Biochar amendments to tropical paddy soil increase rice yields and decrease N_2O emissions by modifying the genes involved in nitrogen cycling

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Abstract

Water management strategies are critical in regulating nitrous oxide (N_2O) emissions from paddy soils under rice cultivation. Biochar is widely used as an amendment to decrease soil N_2O emissions. However, the impacts of biochar amendment on N_2O emissions under different water management strategies in paddy soils have not been investigated thoroughly, and the underlying mechanisms remain poorly understood. In particular, the effects of mid-season water drainage, a strategy used for water conservation, need to be better understood. In this study, a pot experiment was conducted including six treatments: alternating dry-wet conditions during the mid-season period without and with 2% (w/w) biochar (AWD1 and AWD2, respectively), continuous flooding during the mid-season period without (CF1) and with 2% (CF2) biochar, and regular mid-season drainage during the mid-season period without (CON1) and with 2% (CON2) biochar. All treatments received inorganic nitrogen, phosphorus and potassium fertilizers (NPK) administered in a split application. We found that fluxes of N_2O emission peaked after the two fertilizers' additions. No significant difference in the first N_2O flux peak was found among different treatments. There also was no difference in the second N_2O flux peak in both CF treatments, whereas biochar addition significantly decreased the second N_2O flux peak in AWD2 and CON2 treatments. This was mainly due to increased *nosZ* gene copies and decreased ratios of denitrification and *amoA* genes to *nosZ* gene copies. Biochar amendments resulted in significant stimulation of *nifH* gene copies in the AWD2 treatment. Lastly, biochar increased rice yields in all treatments. Our study suggested that AWD2 was the optimal management strategy for mitigating N_2O emissions and improving rice production in this tropical paddy soil.

Keywords: Water management strategies; nitrification; denitrification; nitrous oxide; greenhouse gas emissions; sustainable agriculture

1. Introduction

Global change, which is mainly caused by greenhouse gas emissions (GHGs), has become an urgent problem (Hussain et al., 2015). Among GHGs, the global warming potential of nitrous oxide (N_2O) and methane (CH_4) is more than 270 and 28 times respectively that of carbon dioxide (CO_2) over a 100-year timeframe (Neubauer and Megonigal, 2015). Moreover, N_2O is a known driver of stratospheric ozone (O_3) depletion (Hu et al., 2016). Globally, about 50% of global anthropogenic N_2O emissions are from agricultural soils (Carlson et al., 2017) as a result of inorganic nitrogen (N) fertilizers and manure application and the associated changes in N cycling processes (Shang et al., 2019; Wrage et al., 2001).

Nitrification and denitrification are the most crucial microbial processes of N_2O production in soils (Khalil et al., 2004; Wrage et al., 2004). It has been proved that autotrophic nitrification is the primary source of N_2O at low soil water potentials (30-60% waterfilled pore space (WFPS) and equivalent to < -50 kPa) (Li et al., 2016). Nevertheless, nitrification is generally considered to be a minor source of N_2O compared to denitrification (Bateman and Baggs, 2005). The emissions of N_2O due to soil denitrification have been predicted to reach 14.2 Tg (1 Tg = 10^{12} g) N $year^{-1}$ globally by 2050 (Bouwman et al., 2013). Denitrification mainly occurs under conditions of high soil moisture content or anaerobic conditions (Zumft and Kroneck 2006; Shen et al., 2021b). Therefore, in addition to fertilizer N inputs, soil moisture is a primary driver of soil N_2O emissions (Weitz et al., 2001; Shen et al., 2021a).

Paddy soils are usually subjected to artificial changes in soil moisture during the growing season (Liu et al., 2019a), which have strong effects on N cycling and GHG emissions in these soils (Nishimura et al., 2004; Yang et al., 2012). Several water management strategies are used during the rice-growing season; the traditional continuous flooding is one of the regular water management strategies, which leads to large amounts of CH_4 emissions from paddy soils (Setyanto et al., 2000; Li et al., 2005). In contrast, alternate wetting and drying (AWD) and mid-season drainage, which have been widely applied as water-saving strategies, lead to anaerobic-aerobic cycling and decreased CH_4 emissions in paddy soils (Tyagi et al., 2010; Li et al., 2021). However, AWD and mid-season drainage can promote N_2O emissions in paddy soils (e.g., Yang et al., 2012; Liao et al., 2020; Zhang et al., 2022), likely due to

the alternating oxic and anoxic conditions that favor both nitrification and denitrification (Li et al., 2005). For instance, Zeng et al. (2019) noted a significant increase in N_2O emissions as a result of mid-season drainage which could be attributed to the increase of ammonia-oxidizing bacteria (AOB). Likewise, Meijide et al. (2017) observed that the mid-season drainage created more aerobic conditions, thereby favoring N_2O emissions via incomplete denitrification or nitrification. Thus, a primary concern in rice cultivation lies in finding ways to both save water and effectively mitigate paddy soil N_2O emissions through AWD or mid-season drainage.

Numerous studies have provided evidence that biochar has the potential to decrease N_2O emissions from agricultural soil (Case et al., 2012; Brassard et al., 2016; Xu et al., 2020). Moreover, previous studies reported that biochar amendment significantly decreased soil N_2O emissions while promoting rice yield(Zhang et al., 2010; Sun et al., 2019; Feng et al., 2020). For instance, Sun et al. (2019) showed that biochar application combined with controlled irrigation (i.e., only flooding in regreening stage and 60–80% of saturated soil moisture content in other stages) not only decreased N_2O emissions by 21.5% but also increased rice yield by 20% compared to non-biochar treatment. Feng et al. (2020) observed 19.7-35.7% reductions in N_2O emissions following the application of biochar amendment in paddy soils with mid-season drainage compared to non-amendment. Therefore, there is a consensus that biochar amendment mitigates N_2O emissions in paddy soils.

Despite the evidence showing that biochar could decrease N_2O emissions, the mechanisms behind are still not clear. Some studies attributed the decrease in N_2O emissions to increasing soil pH, or to the fact that a high C/N ratio in biochar can promote microbial immobilization of N, thus decreasing nitrification or denitrification rates (Wang et al., 2012; Li et al., 2023). Recent studies suggest that the reduction of N_2O emissions with biochar is predominantly regulated by biotic mechanisms (Brassard et al., 2016; Li et al., 2021). For instance, biochar additions suppressed soil N_2O emissions from subtropical paddy soils due to increased *nosZ* gene copy numbers (Aamer et al., 2020; Han et al., 2022), and a reduction in the ratio of (*nirS* + *nirK*)/ *nosZ* (Sun et al., 2022). Li et al. (2021) also found that biochar amendments significantly decreased N_2O emissions by increasing *nosZ* gene copies in temperate paddy soil during the drying process conditions. Although numerous above studies have investigated the impact of biochar supplementation on subtropical or temperate

paddy soils, only a limited number of studies have addressed its potential for enhancing rice production (Van Long and Van Dung, 2023; Evy et al., 2019) and mitigating soil N_2O emissions in tropical regions (Nurida et al., 2021). In China, mid-season drainage is the most prevalent water regime in rice fields (Zhang et al., 2022), especially in tropical rice-growing regions (Wang et al., 2021). Of note, limited research has been conducted to specifically investigate the effect of water management during the mid-season period on soil N_2O emissions, rice yield and the mechanisms involved in tropical paddy soil. Therefore, in this study, we compared three different water management strategies (alternating dry-wet conditions (AWD), continuous flooding (CF), and regular mid-season drainage (CON)) during the mid-season period in tropical paddy soil. Given its great potential for mitigating N_2O emissions, we applied biochar as an amendment in our study to evaluate its impact on N_2O emissions in tropical soil under different water management strategies during the mid-season period over an entire growing season. The main goals of this study were: 1) to understand the effects of various water management during the mid-season period on tropical paddy soil N_2O emissions, 2) determine whether the combination of these water management with biochar amendment mitigates N_2O emissions, and 3) to investigate the underlying N_2O production pathways in a specific paddy soil by monitoring soil N-cycling genes.

We hypothesized that (I) whether CF during the mid-season period has the lowest cumulative N_2O with or without biochar amendment compared to others; (II) whether the amendment of biochar would drastically decrease N_2O emissions in all different water management strategies; (III) whether biochar amendment mitigates soil N_2O emissions by increasing the abundance of functional genes of N_2O-reducing microorganisms, regardless of the water management. Quantitative polymerase chain reaction (qPCR) was used to determine the abundance of N cycling functional genes, including ammonia-oxidizing archaea (AOA) *amoA*, AOB *amoA*, *nirS*, fungal and bacterial *nirK*, *nifH* and *nosZ* genes, in order to test our hypotheses.

2. Materials and methods

2.1. Soil, biochar, and rice seedlings details

Soil (0-20 cm depth) was collected from a paddy rice field in Chengmai County, Hainan Province, China (19°55′N, 109°57′E). This area is characterized by a tropical

monsoon climate with a mean annual temperature of 23.8℃ and total annual precipitation of 1786 mm. The paddy soil, with a pH (H_2O) of 5.74 ± 0.30, bulk density of 1.12 ± 0.20 g cm^{-3} and cation exchange capacity (CEC) of 3.99 ± 0.05 cmolc kg^{-1}, contained 2.79 ± 0.03 g kg^{-1} total N, 112 ± 2.30 mg kg^{-1} available phosphorus (P), 56.4 ± 1.50 mg kg^{-1} available potassium (K), and 22.2 ± 1.30 g kg^{-1} organic carbon (C).

The biochar used as a soil amendment was produced from maize stover and heated at a temperature of 400℃ for 2 h under "no-oxygen" conditions in a muffle furnace (Wang et al., 2021). After cooling, the biochar was milled < 0.25-mm. It had a pH of 7.58 ± 0.03, organic C content of 49.32 ± 0.26 %, total N content of 1.79 ± 0.02 %, P content of 0.048 ± 0.01 % and K content of 2.29 ± 0.03 %. The surface area was 3.75 ± 0.05 m^2 g^{-1}, Zeta electric potential was 23.1 ± 0.53 mv and CEC was 46.9 ± 1.30 cmolc kg^{-1} (Wang et al., 2021).

Rice (*Oryza sativa*) seedlings from the new high-yielding rice variety "Nangui Zhan" were used in this experiment, which is the leading cultivars and productive seedling in South China (Zhou et al., 2022). Three seedlings were transplanted per pot at 30 cm shoot height.

2.2. Experimental design

The paddy soil was sieved <2 mm, preincubated for one week at 60% WFPS, then added to the pots (30 cm in length, 20 cm in diameter). Each pot contained 5.00 kg (equal to oven-dry weight) of fresh soil. The experiment included six treatments, with four replicates per treatment (24 pots total): 1) Alternating dry-wet conditions during the mid-season period (defined as October 17th to November 13th) without biochar amendment (AWD1); 2) Alternating dry-wet conditions during the mid-season period with 2% (w/w) biochar amendment (AWD2); 3) Continuous flooding during the mid-season period without biochar amendment (CF1); 4) Continuous flooding during the mid-season period with 2% (w/w) biochar amendment (CF2); 5) Regular mid-season drainage without biochar amendment (CON1); and 6) Regular mid-season drainage with 2% (w/w) biochar amendment (CON2). The biochar amendment rate was based on our previous work by Wang et al. (2021) which showed that a 2% (w/w) of biochar application rate was optimal for decreased N_2O emission in tropical paddy soils. Seedlings were transplanted on September 13th (day 0) and

harvested three months later, on December 13th (day 94).

The continuous flooding (CF) treatment had a 5 cm water layer during the mid-season (from October 17th to November 13th), while the pots under the alternating dry-wet conditions (AWD) treatment had two water drawdowns, each with an interval of 14 days during the mid-season; the first started after October 17th to October 31st, and the second (from wet to dry condition) from October 31st to November 13th. Regular mid-season drainage started on October 17th and lasted 14 days to October 31st, after that, kept in 5 cm depth water until the end of the mid-season. The pots were flooded using deionized water to 5-cm depth until November 13th. Water depth in the pots was measured manually with a ruler at 1- to 2-day intervals during the flooded period.

All treatments received NPK fertilizer in two split applications (September 23rd and October 24th). The first fertilizer application consisted of 0.03 g N-urea kg^{-1}, 0.02 g P_2O_5-P kg^{-1} and 0.02 g K_2O-K kg^{-1}, and the second fertilizer application was of 0.02 g urea-N kg^{-1} and 0.015 g K_2O-K kg^{-1}.

