart). Further fitting of N fixation abundance as a function of years of remediation revealed an “increase-peak-decline” pattern ($R^2 = 0.34$, $P < 0.05$; Fig. 3c). Notably, the ratio of nitrification to denitrification increased with years of remediation ($R^2 = 0.58$, $P < 0.05$, Slope > 0; Fig. 3c). Correlation analysis (Fig. 3d) showed that the dynamics in N-cycle-related functions were significantly correlated with the changes in

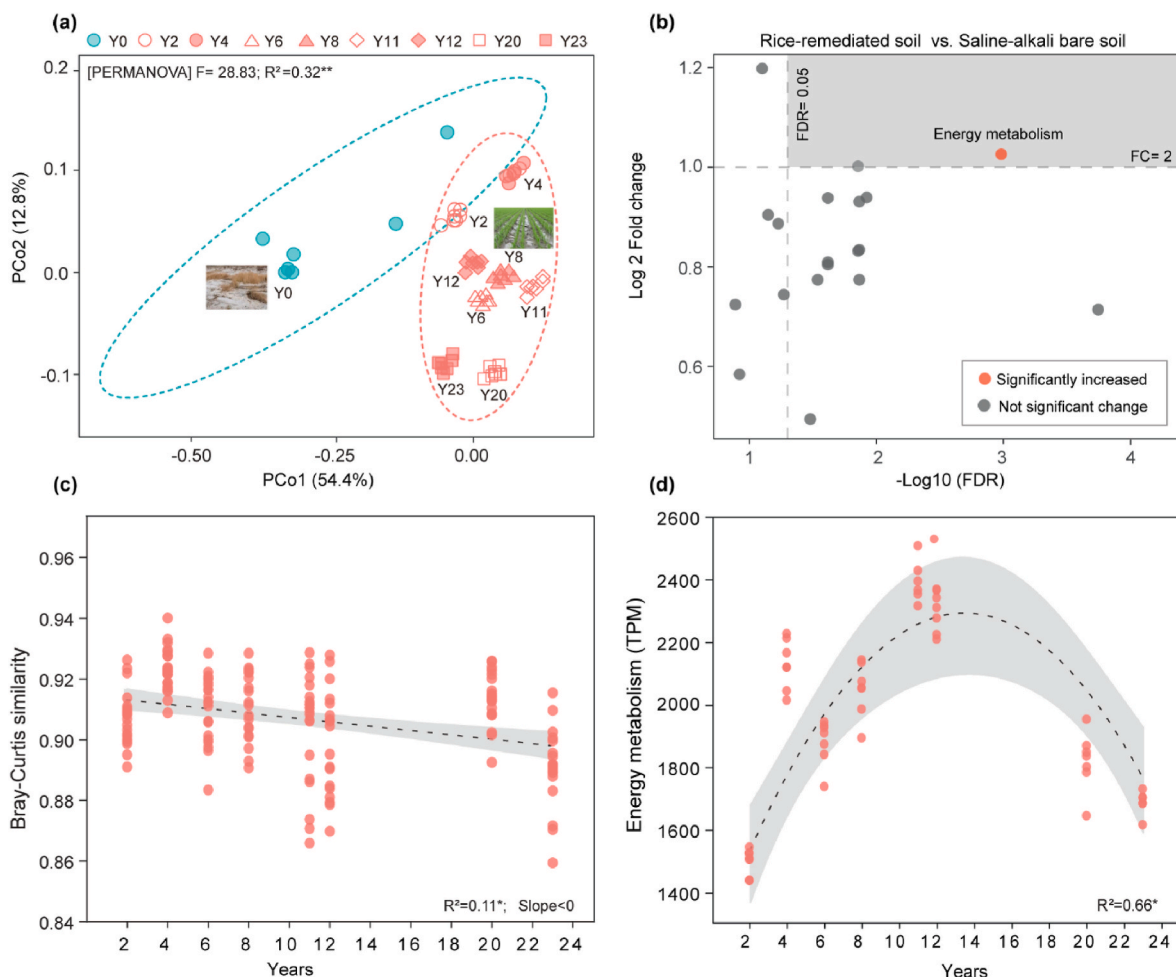


Fig. 2. Overview of microbial functional structure succession in saline-alkaline soils. (a) Principal coordinate analysis (PCoA) of microbial function based on metagenomic sequencing (KO gene abundance based on transcripts per million (TPM) metric; $n = 63$ samples), according to the Bray-Curtis distance. (b) Functional differences between rice-remediated soil and saline-alkali bare soil. Points represent annotated to different functions (first category maps) via the KEGG pathway database. The significance (x-axis; FDR: adjusted P -value) and the fold-change (y-axis) are converted to $-\log_{10}$ (FDR) and \log_2 (fold change), respectively. The vertical and horizontal dotted lines show the cut-off of $FC = 2$, and of $FDR = 0.05$, respectively. There was a functional pathway (energy metabolism) that is significantly ($FC > 2$ and $FDR < 0.05$) increased after remediation by rice cultivation (upper-right, dots colored red). (c) Relationship between Bray-Curtis similarity and saline-alkali soil remediation years. (d) Dynamic varies of energy metabolism in different remediation years in saline-alkali soil. Trend lines display the least squares linear regression model, shaded region indicates the standard deviations ((c), (d), $n = 56$ samples). *and ** indicate that means were significantly different at $P < 0.05$ and $P < 0.01$, respectively.

