



# Article Biochar and Flooding Increase and Change the Diazotroph Communities in Tropical Paddy Fields

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Abstract: Biological nitrogen fixation (BNF) can reduce synthetic N fertilizer application and improve N-use efficiency. However, knowledge about the effect of biochar and water management regimes on soil diazotrophic microorganisms in tropical paddy fields remains only rudimentary. A field trial was started in the early rice season in 2019 and ended in the late rice season in 2020. We studied the effects of five treatments comprising different water management and biochar applications on the diazotrophic abundance and community composition: no N fertilizer + conventional water management, conventional fertilization + conventional water management, no N fertilizer + flooding, conventional fertilization + flooding, and application of 40 t  $ha^{-1}$ biochar + conventional fertilization + conventional water management. According to the results, biochar increased soil pH and organic carbon (SOC), whereas flooding decreased the soil available phosphorus (P) content. However, the addition of biochar and flooding as well as N application treatments increased *nifH* abundance. The *nifH* abundance negatively correlated with available N and P, whereas it significantly positively correlated with SOC (p < 0.05). The results of redundancy analysis unveiled that biochar stimulated the relative abundance of Pelomonas and changed the diazotrophic microbial community structure by increasing soil pH, while flooding stimulated the relative abundance of Azospirllum. Conclusively, both flooding and biochar affect soil diazotrophic microbial community and abundance in paddy fields. Reducing N and P fertilizer application clubbed with biochar amendment and flooding may be beneficial for soil N-fixing in tropical paddy fields.

Keywords: biochar; water management; nifH gene; diazotrophic

# 1. Introduction

Nitrogen (N) is a major atmospheric component and is an essential nutrient for plant growth. To meet the rising human food demand, large amounts of N fertilizers are applied in paddy fields for increasing rice production. In China, the N-use efficiency (NUE) of rice is low because only 20–40% of applied N fertilizers are taken up by the plants. [1–3]. The ecosystem N retention in China is 10–35% in paddy fields, and this creates several environmental problems, such as soil acidification [4] and greenhouse gas emission [5]. Therefore, we must identify a reasonable way to reduce the amount of synthetic N fertilizers used to achieve sustainable agricultural development.

Dinitrogen (N<sub>2</sub>) is available abundantly in the atmosphere, but plants cannot use it directly. N<sub>2</sub> must first be converted into inorganic N through lightning or biological N fixation (BNF) [6]. The BNF has been estimated to be  $52 \sim 130$  Tg (1 Tg =  $10^{12}$  g) N year<sup>-1</sup> in the global natural ecosystem [7]. Thus, unfertilized crops can achieve a certain yield through BNF [8]. In addition to common legume crops, non-symbiotic N-fixing microorganisms are also present in rice roots and provide N for rice growth [9]. Herridge et al. [10] found that BNF in paddy soils can provide 22–30 kg N ha<sup>-1</sup> year<sup>-1</sup>. Diazotrophs catalyze BNF using the highly conserved nitrogenase enzyme [11]. The *nifH* gene is the most



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**Copyright:** © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). common biomarker used for determining the evolution and ecology of N-fixing bacteria. This biomarker has been extensively employed to study diazotrophic microbial communities in paddy soils [12]. The *nifH* gene has also been detected in many microbial genera (e.g., *Geobacter* and *Pelomonas*) [13,14]. Edaphic and environmental factors, such as climate, cropping systems, soil type, nutrient availability, and vegetation, influence BNF activity and diazotrophic microbial communities [15,16]. Edaphic and environmental factors vary across climatic zones [17], which may cause variations in the BNF activity and diazotrophic microbial communities in paddy fields across these zones [18,19]. For instance, soil acidification is more severe in the tropics than in other climatic zones [20]. Soil aluminum determines N and diazotrophic communities in rice fields, which leads to changes in BNF activity and diazotrophic communities in these fields in the tropics relative to the other regions [21]. Moreover, changes in diazotrophic microbial communities, which are mainly caused by temperature and rainfall [22,23], also occur with seasons; different results were obtained at different times in the same location [24]. Therefore, multi-seasonal studies are warranted to elucidate the factors influencing diazotrophic microbial communities in the tropics so as to augment BNF activity in these regions.