2.3. Gas sampling and analysis

Greenhouse gas (CH_4 and N_2O) fluxes were collected using a manual static chamber (Fig. 1), made of plexiglass with a cross-sectional area of 0.25 m^2 (L × W × H = 50 cm × 50 cm × 100 cm). Gas samples were taken once a week throughout the three-month experiment. The sampling frequency was increased after fertilizer application and during mid-season drainage. Prior to each gas sampling, the water storage tank was filled with water, and the gas extraction box was placed on top covering the pot. Gas samples (30 mL) were collected after 0, 15, and 30 min and injected into a 20 mL pre-evacuated vials (Nichiden-Rika GLASS CO. LTD). Concentrations of CH_4 and N_2O in the gas samples were analyzed by a methane conversion furnace, flame ionization detector (FID), and electron capture detector (ECD), respectively (Shen et al., 2018). The flux of gas emissions (F) was calculated according to the following equation (Liao et al., 2020):

$$F = \rho \times h \times \Delta C/\Delta t \times 273.15/ (273.15 + T)$$

Where F is the emission flux of N_2O and CH_4 (the unit of N_2O and CH_4 emissions was μg m^{-2} h^{-1} and mg m^{-2} h^{-1}, respectively), ρ is the gas density at standard state, h is the height of the chamber above the soil (m). The ratio $\Delta C/\Delta t$

was the change in gas concentration in the chamber during gas sampling, and T was the mean air temperature inside the chamber during sampling.

The cumulative gas emissions during the experiment were determined by Singh et al. (1996).

The global warming potential (GWP) was calculated by CH_4 and N_2O emissions (Li et al., 2018a) based on the following equation:

GWP (kg CO_2 eq ha^{-1}) = 298 × N_2O (kg N_2O ha^{-1}) + 25 × CH_4 (kg CH_4 ha^{-1})

The greenhouse gas intensity (GHGI) was calculated by dividing GWP by rice grain yield (Hwang et al., 2017).

GHGI (kg CO_2 equivalent kg^{-1} grain)

= GWP (kg CO_2 eq ha^{-1}) /grain yield (kg ha^{-1})

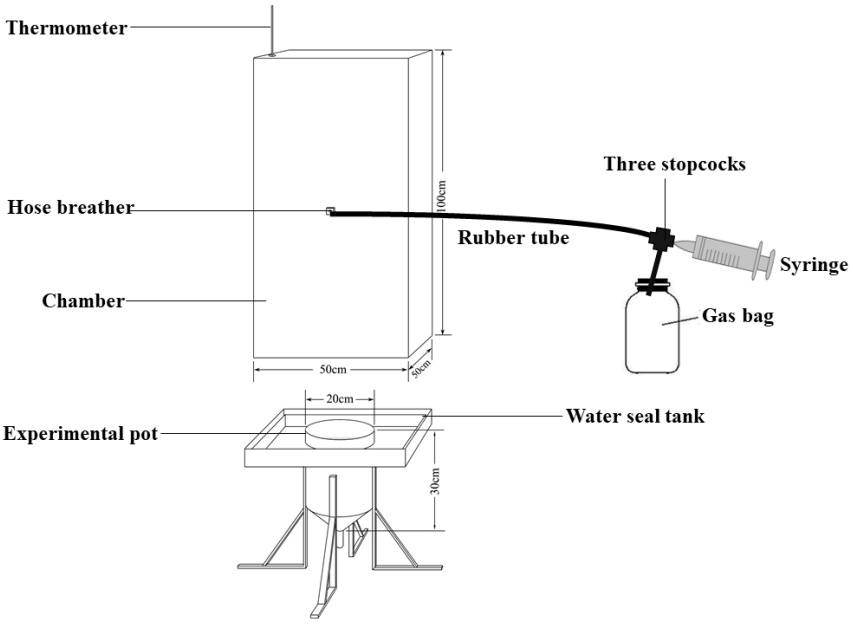


Fig. 1 Details of the experimental pot (25-cm diameter) and static chamber box used to measure gas fluxes (L× W × H =50 cm× 50 cm ×50 cm)

2.4. Soil sampling and analysis

Soil samples were collected on September 17th and 27th, October 8th, 18th and 28th, November 8th and 22nd, and December 13th, to be analyzed for exchangeable ammonium (NH_4^+) and nitrate (NO_3^-) concentrations, soil moisture, and soil pH. Subsamples were kept frozen at -80°C prior to preparation for DNA extraction and qPCR analysis. Soil pH was determined at a 1:2.5 soil: water ratio. Soil redox potential (Eh) was measured with platinum-tipped electrodes (Nishimura et al., 2004). Soil inorganic N (exchangeable NH_4^+ and NO_3^-) was extracted with 2M KCl at a

soil/water ratio of 1:5 and determined by a Sanalar Continuous Flow Analyzer (Skalar, The Netherlands) (Shen et al., 2021a). Soil moisture content was determined by over-drying 10 g of fresh soil at 105 °C for 48 h or until the weight was stable. Rice yield was measured in each pot after harvest. The filled grain was manually separated from the panicles and oven-dried for the measurement of grain weight (Yang et al., 2012). All data are expressed on an oven-dry basis (105 °C, 24 h).

2.5. Soil DNA extraction and qPCR analysis

The DNA was extracted from 0.5 g of fresh soil samples collected, on September 17th and 27th, October 28th, and December 13th, using the FastDNA SPIN Kit (PowerSoil^{TM} DNA Isolation Kit, Mo Bio Laboratories Inc. CA) following the manufacturer's instructions and subsequently stored at -20°C. The quality and concentration of extracted DNA were determined by gel electrophoresis and a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) (Shen et al., 2023). All qPCR reactions were carried out with a LightCycler 480 real-time PCR machine (Roche, Germany) in wells containing 10 µl SYBR@Premix ExTaq (TaKaRa, Japan), including 1 µL of each primer, 1 µL of DNA template adjusted to a final volume of 20 µL with double deionized water. This was followed by the spanning of 10^8–10^1 and a 10-times dilution after estimating the concentrations of plasmids by NanoDrop® ND-2000 UV–Vis to generate the standard curves (Shen et al., 2021a). Quantification of *amoA* for AOA and AOB was performed by the primer pairs Arch-amoAF/Arch-amoAR. The *nirS*, *nirK*-bacteria, *nirK*-fungi, *nosZ*, and *nifH* genes identified by quantitative PCR analysis were determined by gene-specific primers and conditions (Table S5). High efficiencies from 95 to 105% were obtained at all amplifications, with R^2 values ranging between 0.98 to 1.00.

2.6. Data analysis

Statistical analyses were performed using Origin 2018 (Origin Lab Corp. USA), SPSS 26.0 software (IBM Corp. USA) and R v. 4.5.0. Statistically significant differences among treatments were determined by One-way ANOVA and least significant difference (LSD) calculations at a 5% confidence level. A two-way ANOVA was used to compare differences between water management (AWD, CON, and CF), biochar treatment (no biochar and 2% biochar), and their interactions. Treatment differences at $p < 0.05$ were considered to be statistically significant.

Relationships between gene abundance and environmental factors were assessed using non-parametric Spearman correlation analysis. Structural equation modeling (SEM) was done using the "lavaan package" in R (Schleuss et al., 2019). The SEM was used to test causal relationships among biochar amendment, water management, cumulative N_2O emission, cumulative CH_4 emission, rice production and Global warming potential in the conceptual model. The SEM fit was evaluated by using Root Mean Square Error of Approximation (RMSEA), chi square ($\chi 2$), df ($\chi 2$), and the *p*-value of $\chi 2$, when Gfi > 0.9, srmr < 0.08, RMSEA < 0.05 and 0.10 < p < 1.00 indicates a good model (Schermelleh-Engel et al., 2003). Spearman correlation analysis was performed using the R version 4.5.0, and *p* values were adjusted by the multiple testing with the Bonferroni-Holm method.

3. Results

3.1. Changes in soil inorganic N

There was a distinct peak in soil exchangeable NH_4^+-N concentrations in all treatments on September 27th after the first fertilizer application (Fig. 2a). At this time, biochar addition had the main cause of NH_4^+-N concentration shifts (Table S4; p < 0.01). The highest NH_4^+-N concentration was found with AWD1 (160 mg kg^{-1} soil) (p < 0.05), followed by CON1 (153 mg kg^{-1} soil) (p <0.05), CF1 (152 mg kg^{-1} soil) (p < 0.05), AWD2 (143 mg kg^{-1} soil) (p < 0.05), CON2 (133 mg kg^{-1} soil) (p > 0.05) and CF2 (129 mg kg^{-1} soil) (p > 0.05). Following this, NH_4^+-N rapidly decreased until October 8th with the biochar treatment but was still. The difference in soil NH_4^+-N content between biochar treatments (16 mg kg^{-1} soil) and unamended treatments (54 mg kg^{-1} soil) was significant (p < 0.001) regardless of water management. Thereafter, soil NH_4^+-N concentrations continued to gradually decrease and remained stable in all treatments, except CF1 which had a distinct peak (90 mg kg^{-1} soil) on October 28th after the second fertilizer application (Fig. 2a).

The changes in soil NO_3^--N and NH_4^+-N concentrations followed similar trends. There was a rapid increase in all treatments on October 8th after the first fertilizer application (Fig. 2b). Biochar (Table S4; p < 0.001) and water management (Table S4; p < 0.05) were main causes of NO_3^--N concentration shifts. The highest concentration was observed in AWD1 (245 mg kg^{-1} soil) (p < 0.05), followed by CON1 (233 mg kg^{-1} soil) (p < 0.05), AWD2 (203 mg kg^{-1} soil) (p < 0.05), CF1 (199 mg kg^{-1} soil) (p < 0.05), CF2 (166 mg kg^{-1} soil) (p > 0.05), and CON2 (140 mg kg^{-1} soil) (p > 0.05).

Soil NO_3^--N concentration decreased in all treatments on October 18th and remained at ~5 mg kg^{-1} soil for the duration of the experiment, with no significant.

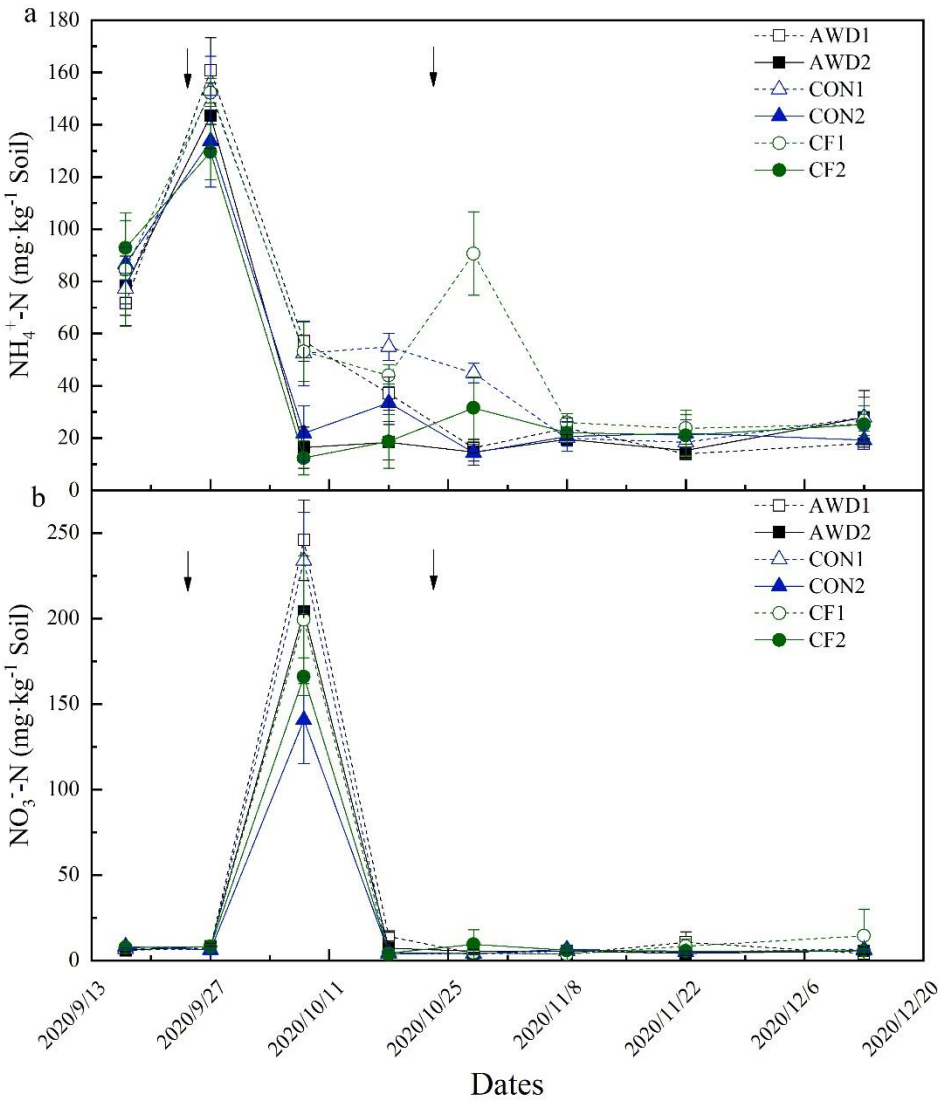


Fig. 2 Changes in soil exchangeable NH_4^+ (a) and NO_3^- (b) at different cultivation times Error bars represent standard errors of means (n=4) AWD1: Alternating dry-wet conditions during the mid-season period without biochar amendment; AWD2: Alternating dry-wet conditions during the mid-season period with 2% (w/w) biochar amendment; CF1: Continuous flooding during mid-season period without biochar amendment; CF2: Continuous flooding during mid-season period with 2% (w/w) biochar amendment;CON1: Regular mid-season drainage during mid-season period without biochar amendment; CON2: Regular mid-season drainage during mid-season period with 2% (w/w) biochar amendment