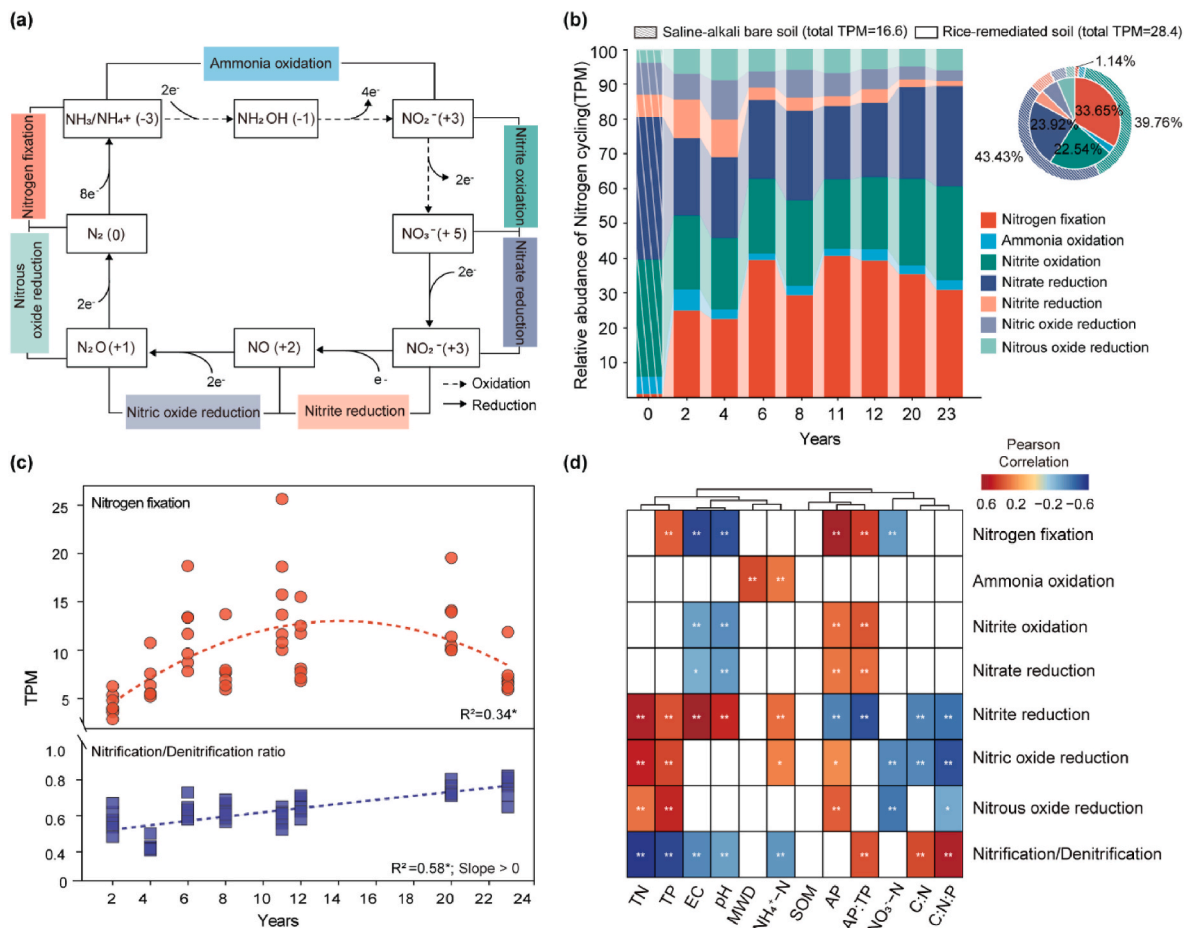


Fig. 3. Succession of nitrogen cycle (N) in saline-alkali soil during different years of rice remediation. (a) Key process of N cycle. The value in each bracket means the valence states of N-element. Dashed lines between compounds represent the oxidation reaction, while the solid lines indicate reduction reactions. (b) Relative abundance of the main process in N-cycle-related function at different remediation years. The colors in the bar and pie charts correspond to different processes in N-cycle. (c) Dynamic change of nitrogen fixation and the ratio of nitrification to denitrification with different remediation years in saline-alkali soil. Trend lines display the least squares linear regression model ($n = 7$ samples per remediation years). (d) Heat map of Pearson's correlation analysis between the N-cycle-related functions and soil environmental variables. The color scale on the right shows the color partitioning of the different r values. *and ** indicate that means were significantly different at $P < 0.05$ and $P < 0.01$, respectively.

soil variables. Such as, nitrogen fixation, nitrite oxidation, and nitrate reduction were significantly positively correlated with AP, and the AP:TP ratio and negatively correlated with pH and EC. By contrast, nitrite reduction was positively correlated with pH and EC and strongly negatively correlated with the AP:TP ratio. In addition, the changes in ammonia oxidation were mainly positively correlated with soil aggregate stability (MWD) and NH₄⁺-N. Finally, the ratio of nitrification to denitrification was positively correlated with the AP:TP, C:N and C:N:P ratios.

3.2.2. Effects of saline-alkali soil remediated by rice cultivation on the carbon cycle

Among C-cycle-related functional processes in energy metabolism, microorganisms primarily participate in photosynthetic C fixation (Calvin cycle), prokaryotic C fixation, organic C oxidation, and methanogenesis (Fig. 4a). In general, C fixation by prokaryotes dominated C-cycle processes in bare saline-alkali soil (Fig. 