Diazotrophs are sensitive to water [25]. Therefore, changes in the soil moisture content can affect BNF activity by altering soil redox conditions, particularly in paddy fields [26]. Das et al. [27] found that BNF activity in paddy soils was lower under non-flooding conditions than under flooding conditions. BNF activity in soil increased when paddy soil changed from dry to wet, whereas it decreased when the soil changed from wet to dry [28]. Changes in water availability in paddy soils can also affect the soil microbial community structure by affecting soil properties, such as pH and nutrient content [29,30], which are crucial factors affecting BNF activity [31]. In tropical regions, water changes dramatically, and diazotrophic communities may be accustomed to such changes [32]. Other factors (except O<sub>2</sub> concentration, redox conditions) may impact BNF activity in tropical paddy fields through water management. Yet, how different irrigation practices affect BNF activity in tropical paddy soils remains poorly known.

Different fertilization management practices also affect the structure of soil microbial communities in paddy fields [33] by affecting soil properties, thereby affecting soil N-fixing microbial diversity and community structure in these fields [34]. Because soil N-fixing microorganisms are more sensitive to the available N content in the soil [25], the soil N-fixing activity of the paddy field without any added N fertilizer was significantly higher than that of the field under long-term N fertilization, and the soil microbial community structure was also changed [35]. However, in some studies, N application had no significant effects on BNF activity and the diazotrophic microbial community composition in paddy soils [36]. Some studies have also reported that phosphorus (P) and potassium (K) deficiency caused a decrease in *nifH* expression and the N<sub>2</sub> fixation ability [37]. Although these nutrients (NPK) can affect the BNF of paddy soils, their effects on the diazotrophic microbial community structure in tropical areas remains unclear and needs further research.

Biochar, as a soil amendment, has recently been investigated extensively because of its great potential in mitigating greenhouse gas emission [38]. Furthermore, biochar has recently exhibited its effects on diazotrophic communities and BNF activity [39]. For example, Liu et al. [40] observed that biochar affects diazotrophic communities and BNF activity by increasing labile carbon (C) in soil. Biochar can also change the characteristics of the diazotrophic community by changing soil C/N [41]. However, according to Zhao et al. [42], biochar affects the soil diazotrophic community mainly by increasing soil pH. In some studies, biochar increased the abundance of diazotrophic microbes by increasing soil Olsen-P [43]. In addition, biochar can affect BNF activity by changing inorganic N availability in paddy soils through abiotic processes such as physical adsorption [44]. Biochar application in fields also favor the biochar weathering process in paddy fields, particularly in tropical areas that receive plenty of rain and heat [45]. Although many studies in temperate and subtropical regions have explored the effects of biochar application and different water management practices on BNF activity in paddy fields [46–48], the effects in tropical paddy soils remain unclear.

This study investigated the effect of fertilization and irrigation practices on the diazotrophic community diversity and composition in tropical paddy soils. We hypothesized that environmental influencing factors have distinct effects on diazotrophic microorganisms in tropical paddy soils compared with those in other climatic zones. Biochar application and water management practices would affect diazotrophic microbial communities by influencing various environmental factors.

## 2. Materials and Methods

# 2.1. Experiment Site

The field experiment was conducted in the Xi'an village Qiaotou Town ( $109^{\circ}56'01''$  E,  $19^{\circ}56'58''$  N), Chengmai County, Hainan Province, China. This village has a tropical monsoon climate (average annual temperature: 23.8 °C; average annual precipitation: 1786 mm). We used sandy loam soil in the experiment (62.7% sand, 20.5% silt, and 16.8% clay). This soil has a pH of 5.9 in soil as well as the following properties: water suspension (1:2.5), soil organic carbon (SOC) of 15.7 g kg<sup>-1</sup>, total N of 2.80 g kg<sup>-1</sup>, available N of 148 mg kg<sup>-1</sup>, available P of 123 mg kg<sup>-1</sup>, available K of 49.6 mg kg<sup>-1</sup>, and cation exchange capacity (CEC) of 4.0 cmolc kg<sup>-1</sup>.