3.2. Changes in emissions of N_2O and CH_4

The daily N_2O fluxes were low, at 20-40 μg m^{-2} h^{-1}, in all treatments before the

first fertilizer application (September 23rd) (Fig. 3a). The first peak of N_2O fluxes in all treatments was on September 27th, except for treatment CON1 which occurred on October 1st (Fig. 3a). During this first peak, the highest flux was from treatments CF1 (1354 μg m^{-2} h^{-1}), followed by CF2, AWD2, CON1 and CON2 (444, 429, 362 and 305 μg m^{-2} h^{-1}). After that, all treatments were fluxes until the second fertilizer application when the highest N_2O fluxes were detected on October 28th. During this second peak, the fluxes in treatments AWD1 (1354 μg m^{-2} h^{-1}) and CON1 (1098 μg m^{-2} h^{-1}) were significantly higher than those in treatments AWD2 (553 μg m^{-2} h^{-1}) and CON2 (440 μg m^{-2} h^{-1}) ($p < 0.001$). The fluxes in CF1 (84 μg m^{-2} h^{-1}) and CF2 (135 μg m^{-2} h^{-1}) treatments were significantly lower than in the other treatments ($p < 0.05$). After November 8th, the N_2O fluxes subsided and remained stable in all treatments (around 15-30 μg m^{-2} h^{-1}) until the end of the experiment (Fig. 3a).

Notably, biochar-amended treatments had significantly decreased total cumulative N_2O emissions in the (Table S1; $p < 0.01$) (Fig. 3c). The highest cumulative emission was in CON1 (2.68 kg N ha^{-1}), followed by AWD1 (2.56 kg N ha^{-1}), compared with 1.87 kg N ha^{-1} in AWD2 and 1.69 kg N ha^{-1} in CON2. Treatment CF2 with 0.85 kg N ha^{-1} had the lowest cumulative emission, followed by treatment CF1 with 1.31 kg N ha^{-1}.

The daily CH_4 fluxes, increased sharply from October 28th to November 8th, then decreased on November 15th (Fig. 3b). The highest peak was in CON2 (10.2 mg m^{-2} h^{-1}) and followed by CON1 (8.91 mg m^{-2} h^{-1}), CF1 (6.75 mg m^{-2} h^{-1}), AWD2 (4.31 mg m^{-2} h^{-1}), and AWD1 (2.32 mg m^{-2} h^{-1}) on November 8th. The highest peak in CF2 (4.64 mg m^{-2} h^{-1}) was on November 1st which was higher than in AWD2 (2.54 mg m^{-2} h^{-1}) (Fig. 3b). By the end of the experiment, treatments CF1 (2.64 mg m^{-2} h^{-1}) and CF2 (1.46 mg m^{-2} h^{-1}) fluxes were still relatively high, but the fluxes in other treatments were much lower (0.2-0.5 mg m^{-2} h^{-1}). Overall, the highest cumulative CH_4 emission was in CF1 (44.6 kg C ha^{-1}) (Fig. 3d), followed by CON2 (37 kg C ha^{-1}), CF2 (36.2 kg C ha^{-1}), and CON1 (30.7 kg C ha^{-1}). The cumulative emission in AWD2 (20.4 kg C ha^{-1}) was significantly higher than in AWD1 (8.03 kg C ha^{-1}). Water management significantly affected cumulative CH_4 emissions(Table S1; $p < 0.01$). The CH_4 fluxes were significantly affected by biochar (Table S2; $p < 0.001$) and the interaction of biochar and water management (Table S2; $p = 0.01$) on October 28th, while on November 8th, only water management had a significant impact (Table S2; $p < 0.001$), the unamended treatments showed a significant difference in CH_4 fluxes: AWD1 <

CON1 < CF1.

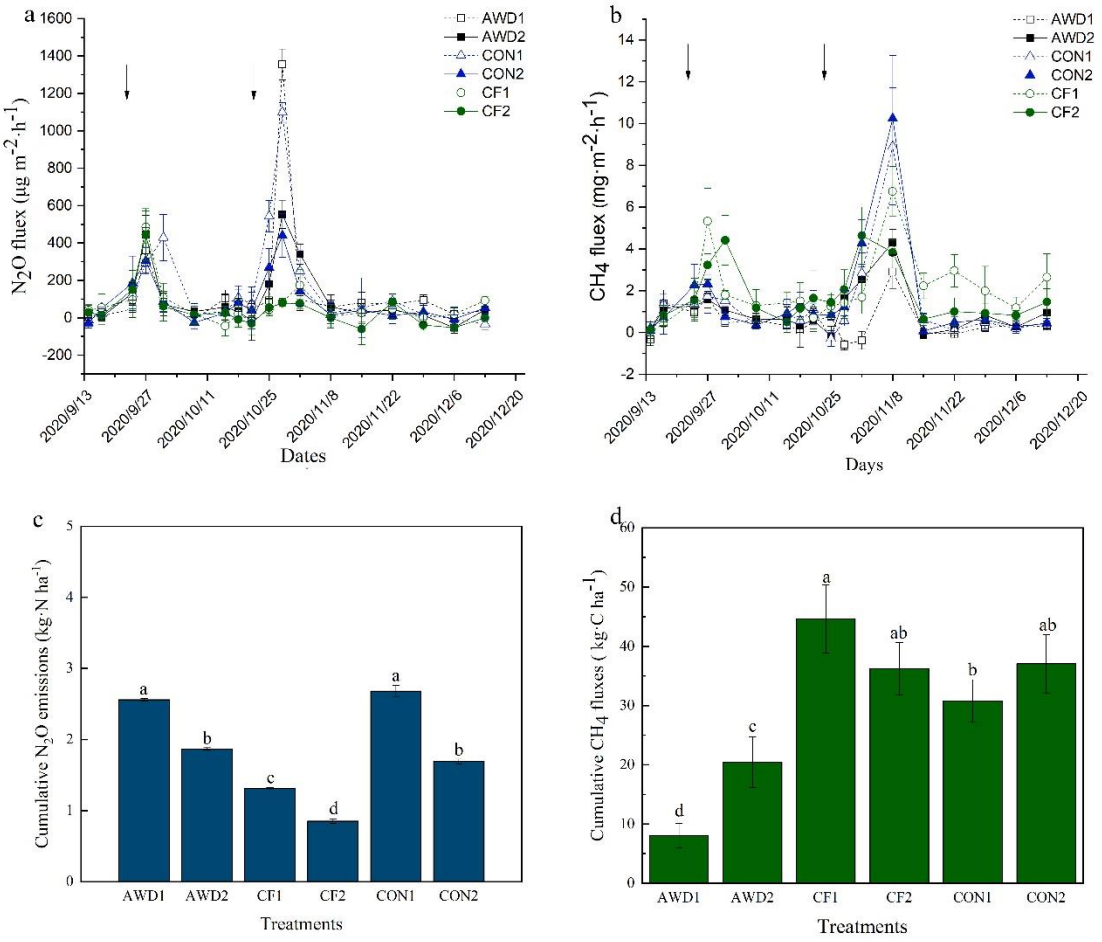


Fig. 3 N_2O fluxes (a), CH_4 fluxes (b), cumulative N_2O emissions (c), and cumulative CH_4 emissions (d) in different treatments during the rice cultivation period Error bars represent standard errors of the means (n=4) Different lowercase letters indicate significant differences among different treatments within the whole cultivation period, which were determined by a one-way ANOVA by a Tukey test for post hoc comparison at $p < 0.05$. For explanation of abbreviations see Fig 2; A two-way ANOVA was used to compare differences between water management (AWD, CON, and CF), biochar treatment (no biochar and 2% biochar), and their interactions, $p < 0.05$ were considered to be statistically significant, shown in Table S1.

3.3. Changes in functional N-cycling genes

The copy numbers of AOA *amoA* gene were significantly higher than those of AOB *amoA* gene in this soil (Fig. 4; $p < 0.05$). On September 27th, AOA *amoA* gene copies significantly increased in all treatments compared with those on September

17th, notably AWD1 and CF1, which were about 57% higher than unamended treatments (Fig. 4a). There was no significant difference between treatments CON1 and CON2 on September 27th (Fig. 4a).

Biochar treatments had significantly fewer AOA *amoA* gene copies than unamended treatments on October 28th, except for treatments CF (Fig. 4a). Significant impacts of biochar, water management and their interaction on AOA *amoA* gene were observed (Table S3; p <0.01), on October 28th, gene copies in CON1 and AWD1 were significantly higher than those in treatment CF1 (Fig. 4a). The significant changes in AOB *amoA* gene copies were only found on October 28th, biochar-amended treatments resulted in significantly increased gene copies compared to unamended treatments (Fig. 4b; $p < 0.05$). Also, the water management significantly affected the AOB *amoA* gene copies simultaneously (Table S3; $p < 0.001$), the highest gene copies in unamended and amended treatments were observed in CON1 and CON2 treatments, respectively. Further, biochar had a greater influence on AOA than on AOB, throughout the experiment (three-times significance vs. once significance; Table S3).

From September 17th to 27th, biochar significantly increased *nirS* gene copies by almost 0.5-fold, 1-fold, and 2-fold CON2, CF2, and AWD2 treatments, respectively (Fig. 5a; $p < 0.05$). On October 28th, water management was the main effect on *nirS* genes (Table S3; $p < 0.001$). At this time, compared to the previous day (September 27th), *nirS* gene abundances in CON1 and CON2 significantly increased ($p < 0.001$), followed by CF2 and CF1, and a minor increase in AWD1, but a significant decrease in AWD2. There was a significant increase in *nirS* gene copies in AWD2, AWD1, and CF1 ($p < 0.05$), but a significant decrease in CON2 ($p < 0.05$) from October 28th to the end of the experiment. The copy numbers in the CON1 treatment were maintained by the end of the experiment.