4b, bar chart). After remediation, the abundance of C fixation in the Calvin cycle increased from 9.77% to 16.69%, and the abundance of methanogenesis increased from 4.60% to 8.81% (Fig. 4b, pie chart). In addition, the patterns of changes in C-cycle-related functions as a function of years of remediation were similar and had a high goodness-of-fit (R^2 from 0.55 to 0.71), with peaks at 12 years of remediation and subsequent declines at 20 and 23 years (Fig. 4c). Further correlation analysis showed that the shift in C-

cycle-related functions was strongly positively correlated with AP and the AP:TP ratio and significantly negatively correlated with EC and NO₃⁻-N (Fig. 4d). The changes in methanogenesis and C fixation were also significantly negatively correlated with soil pH (Fig. 4d).

3.2.3. Effects of saline-alkali soil remediated by rice cultivation on the sulfur cycle

Assimilatory sulfate reduction, dissimilatory sulfate reduction and thiosulfate oxidation are the key components of the biogeochemical S cycle, which mainly converts sulfide (SO₃²⁻) and thiosulfate (S₂O₃²⁻) to sulfate (SO₄²⁻) (Fig. 5a). Remediation significantly enhanced S-cycle-related functions, especially the relative abundance of thiosulfate oxidation, which increased from 4.71% to 25.16% (Fig. 5b, pie chart). Assimilatory sulfate reduction predominated both before and after remediation (Fig. 5b). Assimilatory and dissimilatory sulfate reduction and the ratio of assimilatory sulfate reduction to dissimilatory sulfate reduction gradually increased from 2 to 12 years of remediation and decreased thereafter (R^2 from 0.15 to 0.44, $P < 0.05$; Fig. 5c). Moreover, the ratio of assimilatory sulfate reduction to thiosulfate oxidation increased with the number of years of remediation ($R^2 = 0.32$, $P < 0.05$, Slope > 0 ; Fig. 5c). The analysis of the correlation between S-cycle-related functions and soil environment (Fig. 5d) revealed that assimilatory and dissimilatory sulfate reduction, as well as the ratios of assimilatory sulfate reduction to dissimilatory sulfate reduction and