#### 2.2. Experimental Setup

The field trial was started in the early rice season in 2019 and was ended in the late rice season in 2020. The experiment had a randomized complete block design. The edges of each plot were bedded and covered with tarps. The tarps were buried 50 cm deep in the soil to prevent water and fertilizer circulation between the plots. The rice variety used was 'Teyou 808', which is a temperature-sensitive three-line hybrid rice. Five treatments were established in the experiment: (1) no N fertilizer + conventional water management (CK); (2) conventional fertilization + conventional water management (CON); (3) no N fertilizer + flooding (FCK); (4) conventional fertilization + flooding (FCON); (5) application of 40 t ha<sup>-1</sup> biochar + conventional fertilization + conventional water management (B). Each treatment was set up with three replicates. The application rate of conventional fertilization was determined according to local traditional farming practices, and the fertilization time and application rate are shown in Table 1. Urea, superphosphate, and potassium chloride were applied as N, P, and K fertilizers, respectively. Urea was applied in the early rice season in three splits (5:3:2) at basal, tillering, and ear stage, respectively, while urea was applied in two splits (6:4) at tillering and ear stage, respectively, in the late rice season. Superphosphate and potassium chloride were applied to all plots as basal fertilization in all treatments. The flood treatment maintained flooding throughout the rice growth cycle. Conventional water management practices were implemented according to local traditional water management methods. The rice was dried out for 2 weeks at the tillering stage, after which it was the same as the flood treatment.

Table 1. Field fertilization management and dosage.

Season	Field Management	2019	2020	Nitrogenous Fertilizer/Urea: N 46%	Phosphate Fertilizer /Superphosphate: P <sub>2</sub> O <sub>5</sub> 16%	Potassium Fertilizer /Potassium Chloride: K <sub>2</sub> O 60%
	Seedling transplanting, Base fertilizer	20 April 2019	1 May 2020	$60 \text{ kg} \cdot \text{ha}^{-1}$	90 kg $\cdot$ ha <sup>-1</sup>	90 kg $\cdot$ ha <sup>-1</sup>
Early rice season	Tillering fertilizer	13 May 2019	8 May 2020	36 kg∙ha <sup>-1</sup>		
	Ear fertilizer	13 June 2019	14 June 2020	24 kg∙ha <sup>-1</sup>		
	harvest	20 July 2019	5 August 2020			
	Seedling transplanting, Base fertilizer	30 July 2019	14 August 2020		90 kg $\cdot$ ha <sup>-1</sup>	$120 \text{ kg} \cdot \text{ha}^{-1}$
Late rice season	Tillering fertilizer	12 August 2019	22 August 2020	90 kg∙ha <sup>-1</sup>		
	Ear fertilizer	9 September 2019	21 September 2020	60 kg∙ha <sup>-1</sup>		
	harvest	5 November 2019	8 November 2020	-		

Only phosphorus and potassium fertilizer should be applied in the blank, and all of them should be applied at one time to spread into the field.