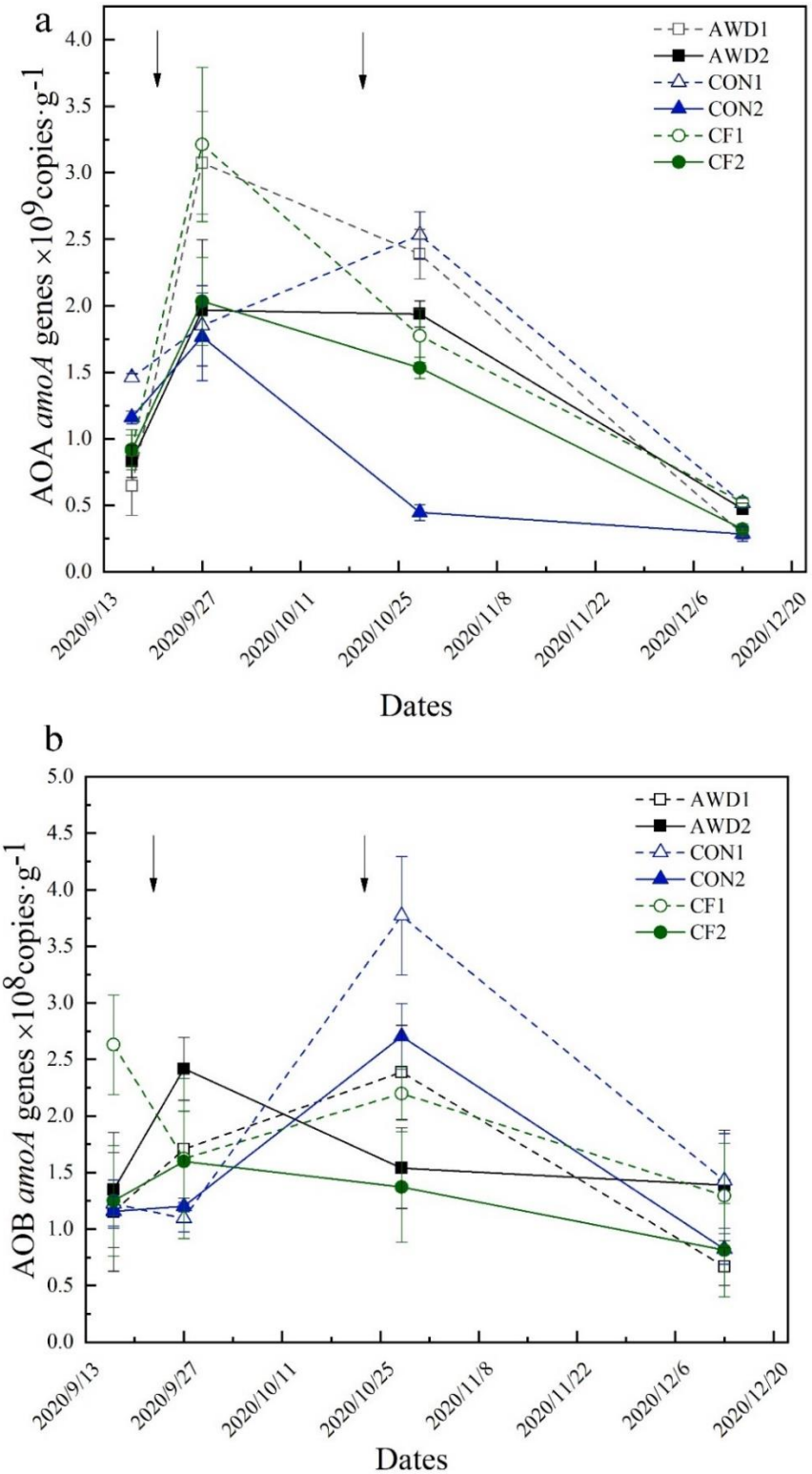
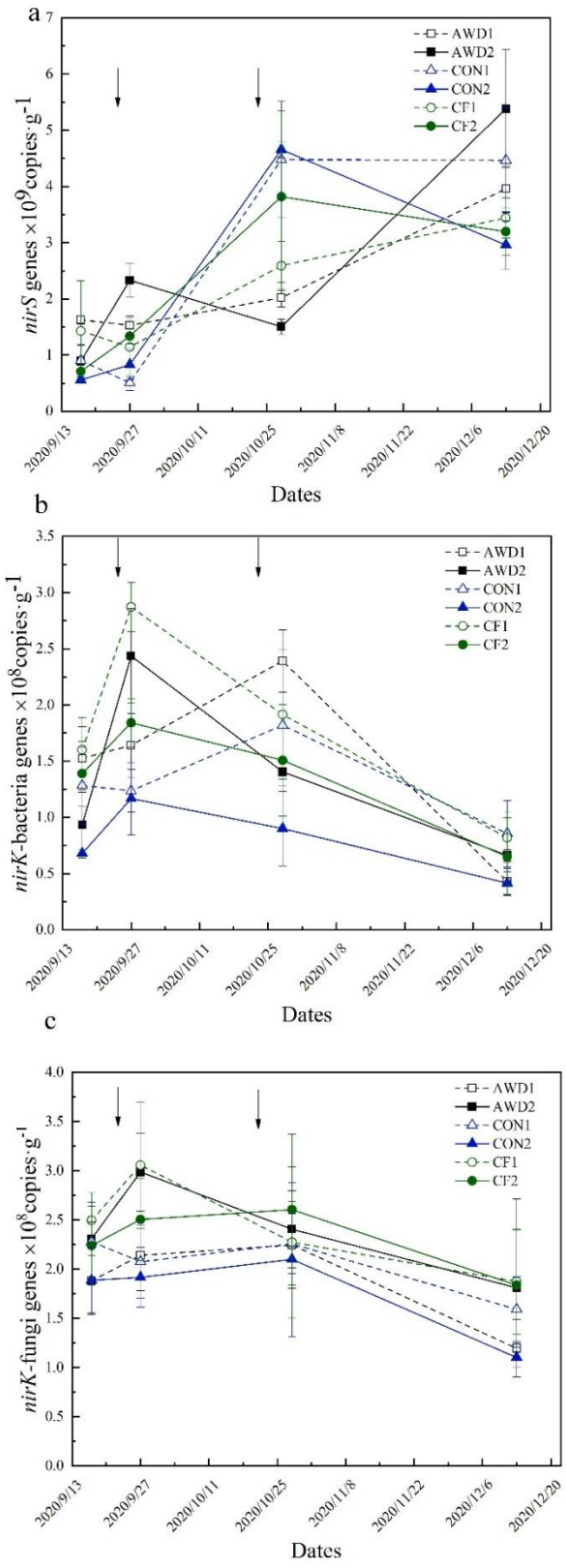


Fig. 4 Changes in AOA (a) and AOB (b) *amoA* genes copy numbers in different treatments during the rice cultivation period. Error bars represent standard errors of the means (n=4). For explanation of abbreviations see Fig. 2.

Regarding *nirK* copy numbers (Fig. 5b and c), fungal-*nirK* gene average copies in all treatments was almost 1.75-fold higher than bacterial-*nirK* gene average copies

in all treatments at day 0 of the experiment ($p < 0.05$). From September 17th to September 27th, the *nirK* gene copy numbers increased almost doubled in CON2, CF1, CF2, AWD2, and AWD1 treatments but decreased in the CON1 treatment. These changes were more rapid with bacterial than fungal *nirK* gene copies.

Immediately on October 28th, compared to the previous day (September 27th), CF1 and AWD2 treatments showed a 40% and 24% decrease in bacterial and fungal *nirK* genes copies respectively ($p < 0.001$), but CON1 and AWD1 treatments showed a 40% and 45% increase in bacterial ($p < 0.001$) *nirK* gene copies and a slight increase in fungal *nirK* gene copies until the end of the experiment. However, the bacterial *nirK* gene copies in CF2 and CON2 treatments significantly decreased by 46% and 43%, respectively, but the fungal ones slightly increased on October 28th, then significantly decreased by about 23% and 40%, respectively until the end of the experiment (Fig. 5b and c). Unlike the *nirS* gene, environmental factors (biochar or water management) had a less overall effect on the *nirK* genes, particularly the *nirK*-fungi genes (Table S3).



441
442 **Fig. 5** Changes in *nirS* (a), *nirK*-bacteria (b), and *nirK*-fungi (c) genes copy numbers
443 in different treatments during the rice cultivation period. Error bars represent standard
444 errors of the means (*n*=4). For explanation of abbreviations see Fig. 2.
445

Each fertilizer application significantly increased the *nosZ* gene copies in amended treatments (Fig. 6a; $p < 0.05$). Biochar addition had a significant impact on *nosZ* gene copies on September 27th ($p < 0.05$), October 28th ($p < 0.001$), and December 13th ($p < 0.01$), although the interaction between biochar and water did not (Table S3). Notably, after the second fertilizer application, biochar treatments had 2-fold more gene copies than unamended treatments (Fig. 6a). The AWD2 treatment maintained high *nosZ* gene copies following the first fertilizer application until the end of the experiment. After the first fertilizer application, biochar addition significantly increased *nosZ* gene copies by about 4.3, 2.3, and 2.5 times in AWD2, CF2, and CON2 treatments, respectively (Fig. 6a), and 1.3 times in CON1. The *nosZ* gene copies in both CON1 and AWD2 treatments decreased significantly by 50% from the October 28th ($p < 0.001$) and 34% in AWD1 from September 17th until the end of the experiment ($p < 0.05$). The *nosZ* gene copies in biochar treatments (CF2 and AWD2) were significantly higher than those in unamended treatments (CF1 and AWD1) at the same period ($p < 0.05$). The *nifH* gene copies in unamended treatments increased following both fertilizer input and subsequently steadily decreased. Notably, the abundance of *nifH* gene copies in biochar treatments was significantly 2-fold higher than those in the unamended treatments on October 28th (Fig. 6b; $p < 0.01$).

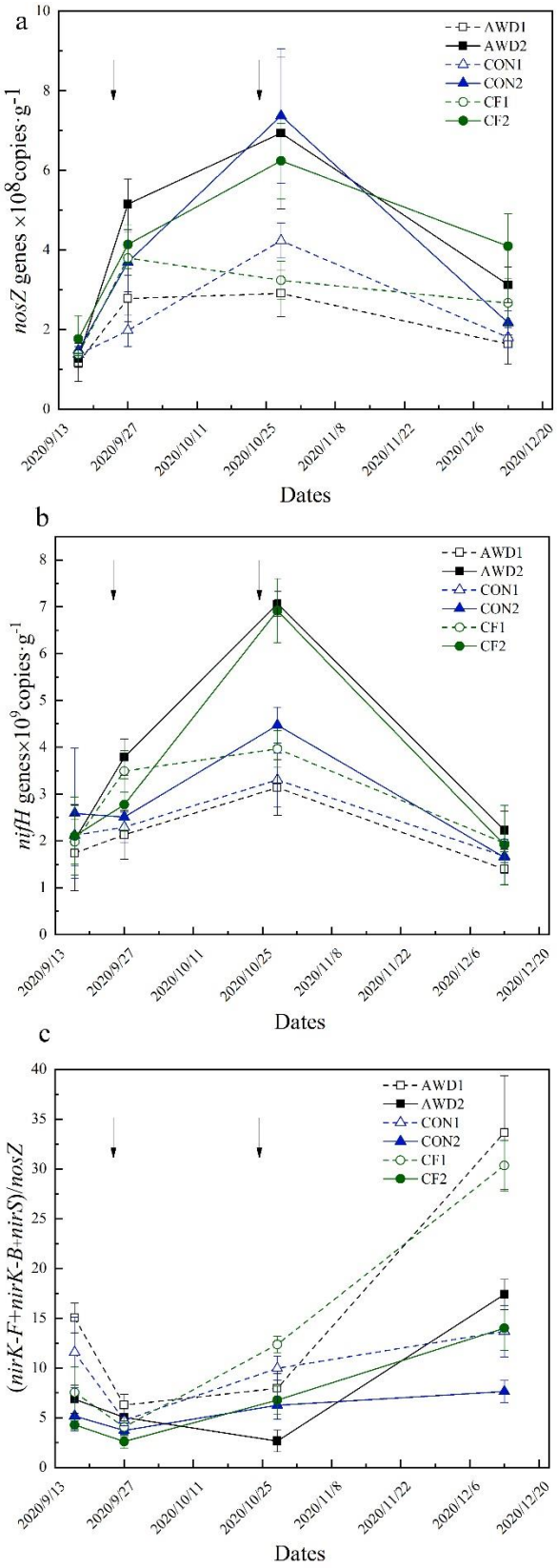


Fig. 6 Changes in *nosZ* (a) and *nifH* (b) genes copy numbers, and ratio of (*nirS*+ *nirK*-F +*nirK*-B)/*nosZ* (c) in different treatments during the rice cultivation period. Error bars represent standard errors of the means (n=4). For explanation of abbreviations see Fig. 2.

Biochar had the greatest impact on the ratios of [*nirK-F*+*nirK-B*+*nirS*]/*nosZ* (denitrification genes ratios) (Fig. 6c; Table S3), especially on October 28th. It significantly decreased the ratio by 50% and significantly increased it in CF1 by 40% and 24% in AWD1 and CON1, respectively. By the end of the experiment, water management did not affect this ratio and showed the long-term impact on the biochar treatments (Table S3; $p < 0.001$): the above ratio in the AWD2 and CF2 treatments was decreased by about 48% compared to AWD1 and CF1 treatments, and by 55% in CON2 compared to CON1 (Fig. 6c).