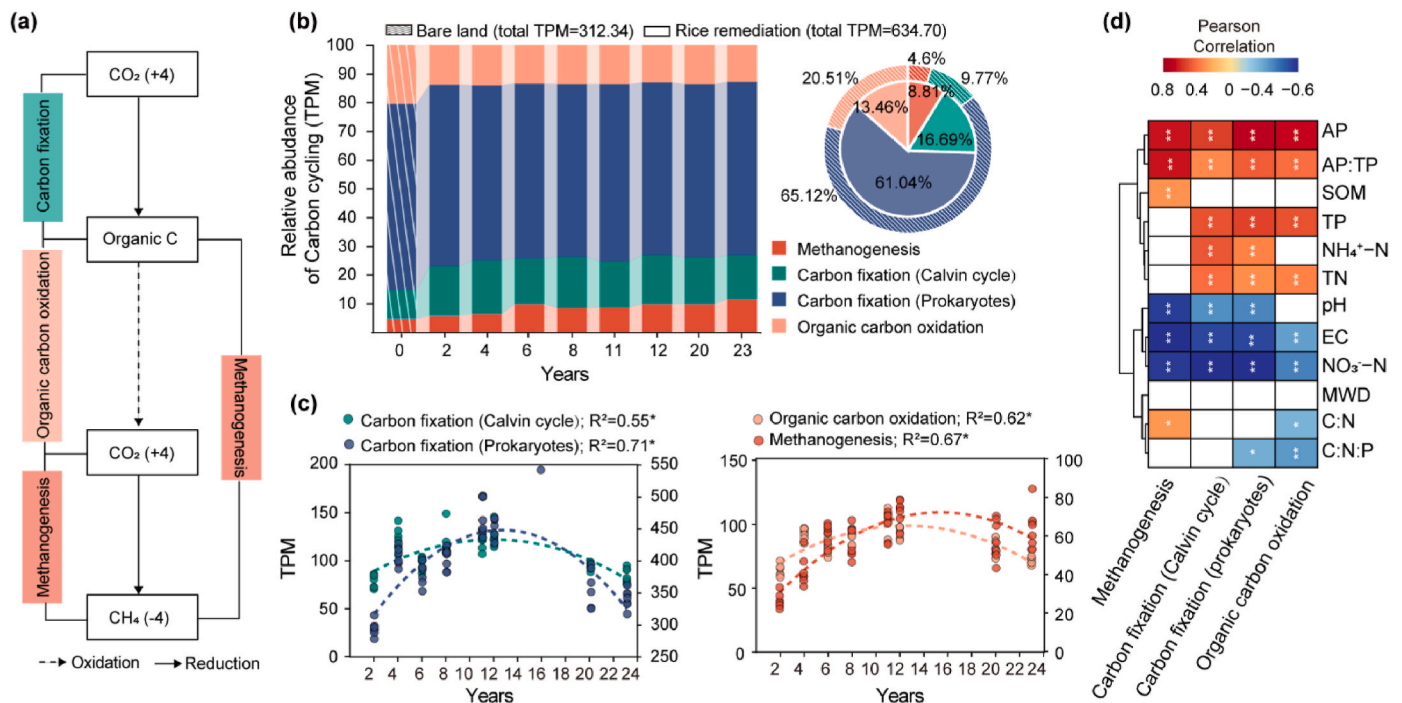


Fig. 4. Succession of carbon (C) cycle in saline-alkali soil during different years of rice remediation. (a) Simplified C-cycle-related functional schematic. The value in each bracket means the valence states of C-element. Dashed lines between compounds represent the oxidation reaction, while the solid lines indicate reduction reactions. (b) Relative abundance of C-related functions at the different remediation years. The colors in the bar and pie charts correspond to different processes in C-cycle. (c) Dynamic changes of C-cycle-related functions in different remediation years. Trend lines display the least squares linear regression model ($n = 7$ samples per remediation years). (d) Heat map of Pearson's correlation analysis between the C-cycle-related functions and soil environmental variables. The color scale on the right shows the color partitioning of the different r values. * and ** indicate that means were significantly different at $P < 0.05$ and $P < 0.01$, respectively.

assimilatory sulfate reduction to thiosulfate oxidation, exhibited significant positive correlations with P (including TP, AP, and the AP:TP ratio).