Biochar application to the soil by spreading and ploughing was only practiced during the first year of the rice season. Biochar was obtained through coconut husk pyrolysis at 600 °C. The physicochemical properties of the biochar were determined according to the methods of Harter et al. [39]. In this study, biochar had a pH of 9.7, total C content of 676.78 g kg<sup>-1</sup>, total N of 3.0 g kg<sup>-1</sup>, available P of 99.6 mg kg<sup>-1</sup>, available K of 65.1 mg kg<sup>-1</sup>, CEC of 8.2 cmol kg<sup>-1</sup>, and specific surface area of 5.8 m<sup>2</sup> g<sup>-1</sup>. In accordance with the local paddy field management methods, flood water in the rice field was drained in the tillering stage and flooding was performed again at the filling stage. Flooding treatment maintained the water level in the field after rice transplanting until the filling stage. Based on the five-point sampling method, soil samples (0–20 cm depth) were collected from each plot in each treatment at each rice harvest, and then mixed into one composite sample. A part of the soil sample was refrigerated at -80 °C for determining soil microbial activity. The other part of the soil sample was naturally air-dried and sieved for determining soil physical and chemical properties.

#### 2.3. Soil Physicochemical Properties

Soil physicochemical properties were determined according to Bao [49]. Soil pH was determined using suspensions prepared at a soil: water ratio of 1:2.5. Available N was determined using a microdiffusion technique following alkaline hydrolysis. The soil available P was extracted with acid ammonium fluoride solution and measured using the molybdenum blue colorimetric method. The soil available K was extracted with NH<sub>4</sub>OAc (1 M, pH 7.0) and determined using a flame photometer. Soil CEC was determined according to the ammonium acetate extraction method [50]. SOC was estimated using the K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> oxidation method.

#### 2.4. Soil DNA Extraction and Real-Time PCR

Soil DNA was extracted from 0.5 g of soil by using the soil DNA extraction kit DNeasy PowerSoil Kit(100)12888-100. Following extraction, the nucleic acid concentration and quality were determined using the micro ultraviolet spectrophotometer. An appropriate amount of the extracted DNA sample was diluted and used as a qPCR template for amplification using Cytology  $2 \times T5$  Fast qPCR Mix (SYBR Green I) and the following primers: PolF (FAAAG-GYGGWATCGGYAARTCCACCAC)/PolR(RTTGTTSGCSGCRTACATSGCCATCAT) [51]. The reaction system was 20 µL, including 10 µL  $2 \times T5$  Fast qPCR Mix (SYBR Green I), 1 µL positive and negative primers, 1 µL DNA template, and the remaining volume was supplemented with sterile water. The PCR amplification procedure was as follows: 95 °C for 12 mir; 94 °C for 1 mir; 55 °C for 30 s; 72 °C for 1 mir; and 39 cycles. The plasmid concentration of eight target gene fragments was prepared, and the standard curve was plotted with the logarithm of the initial template DNA copy number as the horizontal coordinate and the Ct value. Based on the Ct value of the tested samples, *nifH* gene abundance was estimated using the standard curve. The gradient dilution standard plasmid was amplified through fluorescent quantitative PCR to calculate *nifH* abundance.

### 2.5. High-Throughput Sequencing Analysis

The original sequence was analyzed using QIIME (version 1.8.0) and USEARCH (version 10.0) to remove primers and low-quality sequences to obtain the final valid sequence. Effective sequences were converted to amino acid sequences by FunGene Pipeline. The translation protein does not match the *nifH* protein or the sequence containing the stop codon is discarded. High-quality sequences were clustered into operational taxonomic units (OTUs) with 97% similarity and all singleton OTUs were deleted. MEGAN (version 5.8.1) software was used for sequence comparison in the NCBI database (serial number PRJNA 836297) to determine the classification of *nifH* OUT. Alpha diversity was evaluated by Abundance-based Coverage Estimator (ACE), Chao1, and Shannon and Simpson indices [52].

## 2.6. Data Analysis

Data were collected and processed using Excel 2019. SPSS 23.0 was used for analyzing the data for ANOVA. Duncan multiple comparison was performed for one-way ANOVA to compare differences among the various treatments. The effects of seasonal change, fertilization management, and water management practices on *nifH* abundance were analyzed through two- and three-factor ANOVA. The Pearson correlation coefficient (p < 0.05) was used to analyze the correlation between *nifH* abundance and soil physico-chemical properties. Corresponding graphs were drawn using Origin 2021. The redundancy analysis (RDA) of the community structure of N-fixing microbes and environmental factors was performed using CANOCO 5 software.