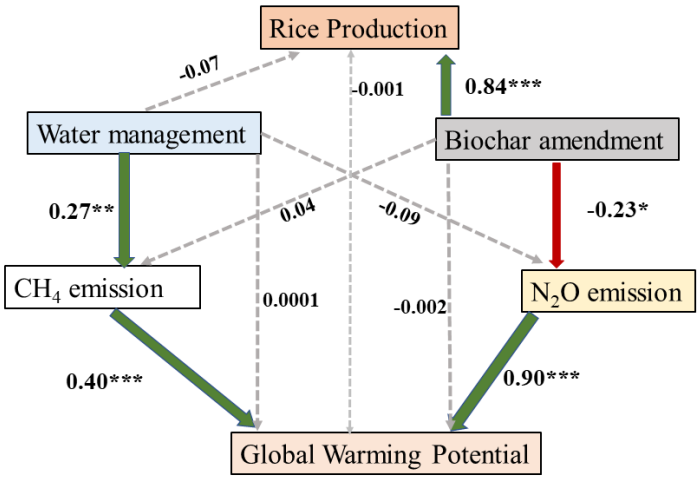
3.4. Global warming potential (GWP) and greenhouse gas intensity (GHGI)

Water management differences had the only significant effect on GWP (Table S1; $p < 0.001$). Biochar significantly decreased GWP in CF2 by about 23% compared to CF1 ($p < 0.05$), but did not affect GWP in CON and AWD treatments (Table 1). The GWP in AWD1 was significantly lower than ($p < 0.05$) in treatments CF1 and CON1, respectively (Table 1). Biochar ($p < 0.01$) and water management ($p < 0.001$) significantly affected GHGI (Table S1). Biochar addition significantly decreased GHGI in the CF treatment, and GHGI in the CF1 treatment was significantly higher than in the treatment AWD1 by about 50% (Table 1). The differences in GHGI followed the same trends as GWP in the treatments, with CF1 having the highest GHGI ($p < 0.001$), followed by CON1 ($p < 0.001$), CON2 ($p < 0.05$), CF2 ($p > 0.05$), AWD1 ($p > 0.05$), and AWD2 ($p > 0.05$) (Table 1). The GHGI in treatments CF2 and CON2 were 39.5% and 15.4% times less than in treatments CF1 and CON1 ($p < 0.05$), respectively, but there was no difference between treatments AWD1 and AWD2. Biochar application significantly increased rice yield by 20% (Fig. S1; $p < 0.05$), with the interaction between biochar and water management also contributing (Table S1; $p = 0.005$). Although water management had no effect on rice yield, the yield in AWD1 and CON1 were significantly greater than in the CF1 treatment.

Table 1 The values of global warming potential (GWP) and greenhouse gas intensity (GHGI) at different treatments**.** The values within the same column followed by different lowercase letters indicate significant differences at $p < 0.05$. A two-way ANOVA was used to compare differences between water management (AWD, CON, and CF), biochar treatment (no biochar and 2% biochar), and their interactions. $p <$

0.05 was considered to be statistically significant as shown in Table S1.

Treatment	GWP kg CO_2-eq ha^{-1}	GHGI kg CO_2-eq kg^{-1} yield
AWD1	903±44.6 d	0.22±0.13 d
AWD2	1068±146.2 cd	0.22±0.03 d
CF1	1597±264.6 a	0.43±0.06 a
CF2	1240±211 bc	0.26±0.04 cd
CON1	1572±192 ab	0.39±0.05 ab
CON2	1485±183 ab	0.33±0.04 bc



Cfi=1, df=2, Gfi=1 Chisq=1.45, Srmr=0.009, RMR=0.009, RMSEA=0, *P*=0.77

Fig 7 Structural equation modeling showing the effects of biochar amendment, water management, rice production, cumulative CH_4 emissions, cumulative N_2O emissions and global warming potential. The model was satisfactorily fitted to the present data, significant paths are illustrated by the gray arrow (no effect), red arrow (negative) and green arrow (positive) with standardized path coefficients. Significant (*p < 005; **p < 001; ***p < 0001)

3.5. SEM and Spearman analysis

The SEM showed that biochar addition significantly and negatively affected cumulative N_2O emissions (p < 0.05), while water management had no significant effect regardless of timing (Fig. 7). However, cumulative CH_4 emissions were significantly and positively affected by water management (p < 0.01). Global warming potential was primarily related to both CH_4 (p < 0.001) and N_2O emissions (p < 0.001). In addition, on September 27th, a significant positive correlation between biochar and *nosZ* gene was observed in AWD and CON treatments, but not in the CF treatments (Fig. S3). On October 28th, spearman's correlation showed that N_2O fluxes had significant and negative relationships with biochar and *nosZ* copies (p < 0.001) in

AWD and CON treatments (Fig. 8a and b). In CF treatments, biochar had no correlation with N_2O fluxes, but it had a significant and positive relationship with *nosZ* gene copies (Fig. 8c).

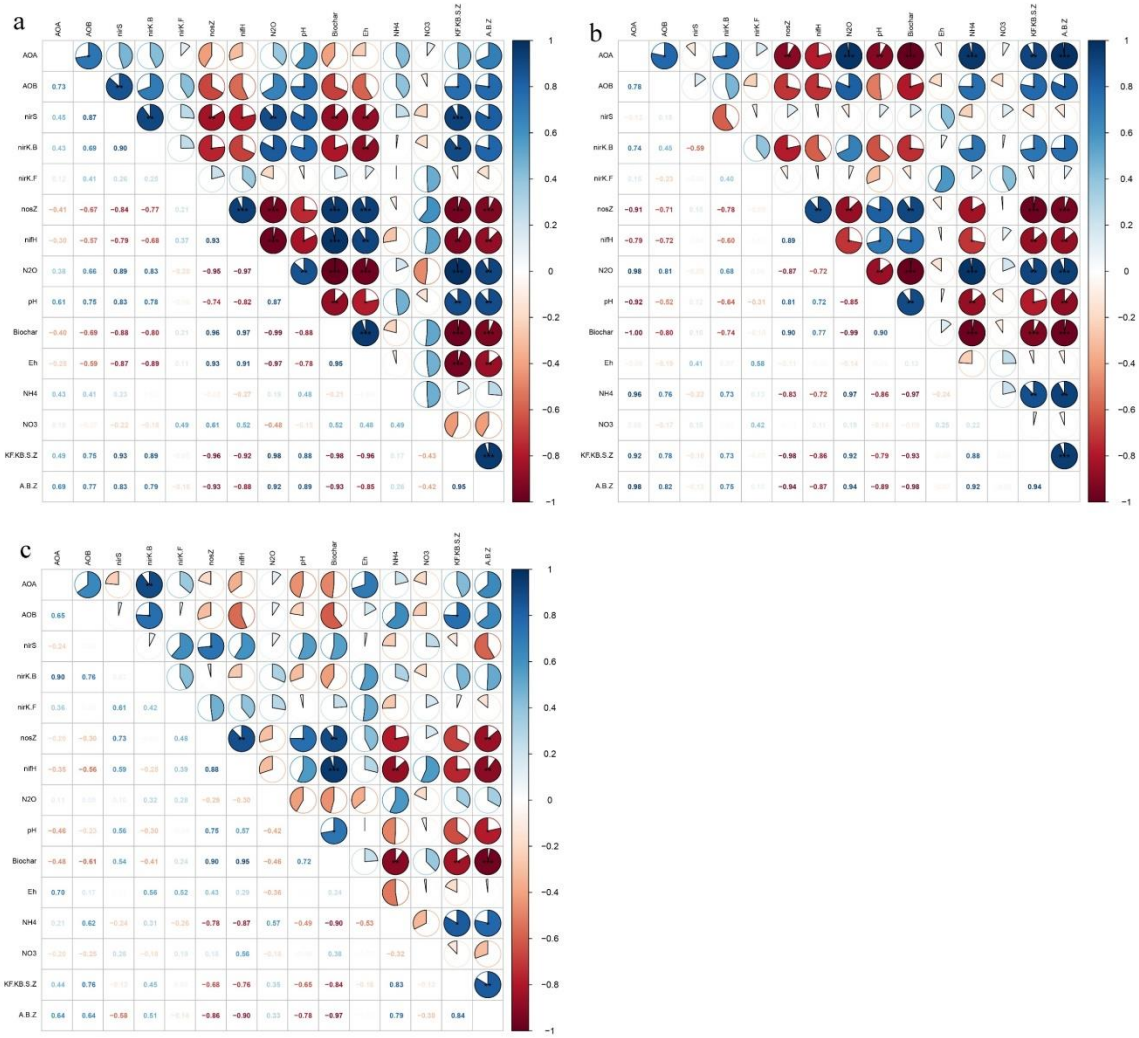


Fig. 8 Spearman's correlation coefficients among biochar, water management, the abundance of N cycling genes (AOA and AOB *amoA* genes, *nirS*, *nirK* (fungal and bacterial), *nosZ* and *nifH*, and the ratio of [*nirS*+ *nirK*-bacteria +*nirK*-fungi]/*nosZ*, N_2O fluxes (N_2O) and different edaphic factors (Eh, Ec, NH_4^+-N, NO_3^-N, and pH). And a, b and c represent AWD, CON and CF treatments on October 28th respectively. Abbreviation: AOB, *amoA* belonging to ammonia-oxidizing bacteria; AOA, *amoA* belonging to ammonia-oxidizing archaea; KFKBSZ, the ratio of [*nirK*-bacteria+*nirK*-fungi+*nirS*]/*nosZ*; ABZ, the ratio of *amoA* /*nosZ*. Red and blue indicate negative and positive correlations, and color depth indicates stronger correlations. Asterisks indicate significant effect ("***" $p < 0001$, "**" $p < 001$, "*" $p < 005$), after *p*-value was corrected by the Bonferroni method.

4. Discussion

4.1. Changes in soil inorganic N

Biochar addition significantly decreased inorganic NO_3^--N and NH_4^+-N contents

which can be attributed to an enhanced soil-specific surface area and NH_4^+ sorption capacity of the soil (Duan et al., 2018). Additionally, biochar may promote N immobilization by soil microbes in agreement with past studies (Zheng et al., 2013; Novak et al., 2010), showing decreased exchangeable NH_4^+ and NO_3^- concentrations. Wu et al. (2017) reported that nitrification converted exchangeable NH_4^+-N to NO_3^--N within a 10-day incubation in accordance with our results. After the second fertilizer input, soil exchangeable NH_4^+-N in both CF treatments increased, and was especially significant in the CF1 treatment, indicating that nitrification was inhibited under continuous waterlogged conditions. Soil NO_3^--N contents in all treatments were low and stable whereas exchangeable NH_4^+-N contents significantly decreased in both AWD and CON treatments but in contrast with CF in which a significant increase in exchangeable NH_4^+-N content after October 18th, suggesting that the denitrification rate was higher than the nitrification rate in both AWD and both CON treatments.

4.2. Greenhouse gas emissions from soil

The lowest cumulative N_2O emission was observed in the CF1 treatment, showing that continuous flooding significantly reduced N_2O emissions in paddy soil compared with those emissions under a fluctuating water regime (Chidthaisong et al., 2018; Khalid et al., 2019), this result supports our first hypothesis. The N_2O emissions from both AWD treatments were significantly greater than those in both CFs, in agreement with Liao et al. (2020), indicating that waterlogged conditions favor more complete denitrification (Shaaban et al., 2018). Lager N_2O emissions evolved from paddy soils during drying-rewetting cycles rather than continuously one's conditions (Verhoeven et al., 2018).

Yu et al. (2007) demonstrated that nitrification can contribute to N_2O production when the Eh was more than 500 mV, a possible critical Eh for the initiation of nitrification, yet the soil Eh in our study was about -150 mV on September 27th (Fig. S2b), suggesting that the fluxes were mainly controlled by denitrifications. Also, higher ratios of [*nirK*+*nirS*]/*nosZ* cause higher N_2O emissions (Shen et al., 2021a and b). Again, there were no significant differences in the ratios of denitrification genes (Fig. 6c), so this was not a factor causing the flux peaks on September 27th. Furthermore, low soil NO_3^--N combined with high exchangeable NH_4^+-N contents occurred in all treatments on September 27th. As the soils were anaerobic, any NO_3^- or NO_2^- which occurred would be rapidly reduced to N_2 by nitrifier denitrification or

denitrifiers (Verhoeven et al., 2018).