4. Discussion

Phytoremediation is widely recognized for its environmentally friendly and ecologically responsible characteristics, and its ability to stimulate synergies among plants, soil properties, and microbes (Qadir et al., 2007; Kim and Owens 2010; Ali et al., 2013). However, the lack of integrated understanding on how ecological functions and soil properties related during soil remediation through rice cultivation has limited its capacity to improve soil quality (Cui et al., 2018). It should be noted that the unique characteristics of rice cultivation for the remediation of saline-alkali soil involve complex agronomic management practices, including tillage and fertilization, which can have a significant impact on microbial functions. Therefore, we sought to address this knowledge gap, by investigating the changes in soil microbial functions and soil physicochemical properties at successive stages of saline-alkali soil remediation through rice cultivation, spanning periods of 2, 4, 6, 8, 11, 12, 20, and 23 years. Our study provides a more comprehensive understanding of the peaks of soil quality and microbial functioning during the process of rice-mediated remediation in saline-alkali soils.

4.1. Rice-mediated remediation improves soil fertility and reduced soil salinity

The implementation of saline soil remediation by rice cultivation can result in a noteworthy enhancement in soil nutrients, such as soil TP, TN, SOM, AP, and $\text{NH}_4^+\text{-N}$, as well as a significant reduction in soil pH and EC compared to saline bare soil without remediation (Fig. 1). In line with our findings, Xu et al. (2020) reported that rice remediation for 1 and 3 years decreased the salinity and increased the nutrient content of

saline-alkali soil. As soil nutrients are coupled to each other during cycling, the positive correlation between AP:TP and C:N ratios with nutrient availability supports the effectiveness of rice restoration strategies (Yu and Chi 2019). Moreover, remediation mediated by rice improves soil aggregate structure, as evidenced by significantly higher MWD (Wang et al., 2022), which is essential for maintaining saline-alkali soil quality (Xie et al., 2020; Chen et al., 2021). The positive correlation between MWD and $\text{NH}_4^+\text{-N}$ found in the current study (Fig. S1) suggests that the growth of rice roots stimulates water infiltration, air permeability, and nutrient cycling in saline-alkali soils (Xu et al., 2020).

In the present study, $\text{NO}_3^-\text{-N}$ content decreased significantly after remediation, which could be related to the alternating wet and dry conditions of rice cultivation that create aerobic and anaerobic states. NO_3^- is highly mobile and can leach easily to deeper soil layers, hence some of it in the oxidized topsoil layer may be absorbed by the rice roots or diffuse into the underlying reduced layer where it may be denitrified into N_2O or N_2 (Tan et al., 2013). The negative correlation observed between $\text{NO}_3^-\text{-N}$ and nitric oxide reduction (reducing NO to N_2O) and nitrous oxide reduction (reducing N_2O to N_2) (Fig. 3d) lends further support to these possibilities. Additionally, microbes use NO_2^- , NO and N_2O as terminal electron acceptors to generate N_2 due to NO_3^- limitation, which leads to the loss of N. This may result in the efficiency of N use in paddy soil being less than half of what is typically observed in other agricultural systems (Anas et al., 2020). Therefore, to better regulate the effects of the N-cycle on paddy soil or the effectiveness and efficiency of agriculture or restoration, it may be necessary to give more attention to dry and wet irrigation practices.

It is unsurprising that the fertility and soil aggregate stability increased and salinity decreased in paddy soil as result of rice cultivation and specific soil management practices like tillage and fertilization (Fageria et al., 2011, IUSS Working Group, 2014). However, by covering a 23-year long period of remediation, it was observed that a quadratic