#### 3. Results

## 3.1. Soil Physicochemical Properties

Following four seasons of rice planting, the applied N fertilizer reduced soil pH. However, under the same fertilization condition, flooding treatment exerted no significant effect on soil pH (Table 2). Compared with other treatments, biochar addition significantly increased soil pH and significantly increased SOC. Under the same fertilization condition, flooding treatment increased the soil available N content (except in the early rice season in the second year for FCON and in the late rice season in the first year for FCK and FCON), and the increase rates of the FCK and FCON treatments were 12.06% to 14.64% and 8.61% to 20.27%, respectively (Table 2). By contrast, biochar only significantly decreased the available N content in the early rice season in 2019, and then, the effect disappeared. Except for the second year of the early rice season, flooding treatment reduced the soil available P content. Compared with CON, biochar alone reduced the available P content in the first year, but had no effect on the content in the second year. In addition to the early rice season in 2020, the available K content following in the FCK and FCON treatments increased by 17–70% and 3.0–81%, respectively, compared with the CK treatment. Additionally, soil available N and P contents were higher after the first year of rice planting than after the second year.

Season	Treatments	pH	$SOC/g \cdot kg^{-1}$	Available N/mg∙kg <sup>−1</sup>	Available P/mg·kg <sup>-1</sup>	Available K/mg·kg <sup>-1</sup>
	СК	$5.5\pm0.19$ b $^{+}$	$16.3\pm0.40~\mathrm{b}$	$216.8 \pm 14.31$ a	$101.1 \pm 17.05$ ab	$29.8\pm2.39\mathrm{b}$
	CON	$5.4\pm0.09~\mathrm{b}$	$15.4\pm0.40\mathrm{b}$	$199.8 \pm 25.71$ a	$120.4\pm12.40~\mathrm{a}$	$20.5\pm1.64~\mathrm{c}$
Z19	FCK	$5.3\pm0.06~\mathrm{b}$	$15.6\pm0.34\mathrm{b}$	$186.8\pm4.09~\mathrm{a}$	$89.7\pm5.30\mathrm{b}$	$35.0\pm5.85~\mathrm{ab}$
	FCON	$5.2\pm0.13$ b	$15.8\pm0.11~\mathrm{b}$	$217.0\pm4.86~\mathrm{a}$	$94.3\pm2.50b$	$37.1\pm4.10~\mathrm{ab}$
	В	$6.2\pm0.48~\mathrm{a}$	$19.0\pm0.90~\mathrm{a}$	$128.5\pm8.52b$	$111.8\pm8.53~\mathrm{ab}$	$40.4\pm4.99~\mathrm{a}$
	СК	$5.7\pm0.09~\mathrm{b}$	$17.6\pm0.20~\mathrm{ab}$	$189.5\pm27.42~\mathrm{ab}$	$113.9 \pm 5.49$ a	$30.2\pm8.92\mathrm{b}$
	CON	$5.5\pm0.01~\mathrm{ab}$	$17.6\pm1.0~\mathrm{ab}$	$113.5 \pm 6.19 \text{ d}$	$121.7\pm18.10$ a	$26.8\pm1.12\mathrm{b}$
W19	FCK	$5.4\pm0.16~{ m b}$	$15.4\pm1.15\mathrm{b}$	$217.3\pm16.98~\mathrm{a}$	$64.9\pm1.27\mathrm{b}$	$51.2\pm6.42$ a
	FCON	$5.3\pm0.10\mathrm{b}$	$15.3\pm0.24\mathrm{b}$	$136.5 \pm 2.35 \ dc$	$81.9\pm4.96~\mathrm{b}$	$27.5\pm0.49\mathrm{b}$
	В	$6.5\pm0.44$ a	$19.2\pm2.10~\mathrm{a}$	$165.2\pm16.74bc$	$82.6\pm11.42~\mathrm{b}$	$35.6\pm6.47~b$