There were significantly higher second peaks in the N_2O fluxes in both AWD and CON treatments on October 28th, compared with those in the first peak of N_2O fluxes, including both CF treatments. This indicates that the conversion of flooding to upland contributed to increased N_2O emissions (Wu et al., 2017). This may have increased nitrification and contributed to more but less complete denitrification, with an overall increase in N_2O emissions (Cai et al., 1997, Yu et al., 2007, Devkota et al., 2013). The Spearman's correlation reflects that the *amoA* genes correlated significantly positively with N_2O fluxes in the CON treatments (Fig. 8a), and denitrification genes ratios had significant positive relationships with N_2O fluxes in the AWD treatments on October 28th (Fig. 8b). This suggests that nitrification and denitrification may be mainly responsible for the second N_2O fluxes peak in the AWD and CON treatments respectively, the difference may be caused by different soil water contents. A higher soil Eh was also observed (~100 mV) during the mid-season period, conditions which favour nitrification and partial denitrification thus increasing the N_2O emissions in the AWD and CON treatments. Also, the findings of Spearman's correlation showing that ratios of *amoA* genes to *nosZ* gene and denitrification genes ratios significantly positively correlated with N_2O emissions (Fig. 8d and e), this possibly supports the notion of a direct contribution via coupled nitrification-denitrification. These results agree with other studies showing a close link between nitrification and denitrification in rice systems (Dong et al., 2013; Verhoeven et al., 2018). In addition, a shift from anaerobic to aerobic conditions increased the N_2O fluxes, which may also be due to: i) conversion of flooding to aerobic soils causing increased aeration, favouring N_2O release from soil (Wang et al., 2011a), and ii) increased soil organic matter mineralization due to enhanced soil aeration (Wang et al., 2014). These may cause significantly high emissions in AWD and CON treatments rather than in the CF treatments. Notably, the significant decrease in N_2O fluxes in the AWD2 and CON2 treatments may indicate that biochar addition inhibits N_2O production in paddy soils, which was reported previously (Wang et al., 2011b; Liu et al., 2019b), as shown by correlation results (Figs. 8d and e). However, both nitrifiers and *nirK*-bacteria gene copies were greatly decreased following biochar addition, this is a more likely explanation. In contrast, the N_2O fluxes without and with biochar (CF1 and CF2) were low and no different, as there were no denitrification sources (NO_3^--N) in either CF treatment, NO_3^--N plays a key role in N_2O production as an electron acceptor during

denitrification (Lan et al., 2017), and also the nitrification process was inhibited (Fig. 2).

In comparison to water management, biochar negatively affected the N_2O emissions, as shown by the SEM results (Fig. 7; $p < 0.05$), and it significantly decreased cumulative N_2O emissions in different water managements, which is consistent with the second hypothesis. Soil pH (Fig. S2a) did not affect N_2O emissions even though it increased following biochar addition. Therefore, other factors such as available C had more influence on N_2O emissions (Shen et al., 2021a), which may drive N_2O emission from soil organic matter by priming effect (Li et al., 2022). Soil Eh contributed to the N_2O fluxes (Fig. S2b), increasing sharply from -150 to 150 mv during the mid-season period. Previous studies showed increased conversion of N_2O to N_2 at low Eh (Yu et al., 2001 and 2004), and soil Eh is significantly increased by increasing soil moisture in paddy soils (Xu et al., 2002). Thus, the increased soil Eh caused by decreasing soil moisture in this study may have contributed to increased N_2O emissions, as shown by others (Katayanagi et al., 2012; Chidthaisong et al., 2018).

The largest cumulative CH_4 emission was in the CF1 treatment. This agrees with previous findings that cumulative CH_4 emissions at 100% WHC and flooded soils were significantly higher than at lower water contents (Shen et al., 2021a). Permanent flooding was suggested to promote CH_4 emissions by triggering anaerobic microbial degradation chains in methanogens (Whalen et al., 1990; Gauthier et al., 2015; Lagomarsino et al., 2014). Furthermore, after the mid-season period, CH_4 flux peaks in both CON treatments re-emitted were significantly greater than those in both CF treatments, however, the CH_4 fluxes in both AWD treatments were significantly lower than those in the CF1 treatment. The difference in CH_4 fluxes between AWD and CON treatments may be related to different drying-rewetting cycle modes, the rapid drainage from October 17th in CON treatments caused the comprehensively aerobic conditions promoting soil organic C decomposition (Chow et al., 2006), so after rapid re-flooding (October 31st) the rapid accumulation of available substrate and microbial biomass before the mid-season drainage period would provide CH_4 production source under anaerobic conditions (Ouyang et al., 2013; Liao et al., 2020). In contrast, the mild drying cycling from October 17th in the AWD treatments might not result in the rapid accumulation of available substrate. Additionally, the drying cycle caused increased aerobic conditions after rapid re-flooding (October 31st), which would not

favour CH_4 emissions (Liao et al., 2020).

The effects of biochar additions on CH_4 emissions from paddy soils are contradictory. Some studies showed suppression (Liu et al., 2011; Wang et al., 2019) while others reported stimulation (Zhang et al., 2010) as in this study. Jeffery et al. (2016) showed that biochar amendments decreased CH_4 emissions under flooded conditions, which agrees with our results and the "biofilter" function of CH_4 consumption. Therefore, the lack of an influence or increased CH_4 emissions in both CON and both AWD treatments can be attributed to a decreased ratio of methanogenic archaea to methanotrophs due to the biochar addition (Feng et al., 2012).

4.3. Functional gene shifts

The AOA *amoA* gene copy numbers were significantly higher than AOB *amoA* gene copy numbers in this soil, which is consistent with other studies (Li et al., 2018b; Sarr et al., 2020; Shen et al., 2021a and b). Biochar decreased both *amoA* genes copy numbers implying that it has the potential to inhibit the growth of nitrification gene carriers, possibly because it contains nitrification inhibitors (Sarr et al., 2020; Shen et al., 2021b). This could also explain the significant decreases in N_2O fluxes in biochar-amended treatments.

Water management significantly affected *amoA* genes copy numbers, especially on October 28th (Table S3; $p < 0.01$): *amoA* genes copy numbers in both AWD and CON treatments were significantly higher (33%) than those in both CF treatments. There were greater abundances of both genes in AWD1 and CON2 in comparison with CF1, indicating that low soil moisture content favoured nitrifiers (Liu et al., 2017). This also agrees with Wang et al. (2017).

Fujimura et al. (2020) evaluated the community composition of denitrifying bacteria and fungi across different land-use types. The *nirK*-fungi genes were not found in paddy soils. However, in our study *nirK*-fungi gene copy numbers were initially significantly higher than *nirK*-bacteria gene copy numbers (Fig. 5c), indicating that fungal denitrifiers may largely contribute to denitrification and N_2O production. In addition, fungal denitrifiers play a vital role in N_2O production in soils due to the lack of N_2O reductase (Lourenço et al., 2018; Jiang et al., 2020). Lourenço et al. (2022) also reported that fungal denitrifiers (*nirK*-fungi) were associated with N_2O production in tropical upland soils. During the early period of this study, there was a larger increase in *nirK*-bacteria than *nirK*-fungi which is attributed to the

physiological difference between bacteria and fungi and to an increase in soil pH (Fig. S2a), probably because bacterial denitrifiers favour neutral or slightly alkaline soils (Chen et al., 2015). Wei et al. (2015) found that bacterial denitrifiers generally require lower oxygen conditions for denitrification than fungal denitrifiers. Therefore, waterlogged conditions may favour *nirK*-bacteria denitrifiers compared with *nirK*-fungi denitrifiers.

On October 28th, unlike fungal *nirK* genes, biochar addition caused a significant decrease in bacterial *nirK* gene copies in AWD2 and CON2 treatments, and a slight decrease in the CF2 treatment (Figs. 5b and c; Figs. 8d and e), compared with those on September 27th. These findings suggest that biochar mainly inhibited the growth of *nirK*-bacteria carriers, this may explain the significant decrease in the second N_2O flux peak in AWD2 and CON2 treatments compared to those unamended. Graf et al. (2014) found that *nosZ* gene had a higher frequency of co-occurrence with *nirS* than with *nirK*, and that *nirS*-type denitrifiers were more related to complete denitrification. Therefore, they produced less N_2O than *nirK*-type denitrifiers (Shen et al., 2021a). This could explain the suppression of N_2O emissions caused by biochar. Ji et al. (2022) reported that biochar additions inhibited fungal denitrifier growth and denitrification in upland soils, however, fungal denitrifier abundances in AWD2 were significantly higher than those in the AWD2 treatment on September 27th, attributed to different soil types and environmental conditions.

Wu et al. (2017) have found that the transition period from paddy to an aerobic system resulted in the greatest N_2O emissions, attributed to the mineralization of soil organic matter. In our work, N_2O fluxes significantly increased during the mid-season period (Fig. 3a). However, N_2O emission then decreased due to an increase in *nosZ* gene copies caused by biochar addition, which is in line with our third hypothesis. Biochar also caused a large decrease in denitrification genes ratios in agreement with other work (Aamer et al., 2021), in the treatment of CF2, but there was no difference in N_2O fluxes between the CF1 and CF2 treatments which may indicate that complete denitrification produced less N_2O (Fig. 3a). Biochar was the dominant cause of N_2O emissions, as biochar still significantly increased *nosZ* gene copies by the end of the experiment (Table S3). This implies that biochar additions can play a key role in mitigating N_2O emissions in tropical paddy soils.

Biochar addition resulted in a significant increase in *nifH* gene abundances after the second fertilizer addition, in agreement with previous studies showing that

increases in substrate C availability, such as following biochar addition increases biological N_2 fixation by increasing *nifH* gene abundances (Zhao et al., 2021). Organic fertilizers provide increased accessible C for microbial growth, increasing diazotrophic copy numbers and activity (Perez et al., 2014). These suggest that available C derived from biochar may stimulate diazotroph growth. Furthermore, effect changes in water management from CF to CON and AWD on *nifH* gene copies were significantly negative (Fig. 6b; Table S3).

4.4. Global warming potential contribution and rice yield

The highest GWP and GHGI were with treatment CF1, which was primarily driven by the emission of CH_4, as the previous study revealed that CH_4 emissions dominated the GWPs in waterlogged paddy soils, which was attributed to the waterlogging promoting CH_4 production (Weller et al., 2016). Zhang et al. (2012) found that biochar amendments significantly decreased the GWP and GHGI in paddy soils under continuous flooding, which is consistent with our results. However, biochar had no effect on GWP and GHGI in the AWD and CON treatments (Table 1). We attribute this to the enhanced rice yields resulting from the amendment of biochar, in agreement with previous findings (Wu et al., 2021) as biochar itself provides inorganic nutrients which may stimulate rice production (Zhang et al., 2020). Water management significantly influenced GWP and GHGI (Table S1). Both GWP and GHGI were significantly lower in the AWD1 treatment than in the CF1 treatment, which indicates that water management in rice planting was important for mitigating greenhouse effects. In addition, biochar additions may increase both N_2 fixation and N supply by increasing *nifH* gene abundance and thus promote rice yield. Significantly smaller grain yields occurred with both CF treatments (Fig. S2), indicating that drying-rewetting cycles increase the growth and numbers of tillers in paddy and growth (Tan et al., 2013). The highest yield and lowest GHGI were observed in the AWD2 treatment, implying that biochar amendment in tropical paddy soil with drying-rewetting cycles during the mid-season period may be the best management strategy for rice production and to mitigate GWP.

4.5 The limitations of the study

This study was carried on under controlled greenhouse conditions, which resulted in differences from natural in situ conditions. For example, biochar addition led to a decrease in N_2O emissions under specific greenhouse conditions but this effect was

not consistent across all field conditions (Fidel et al., 2019). It is necessary to note that the outcomes observed in one specific paddy soil type may not be representative of the broader impact of biochar applications on the mitigation of N_2O emissions in all tropical soils, although experimental soil used is typical paddy soil in the tropics of China. Thus, future experiments are necessarily conducted to bridge this gap. Furthermore, previous study has highlighted differences in nitrification and denitrification response to short-term and long-term biochar application (He et al., 2019), suggesting the duration of the experiment may impact the efficiency of N_2O emissions mitigation by biochar. In some cases, this could even lead to increased total N gas loss in long-term periods. Variations in responses to biochar treatments and water content levels may exist among different rice genotypes (Auler et al., 2021; Liu et al., 2022). Therefore, it would be compelling to explore how biochar treatments and different water conditions impact nitrogen cycling across various rice genotypes in future studies. Given the influence of various experimental factors on N_2O emissions, it is essential to approach the conclusions with caution and seek verification in the field conditions in the future (Elrys et al., 2023).