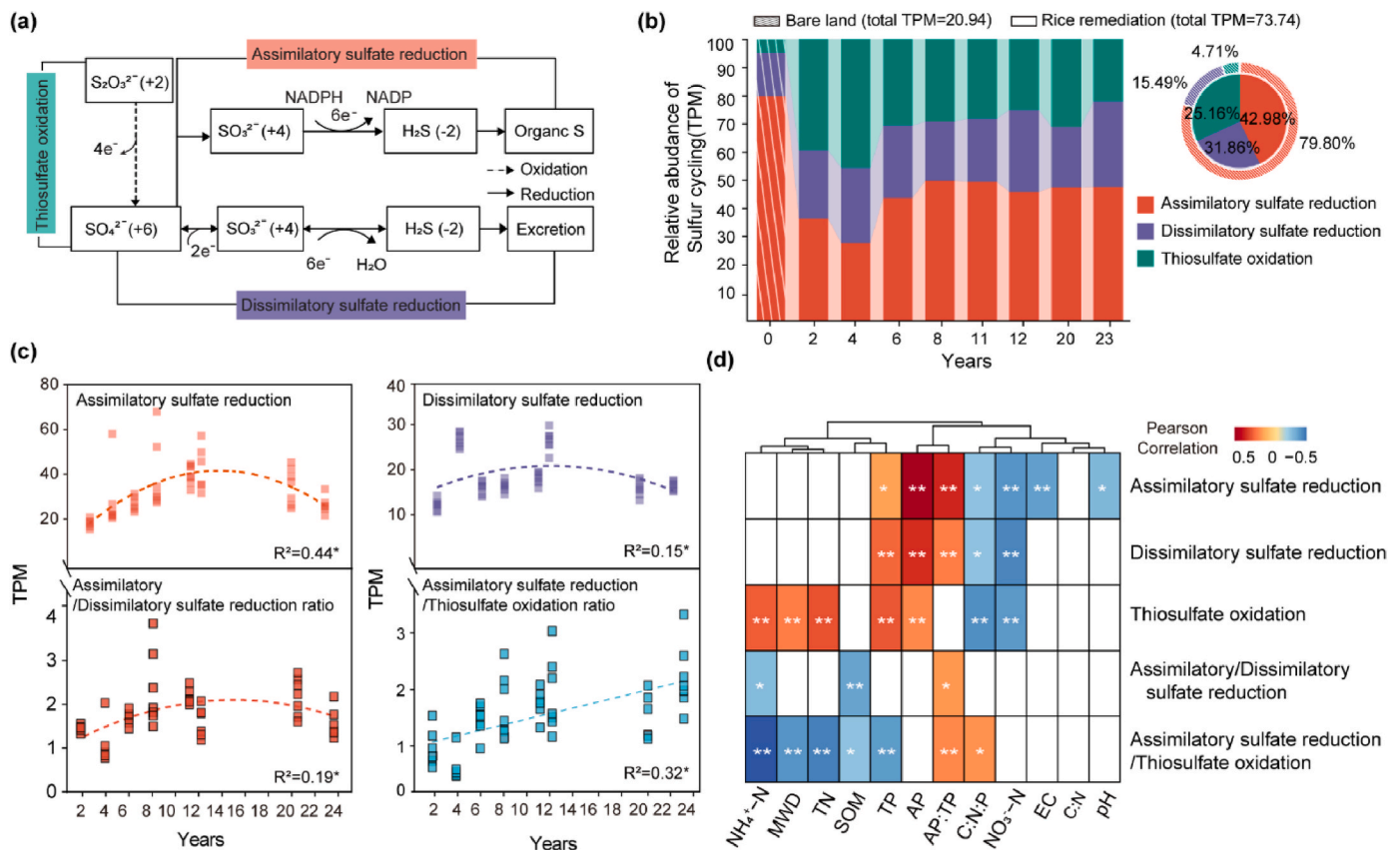


Fig. 5. Succession of sulfur (S) cycle in saline-alkali soil during different years of rice remediation. (a) Simplified schematic diagram of the sulfur cycle. The value in each bracket means the valence states of S-element. Dashed lines between compounds represent the oxidation reaction, while the solid lines indicate reduction reactions. (b) Relative abundance of the main processes in S-cycle-related functions at the different remediation years. The colors in the bar and pie charts correspond to different processes in S-cycle. (c) Dynamic relationships among S-cycle-related functions at different remediation years in saline-alkali soil. Trend lines display the least squares linear regression model ($n = 7$ samples per remediation years). (d) Heat map of Pearson's correlation analysis between the S-cycle-related functions and soil environmental variables. The color scale on the right shows the color partitioning of the different r values. * and ** indicate that means were significantly different at $P < 0.05$ and $P < 0.01$, respectively.

response, particularly changes in P (AP, TP, AP:TP ratio; Fig. 1b, a, c), indicating the remediation strategy reached a peak in the improvement of soil quality between 11 and 16 years of rice cultivation. After 16 years of continuous rice cultivation, the reasons for the decrease of soil TP and AP:TP ratio may be related to various biotic and abiotic factors. Rice requires uptake substantial amounts of nutrients, including the N and P for growth and development (Goswami et al., 2020; Wang et al., 2023). Additionally, the long-term rice monoculture system may be accompanied by loss of topsoil, and a decline in soil fertility over time (Wei et al., 2022). Furthermore, the constant flooding of paddy soil can lead to the formation of Fe- and Al-bound P (Fe/Al-P), which are not readily available to rice, and over time, these insoluble P compounds can accumulate (Bhattacharyya et al., 2015). Moreover, paddy soils undergo long-term flooding conditions that reduce the soil redox potential (Kögel-Knabner et al., 2010), this low redox potential may change in soil pH, microbial communities, and the microbial-mediated redox processes, and these changes related to the dynamics of soil P (Ahmed et al., 2019; Qaswar et al., 2022). The changes in functions related to N, C, and S cycling (i.e., N fixation, C fixation, organic carbon oxidation, methanogenesis, and sulfate reduction), as well as their correlations with P observed in this study, further support the possibility that alterations in microbial processes due to remediation through rice cultivation can impact soil P dynamics (Figs. 3, 4, 5, S1).