	СК	$5.8\pm0.04~\mathrm{b}$	$19.9\pm0.30~\mathrm{a}$	$81.4\pm12.12~\mathrm{c}$	$56.2 \pm 3.21$ a	$148.6\pm12.02~\mathrm{a}$
	CON	$5.6\pm0.07~{ m bc}$	$16.8\pm0.70\mathrm{b}$	$114.1\pm6.64\mathrm{b}$	$48.3\pm2.71~\mathrm{a}$	$99.0\pm12.61\mathrm{b}$
Z20	FCK	$5.7\pm0.23$ bc	$16.3\pm1.14\mathrm{b}$	$92.6\pm1.79~\mathrm{c}$	$52.0\pm8.88$ a	$31.6\pm1.99~\mathrm{c}$
	FCON	$5.5\pm0.16~{ m c}$	$16.5\pm1.18\mathrm{b}$	$84.9\pm2.43\mathrm{c}$	$54.3\pm1.90~\mathrm{a}$	$25.6\pm1.40~\mathrm{c}$
	В	$6.2\pm0.18~\mathrm{a}$	$20.4\pm1.10~\mathrm{a}$	$141.8\pm8.12~\mathrm{a}$	$50.4\pm8.36$ a	$153.5\pm21.84~\mathrm{a}$
W20	СК	$5.6\pm0.09~b$	$18.3\pm0.80\mathrm{b}$	$83.7\pm3.56\mathrm{b}$	$105.0\pm14.66~\mathrm{a}$	$29.8\pm2.39b$
	CON	$5.3\pm0.05\mathrm{b}$	$18.3\pm0.70\mathrm{b}$	$73.7\pm1.78~\mathrm{c}$	$115.9\pm11.96~\mathrm{a}$	$20.5\pm1.64~\mathrm{d}$
	FCK	$5.6\pm0.24\mathrm{b}$	$18.0\pm0.36\mathrm{b}$	$93.8\pm2.92~\mathrm{a}$	$32.3\pm4.46\mathrm{b}$	$39.4\pm2.49~\mathrm{a}$
	FCON	$5.7\pm0.10~\mathrm{ab}$	$18.3\pm0.85\mathrm{b}$	$82.5\pm5.99~\mathrm{b}$	$37.5\pm15.76\mathrm{b}$	$36.0\pm4.39\mathrm{b}$
	В	$5.9\pm0.11~\mathrm{a}$	$19.5\pm0.40~\mathrm{a}$	$82.3\pm10.82~\mathrm{c}$	$111.2\pm3.18~\mathrm{a}$	$24.7\pm2.37~\mathrm{c}$

Table 2. Soil properties after rice planting.

<sup>+</sup> Different letters indicate significant difference between different treatments in the same rice season (n = 3; p < 0.05). The numbers after  $\pm$  symbol are standard errors. Z19, W19, Z20, W20, respectively, denote the early rice season of 2019, the late rice season of 2019, the early rice season of 2020, and the late rice season of 2020. CK: no N fertilizer + conventional water management; CON: conventional fertilization + conventional water management; FCK: no N fertilizer + flooding; FCON: conventional fertilization + flooding; B: application of 40 t ha<sup>-1</sup> biochar + conventional fertilization + conventional water management.

Tables 3 and 4 present the average soil physicochemical properties of the four rice planting seasons. Compared with other treatments, biochar significantly increased soil pH and SOC, whereas flooding treatment significantly decreased soil available P and K contents. Under different water management conditions, N fertilizer application and seasonal changes significantly affected the measured soil properties (p < 0.05), whereas water management only significantly affected SOC and soil available P and available K contents (p < 0.05). Following conventional water management, different fertilization treatments significantly affected pH, SOC, and the available K content, while seasonal changes significantly affected other soil properties (p < 0.05), except pH. However, different fertilization treatments and seasonal changes only exhibited significant interactions with soil available N and K contents.