5. Conclusions

This study indicated that water management during the mid-season had a significant effect on N_2O emissions in tropical paddy soil. Alternating wet-dry cycles and conventional mid-season drainage significantly increased cumulative N_2O emissions and significantly decreased cumulative CH_4 emissions compared to continuous flooding. Biochar additions, however, could effectively mitigate N_2O emissions mainly by increasing *nosZ* gene copies. There was no significant difference among different treatments for the first N_2O flux peak; however, the significant difference in the second higher peak was mainly caused by biochar, which significantly decreased N_2O emissions via increased *nosZ* gene copies in AWD and CON treatments, the ratios of denitrification and *amoA* genes to *nosZ* gene copies regulated N_2O emissions as well. The fluxes in both CF treatments were low may be due to mostly complete denitrification. Biochar addition also caused an increase in *nifH* gene copies and probably promoted N fixation in the paddy soil. Moreover, biochar-amended soils subjected to alternating wet-dry cycles had the lowest GHGI and the highest crop yields. Continuous flooding significantly increased CH_4 emissions and decreased N_2O emissions compared to alternating drying-rewetting

cycles and conventional mid-season drainage. Biochar addition significantly decreased CH_4 emissions under continuously flooded soil conditions.

In summary, biochar applications to tropical paddy soil with drying-rewetting cycles during the mid-season period may be useful for increasing fertilizer N use efficiency in rice production and mitigating GWP. These results are therefore important for field practices with regard to greenhouse gas management and rice production in tropical paddy soils. Further work is required to confirm these findings under field conditions over a long-term period to further investigate functional gene expression and reveal microbial communities carrying related genes in response and adaption to the biochar addition and the changing water management strategies.

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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.

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Supplementary material

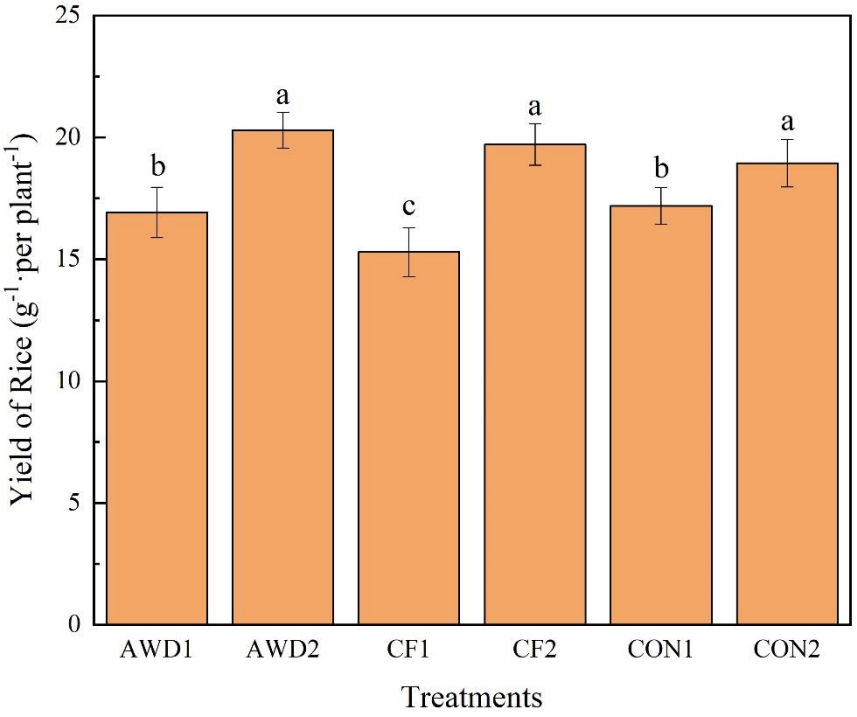

Fig. S1 The total rice yield in different treatments during the rice cultivation period. Error bars represent standard errors of the means (n=4). Different lowercase letters indicate significant differences among different treatments within the whole cultivation time, which were determined by a one-way ANOVA by a Tukey test for post hoc comparison at p <0.05. A two-way ANOVA was used to compare differences between water management (AWD, CON, and CF), biochar treatment (no biochar and 2% biochar), and their interactions, p < 0.05 were considered to be statistically significant, shown in Table S1.

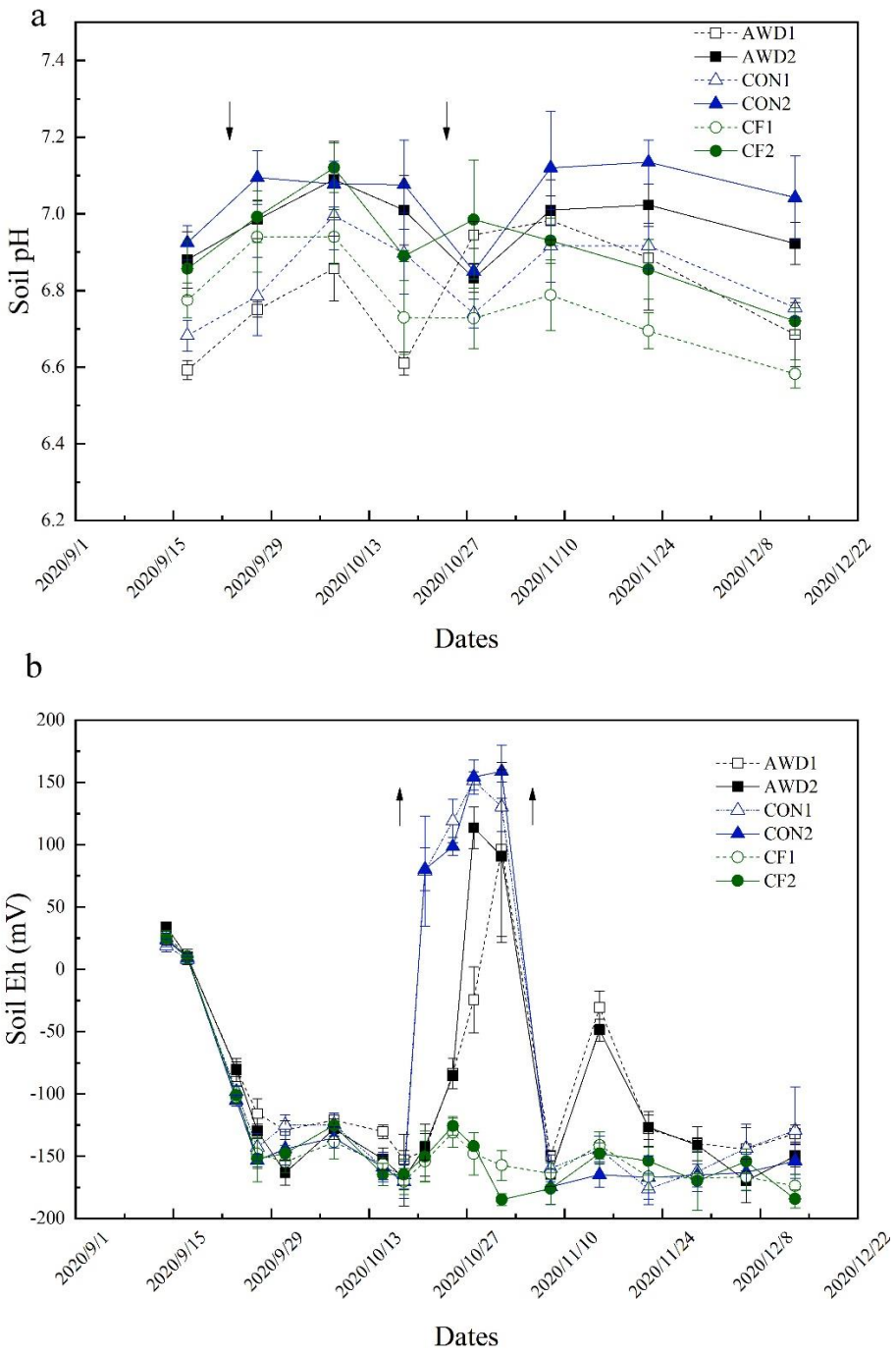

Fig. S2 Changes in soil pH (a) and soil Eh (b) during the rice cultivation period. Error bars represent standard errors of the means (*n*=4). AWD1: Alternating dry-wet conditions during the mid-season period without biochar amendment; AWD2: Alternating dry-wet conditions during the mid-season period with 2% (w/w) biochar amendment; CF1: Continuous flooding during the mid-season period without biochar amendment; CF2: Continuous flooding during the mid-season period with 2% (w/w) biochar amendment; CON1: Regular mid-season drainage during the mid-season period without biochar amendment; CON2: Regular mid-season drainage during the mid-season period with 2% (w/w) biochar amendment.

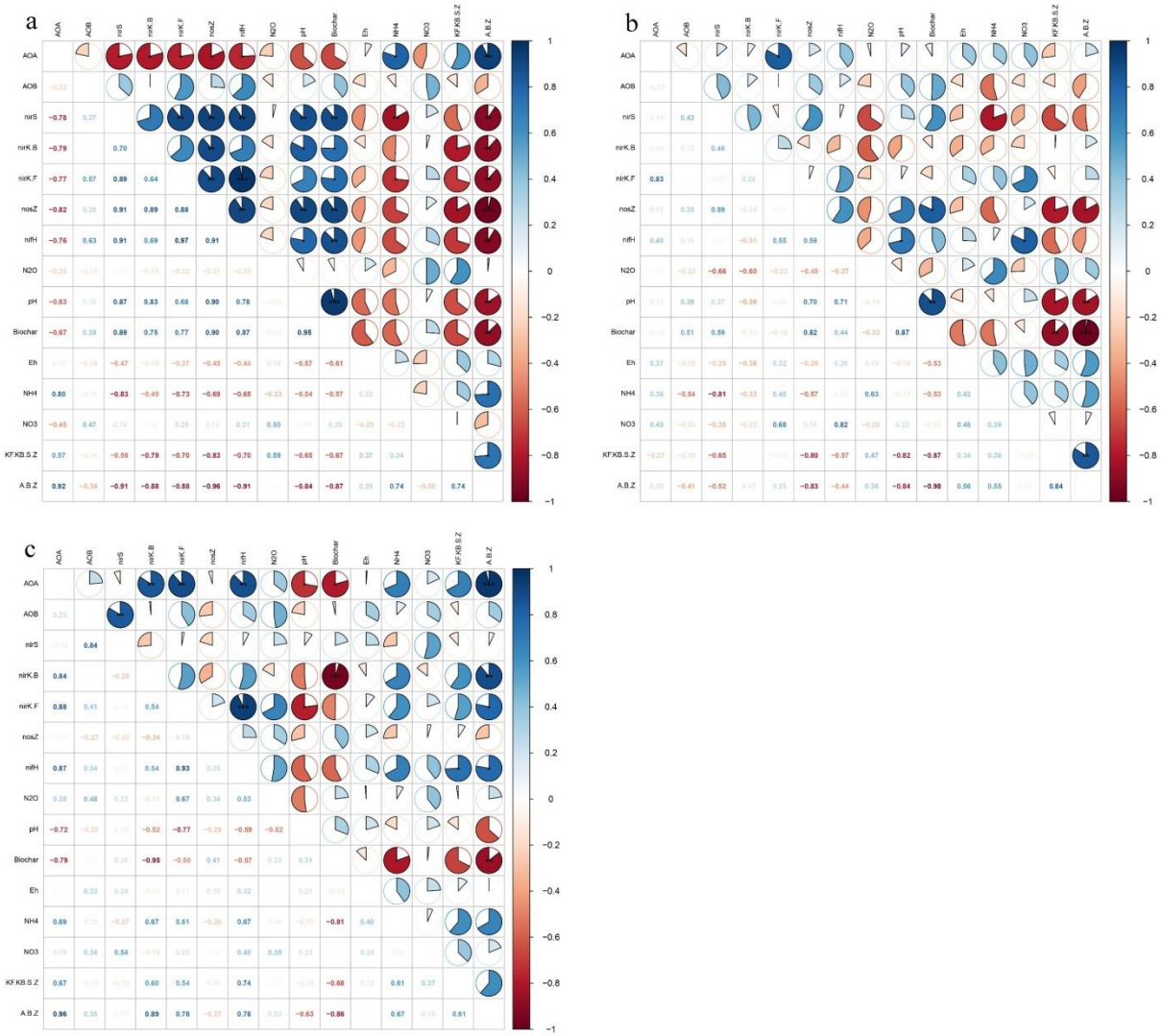

Fig. S3 Spearman's correlation coefficients among biochar, water management, the abundance of N cycling genes (AOA and AOB *amoA* genes, *nirS*, *nirK* (fungal and bacterial), *nosZ* and *nifH*, and the ratio of [*nirS*+ *nirK*-bacteria +*nirK*-fungi]/*nosZ*, N_2O fluxes (N_2O) and different edaphic factors (Eh, Ec, NH_4^+-N, NO_3^-N, and pH). And a, b and c represent AWD, CON and CF treatments on September 27th respectively. Abbreviation: AOB, *amoA* belonging to ammonia-oxidizing bacteria; AOA, *amoA* belonging to ammonia-oxidizing archaea; KFKBSZ, the ratio of [*nirK*-bacteria+*nirK*-fungi+*nirS*]/*nosZ*; ABZ, the ratio of *amoA* /*nosZ*. Red and blue indicate negative and positive correlations, and color depth indicates stronger correlations. Asterisks indicate significant effect ("***" $p < 0001$, "**" $p < 001$, "*" $p < 005$), after *p*-value was corrected by the Bonferroni method

Table S1

The analysis of variance (Two-way ANOVA) table for emissions of N_2O and CH_4 (cumulative totals), N_2O-F (cumulative emissions after the first to before the second fertilizer), CH_4-F, N_2O-S (cumulative emissions after the second fertilizer to the end of the experiment), CH_4-S and Rice Yield, showing the effect of biochar (B) and water management (WM) and their interaction. The variance ratio statistic (*F*) is presented together with its probability level (*p*). "*","**" and "***" significance at 0.05, 0.01 and 0.001, respectively.