4.2. Feedback effects of saline-alkali soil remediated by rice cultivation on microbial function

The shifts in the potential function of the soil microbiome revealed the influence of different succession stages in rice-mediation of saline-alkali soil. The most prominent change was increased energy metabolism (Fig. 2b). The alternating wet and dry conditions in paddy fields result in microbially driven redox shifts between aerobic and anaerobic environments, which regulate nutrient cycling and energy flows. Among energy metabolism functions, rice cultivation increased the abundances of functions related to N, C, and S cycling in saline-alkali soil, especially N fixation, C fixation, methanogenesis, dissimilatory sulfate reduction, and thiosulfate oxidation (Figs. 3c, 4c and 5c). This finding is important because N, C, and S cycles are essential for ecosystem sustainability (Canfield et al., 2010; Basu et al., 2021), which is driven by microbes that facilitate nutrient cycling (Balestrini et al., 2015; Liu et al., 2020) and consequently impact plant growth (Moreau et al., 2019; Naylor et al., 2020; Narayan et al., 2022).

Nitrogen fixation, which reduces N_2 to $\text{NH}_3/\text{NH}_4^+$, is particularly important because N is usually the limiting factor for crop growth and productivity (Sun et al., 2021). In the current study, the abundances of N fixation associated genes were consistent with the changes in overall energy metabolism: it was highest in the middle successional stages, specifically years 11–16, and declined thereafter (Figs. 2d and 3c). As discussed previously, this may be related to the changes in soil properties (Fig. 3d), such as soil pH and EC (Yang et al., 2022) and interestingly were especially limited by AP (Vance et al., 2000; Ford et al., 2016; Wei

et al., 2017). N fixation is an energy-intensive process, and P is required for the synthesis of ATP, the energy currency of cells. Nitrification and denitrification, which convert N in various forms (Lourenço et al. 2018, 2022), also require energy and are stimulated by the presence of P. In addition, there is a growing body of evidence suggesting that P availability plays a crucial role in enhancing plant growth by promoting photosynthesis and stimulating the activity of various microbes involved in redox reactions, including those related to the nitrogen cycle, such as nitrogen-fixing bacteria, nitrifiers, and denitrifiers (Tang et al., 2016; Wei et al., 2017; O'Neill et al., 2022). Our study also supports this notion, as we found that AP was positively correlated with the abundance of genes related to nitrogen fixation, nitrification, and denitrification (Fig. 3d, S1). These findings suggest that maintaining adequate P availability in paddy soils is essential for maintaining microbial functions related to nitrogen cycling and ultimately improving soil fertility.

Moreover, AP and the AP:TP ratio not only responded positively to N-cycle-related functions but were also closely correlated with almost all key processes in the C and S cycles (Figs. 4d, 5d, S1). Given P is second only to N as the most limiting nutrient for plant growth and has myriad essential biological functions as a structural element in phospholipids and nucleic acids (Kumar et al., 2018), in energy metabolism, in the regulation of enzymatic activities, and in signal transduction cascades (Vance et al., 2000; Rausch and Bucher 2002). Despite the lack of direct evidence demonstrating that P availability enhances the cycling of C and S, there is a correlation between certain microbes, such as arbuscular mycorrhizal fungi (AMF), which facilitate the uptake of P, and the promotion of SOM decomposition and transport of SO_4^{2-} (Hodge et al., 2001; Narayan et al., 2022). As a result, these microbes facilitate N, C, and S nutrient cycling and help maintain the balance of nutrients in the paddy ecosystem. Another important finding from the current study is that the ratio of nitrification to denitrification increased with remediation succession stages (Fig. 3c). Specifically, nitrification tended to increase with succession stages (Fig. S2a), whereas denitrification, i.e., nitrite reduction (reducing NO_2^- to NO) and nitric oxide reduction (reducing NO to N_2O), obviously decreased (Figs. S2b and c). This shift towards higher rates of nitrification suggests an increase in the availability of NO_3^- in the soil. This may be beneficial for plant growth, because rice absorbs and assimilates NO_3^- more efficiently than NH_4^+ (Kirk and Kronzucker 2005). In short, our study found that growing rice crop can improve the long-term remediation of saline-alkali soil by enhancing N utilization. Additionally, we suggest that farmers interested in using rice for saline soil remediation or future research in this subject should carefully monitor the effectiveness of soil P nutrients.