GHG	Factor	*F*	*p*
N_2O	**B**	**7.352**	**<0.01****
	WM	0.03	0.95
	B×WM	0.177	0.67
N_2O-F	B	0.067	0.80
	WM	0.254	0.61
	B×WM	**11.5**	**0.002****
N_2O-S	**B**	**5.953**	**0.024***
	WM	0.671	0.423
	B×WM	0.013	0.911
CH_4	B	0.768	0.39
	WM	**153**	**0.0011****
	B×WM	0.238	0.63
CH_4-F	B	1.06	0.32
	WM	**4.54**	**0.045***
	B×WM	0.143	0.709
CH_4-S	B	0.177	0.67
	WM	**12.52**	**0.002****
	B×WM	0.41	0.523
Rice Yield	**B**	**60**	**<0.001****
	WM	1.11	0.304
	B×WM	**10.5**	**0.005****
GWP	B	0.87	0.354
	WM	**1.11**	**<0.001****
	B×WM	1.07	0.31
GHGI	**B**	**8.13**	**<0.01****
	WM	**16.31**	**<0.001****
	B×WM	0.67	0.44

Table S2

The analysis of variance (Two-way ANOVA) table for daily emissions of N_2O and CH_4, showing the effect of biochar (B) and water management (WM) and their interaction. The variance ratio statistic (*F*) is presented together with its probability level (*p*). "*", "**" and "***" significance at 0.05, 0.01 and 0.001, respectively.

Gas	Day	Factor	*F*	*p*
N_2O	9 17	B	1.76	0.20
		WM	4.14	0.1
		B× WM	2.354	0.12
	9 27	B	0.46	0.505
		WM	0.72	0.611
		B× WM	1.16	0.293
	10 28	**B**	**7.176**	**0.0044****
		WM	0.684	0.418
		B× WM	0.103	0.751
	11 08	B	3.71	0.068
		WM	**254**	**<0.001*****
		B× WM	1.764	0.199
	12 13	B	0.002	0.96
		WM	3.054	0.157
		B× WM	**4.688**	**0.043***
CH_4	9 17	B	0.883	0.36
		WM	4.25	0.054
		B× WM	1.879	0.87
	9 27	B	0.418	0.53
		WM	0.45	0.59
		B × WM	0.093	0.76
	10 28	**B**	**15.3**	**<0.001****
		WM	1.83	0.19
		B × WM	**8.30**	**0.01****
	11 08	B	0.014	0.906
		WM	**78.5**	**<0.001****
		B × WM	0.005	0.946
	12 13	B	0.284	0.65
		WM	0.107	0.74
		B × WM	0.577	0.73

Table S3

The analysis of variance (ANOVA) table for functional genes, showing the effect of biochar (B) and water management (WM) and their interaction. The variance ratio statistic (*F*) is presented together with its probability level (*p*). "*", "**" and "***" significance at 0.05, 0.01 and 0.001, respectively.

Genes	Day	Factor	*F*	*p*
AOA *amoA*	9 17	B	0.059	0.95
		WM	0.053	0.65
		B× WM	**11.84**	**<0.001*****
	9 27	**B**	**5.096**	**0.002****
		WM	1.385	0.058
		B× WM	2.737	0.092
	10 28	**B**	**53.178**	**<0.001*****
		WM	**8.567**	**0.002****
		B× WM	**22.85**	**<0.001*****
	12 13	**B**	**24.646**	**<0.001*****
		WM	2.054	0.157
		B× WM	**65.007**	**<0.001*****
AOB *amoA*	9 17	B	4.318	0.54
		WM	5.573	0.051
		B× WM	**5.678**	**0.014***
	9 27	B	0.6	0.46
		WM	0.58	0.07
		B × WM	0.417	0.66
	10 28	**B**	**18.3**	**<0.001*****
		WM	**22.7**	**<0.001*****
		B × WM	0.439	0.652
	12 13	B	0.584	0.455
		WM	0.117	0.89
		B × WM	5.977	0.1
nirS	9 17	**B**	**7.101**	**0.017***
		WM	2.214	0.142
		B × WM	0.397	0.679
	9 27	**B**	**9.813**	**0.006****
		WM	**29**	**<0.001*****
		B × WM	1.85	0.19
	10 28	B	0.90	0.355
		WM	**26.4**	**<0.001*****
		B × WM	2.584	0.103
	12 13	B	0.188	0.67
		WM	**7.89**	**0.003****
		B × WM	**8.60**	**0.002****
nirK-B	9 17	B	1.654	0.215

Genes	Day	Factor	*F*	*p*
		WM	0.856	0.367
		B × WM	0.133	0.72
	9 27	B	0.25	0.6233
		WM	**7.814**	**0.0112***
		B × WM	1.872	0.1864
	10 28	**B**	**14.47**	**<0.001*****
		WM	3.858	0.06355
		B × WM	0.038	0.84
	12 13	B	1.651	0.2135
		WM	0.534	0.47
		B × WM	**7.516**	**0.013***
nirK-F	9 17	B	0.027	0.87
		WM	0	0.98
		B × WM	1.056	0.318
	9 27	B	0.063	0.803
		WM	3.929	0.061
		B × WM	2.951	0.103
	10 28	B	0.224	0.641
		WM	0.18	0.675
		B × WM	0.18	0.675
	12 13	B	0.016	0.9
		WM	0.210	0.65
		B × WM	3.352	0.081
nosZ	9 17	B	1.465	0.242
		WM	0.850	0.369
		B × WM	0.311	0.584
	9 27	**B**	**39.28**	**<0.001*****
		WM	5.727	0.06
		B × WM	0.365	0.55
	10 28	**B**	**67.58**	**<0.001*****
		WM	1.300	0.268
		B × WM	2.734	0.114
	12 13	**B**	**10.65**	**0.0039****
		WM	1.043	0.31
		B × WM	1.770	0.19
nifH	9 17	B	0.559	0.464
		WM	0.762	0.394
		B × WM	0.029	0.866
	9 27	B	2.110	0.162
		WM	2.911	0.104
		B × WM	**4.985**	**0.037***
	10 28	**B**	**82.64**	**<0.001*****
		WM	**11.39**	**0.0031****
		B × WM	**14.38**	**0.0014****
	12 13	B	1.389	0.252

Genes	Day	Factor	*F*	*p*
		WM	0.485	0.494
		B × WM	3.210	0.083
[nirK-F+nirK-B+nirS]/nosZ	9 17	**B**	**125**	**<0.001*****
		WM	**52.79**	**<0.001*****
		B × WM	**12.13**	**0.00265****
	9 27	**B**	**11.11**	**0.0031****
		WM	**28.01**	**<0.001*****
		B × WM	0.227	0.604
	10 28	**B**	**93.18**	**<0.001*****
		WM	**41.22**	**<0.001*****
		B × WM	0.281	0.602
	12 13	**B**	**17.01**	**0.0005*****
		WM	0.766	0.39
		B × WM	0	0.98
amoA/ nosZ	9 17	B	1.421	0.248
		WM	**25.89**	**<0.001*****
		B × WM	**15.17**	**0.0001*****
	9 27	**B**	**11.11**	**<0.001*****
		WM	0.590	0.452
		B × WM	1.282	0.271
	10 28	**B**	**93.18**	**<0.001*****
		WM	**13.87**	**0.0013****
		B × WM	0.181	0.677
	12 13	**B**	**17.01**	**<0.001*****
		WM	3.087	0.094
		B × WM	6.071	0.03*

Table S4

The analysis of variance (Two-way ANOVA) table for NH_4^+-N and NO_3^--N contents, showing the effect of biochar amendment (B) and water management (WM) and their interaction. The variance ratio statistic (*F*) is presented together with its probability level (*p*). "*", "**" and "***" significance at 0.05, 0.01 and 0.001, respectively.

Inorganic N	Day	Factor	*F*	*p*
NH_4^+-N	9 17	B	2.51	0.13
		WM	1.23	0.28
		B× WM	0.051	0.83
	9 27	**B**	**11.92**	**0.003****
		WM	1.494	0.236
		B× WM	0.021	0.88
	10 08	**B**	**71.37**	**0.0001****
		WM	0.781	0.98
		B× WM	0.303	0.91
	10 28	**B**	**9.084**	**0.007****
		WM	1.326	0.26
		B× WM	1.362	0.26
	11 08	B	1.80	0.195
		WM	0.65	0.552
		B× WM	1.19	0.288
	12 13	B	0.017	0.899
		WM	0.032	0.861
		B× WM	0.802	0.053
NO_3^--N	9 17	B	0.992	0.331
		WM	0.79	0.051
		B× WM	0.879	0.49
	9 27	B	0.15	0.69
		WM	3.17	0.09
		B × WM	0.26	0.62
	10 08	**B**	**22.63**	**<0.001*****
		WM	**7.50**	**0.01***
		B × WM	0.43	0.09
	10 28	B	1.32	0.265
		WM	0.13	0.725
		B × WM	0.087	0.771
	11 08	B	0.014	0.906
		WM	0.15	0.051
		B × WM	0.005	0.946
	12 13	B	0.723	0.405
		WM	0.118	0.74
		B × WM	0.100	0.76

1280

1281 **Table S5** Primer pairs and PCR conditions used in real-time qPCR analysis

Target group	Primer name	Sequence/ 5'-3'	Reference
AOA-*amoA*	AOA-23F	ATGGTCTGGCTWAGACG	(Sahan and Muyzer, 2008)
	AOA-616R	GCCATCCATCTGTATGTCCA	
AOB-*amoA*	AOB-1F	GGGGTTTCTACTGGTGGT	(Sahan and Muyzer, 2008)
	AOB-2R	CCCCTCKGSAAAGCCTTCTTC	
nifH	*nifH*-F	AAAGGYGGWATCGGYAARTCCACCAC	(Poly et al., 2008)
	nifH-R	TTGTTSGCSGCRTACATSGCCATCAT	
nirK-bacteria	*nirK*-876F	ATYGGCGGVCAYGGCGA	(Henry et al., 2004)
	nirK-1055R	GCYTCGATCAGRTTRTGGTT	
nirS	*nirS*-cd3aF	GTSAACGTSAAGGARACSGG	(Michotey et al., 2000)
	nirS-R3cd	GASTTCGGRTGSGTCTTGA	
nosZ	*nosZI*-1126F	GGGCTBGGGCCRTTGCA	(Chen et al., 2012)
	nosZI-1381R	GAAGCGRTCCTTSGARAACTTG	
nirK-fungi	fungi *nirK*-2F	GTYCAYATYGCYAACGGSATGTACGG	(Long et al., 2014)
	fungi *nirK*-1R	GCRTGRTCNACMAGNGTRCGTCCC	