With respect to the C cycle, the anaerobic conditions induced by flooding can slow organic matter decomposition and thus promote soil organic C accumulation. However, these conditions can also increase methane emissions (Liu et al., 2021), which may explain the rapid increase in the relative abundances of C fixation and methanogenesis in years 2–16 and their subsequent decline in the later stages of remediation (Fig. 4c). Although direct evidence is lacking, previous studies have shown that fertilization and irrigation can be rate-limiting factors in C fixation and methanogenesis (Yang and Chang 1998; Bar-Even et al., 2010). The availability of electron donors is another important determinant of the dominant reduction process (Chidthaisong and Conrad 2000); methanogenesis becomes the terminal process of organic mineralization only when SO_4^{2-} or NO_3^- (electron acceptors) is depleted (Jørgensen et al., 2019). Moreover, there is accumulating evidence from different ecosystems that the succession of biogeochemical activities in soil follows soil environmental conditions (Schmidt et al., 2011; Hussain et al., 2021).

The S cycle is an essential biogeochemical cycle because S is a structural component of protein disulfide bonds, amino acids, vitamins, and cofactors. Sulfur is especially important for redox-active control in microbial respiration and is required for the adequate growth and development of plants (Zhang et al., 2019; Dahl 2020; Narayan et al., 2022). Most S in soil is present in organic matter and hence not

accessible to plants (Narayan et al., 2022). Organic matter decomposition by microbes mineralizes organic S to SO_4^{2-} , which is available to plants (Blum et al., 2013). Sulfate must be reduced and activated before absorption. Depending on the microorganism, sulfate reduction is assimilatory or dissimilatory (Kushkevych et al., 2020). Assimilatory sulfate reduction uses SO_4^{2-} in amino acid synthesis, whereas dissimilatory sulfate reduction generates energy using SO_4^{2-} as an electron acceptor. Intriguingly, the patterns observed in assimilatory and dissimilatory sulfate reduction were similar to the trends of N and C fixation during saline-alkali soil restoration. In addition, the balance between assimilatory and dissimilatory sulfate reduction, as well as the balance between assimilatory sulfate reduction and thiosulfate oxidation both indicate that organic S accumulated in the soil during remediation (Fig. 5c).

In summary, this study establishes the succession of soil physico-chemical properties and microbial functions in saline-alkali soil remediation through long-term rice cultivation. The findings demonstrate that rice cropping can significantly improve key nutrient contents (TP, TN, SOM, AP, and $\text{NH}_4^+\text{-N}$), as well as soil aggregate stability (MWD), and stoichiometry (AP:TP ratio and C:N ratio). The study also reveals the response of microbial functions related to nutrient cycling (i.e., N fixation, C fixation, methanogenesis, dissimilatory sulfate reduction, and thiosulfate oxidation) at different stages of remediation. Interestingly, the changes in soil properties, particularly P availability (AP and AP:TP), were identified as the primary drivers of the succession of soil microbial N, C, and S functions during saline-alkali soil remediation. Moreover, this study demonstrates that continuous rice cultivation for 11–16 years is sufficient to restore the physicochemical properties of saline-alkali soil and the equilibrium of microbial functions related to N, C, and S cycles without the use of other chemical and physical restoration approaches. Overall, this study provides new insights into the succession of soil properties and nutrient stoichiometry and their links with microbial functions during the remediation of saline-alkali soil by rice cropping.

Ethics approval and consent to participate

Ethics approval was not required for this study.

Consent for publication

Not applicable.

Availability of data and materials

The raw sequences were submitted to the National Center for Biotechnology Information (NCBI; <https://www.ncbi.nlm.nih.gov/>) under study accession number PRJNA924737.

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Authors' contributions

L.J., C.T. and E.E.K. planned and designed the research; L.J. and C.T. conducted the fieldwork. L.J. analyzed the data with supervision of E.E.K. and wrote the manuscript. E.E.K. and C.T. critically reviewed the manuscript. All author(s) read and approved the final manuscript.

Declaration of competing interest

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.soilbio.2023.109125>.

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