**Table 3.** Mean pH, SOC, available N, available P, available K, and *nifH* gene abundance under different water management treatments over a two-year experiment period.

Treatments	рН	$SOC/g \cdot kg^{-1}$	Available N/mg∙kg <sup>-1</sup>	Available P/mg·kg <sup>-1</sup>	Available K/mg∙kg <sup>-1</sup>	lg ( <i>nifH</i> Gene Abundance/Dry Soil)
CK	$5.6\pm0.18$ a $^{+}$	$18.0\pm1.39~\mathrm{a}$	$142.8\pm63.33~\mathrm{a}$	$94.0\pm25.22~\mathrm{a}$	$59.6 \pm 51.96$ a	$7.8\pm0.34~\mathrm{b}$
CON	$5.5\pm0.12~\mathrm{b}$	$17.0\pm1.34~\mathrm{b}$	$125.3\pm48.00\mathrm{b}$	$101.6 \pm 33.29$ a	$41.7\pm33.77~\mathrm{b}$	$7.7\pm0.33$ b
FCK	$5.5\pm0.25~\mathrm{b}$	$16.3\pm1.20~\mathrm{b}$	$146.6 \pm 57.27$ a	$59.7\pm21.58b$	$39.3\pm8.73\mathrm{b}$	$8.0\pm0.48$ a
FCON	$5.4\pm0.21~\mathrm{b}$	$16.5\pm1.36~\mathrm{b}$	$130.2\pm54.71\mathrm{b}$	$67.0\pm23.88~\mathrm{b}$	$31.5\pm5.92~\mathrm{c}$	$7.9\pm0.42$ a
Ν	**	**	**	*	**	*
S	**	**	**	**	**	**
W	ns	**	ns	**	**	**
N  imes S	ns	**	**	ns	**	ns
N  imes W	ns	*	ns	ns	*	ns
S  imes W	*	**	*	**	**	*
$N\times S\times W$	ns	ns	**	ns	**	ns

<sup>†</sup> Different characters in the same column indicate significance at the 0.05 level between different treatments (n = 12) based on the one-way ANOVA. The numbers after  $\pm$  symbol are standard errors. The results of three-way analysis of variance on the effect of N fertilizer (N), season (S), water management (W), and their interactions (N × S; N × W; S × W; and N × S × W) on pH, SOC, available N, available P, available K, and *nifH* gene abundance. \*\* significant at p < 0.01; \* significant at p < 0.05; ns no significant. CK: no N fertilizer + conventional water management; CON: conventional fertilization + conventional water management; FCK: no N fertilizer + flooding; FCON: conventional fertilization + flooding.

**Table 4.** Mean pH, SOC, available N, available P, available K, and *nifH* gene abundance under different fertilizer treatments over a two-year experiment period.

Treatments	рН	SOC/g⋅kg <sup>-1</sup>	Available N/mg∙kg <sup>-1</sup>	Available P/mg·kg <sup>-1</sup>	Available K/mg∙kg <sup>−1</sup>	lg ( <i>nifH</i> Gene Abundance/Dry Soil)
CK	$5.6\pm0.18$ b $^{+}$	$18.0\pm1.39~\mathrm{b}$	$142.8\pm63.33~\mathrm{a}$	$94.0\pm25.22~ab$	$59.6 \pm 51.96$ a	$7.8\pm0.34~\mathrm{b}$
CON	$5.5\pm0.12~\mathrm{b}$	$17.0\pm1.34~\mathrm{c}$	$125.3\pm48.00~\text{ab}$	$101.6\pm33.29~\mathrm{a}$	$41.7\pm33.77~\mathrm{b}$	$7.7\pm0.33$ b
В	$6.2\pm0.39$ a	$19.5\pm1.38~\mathrm{a}$	$129.4\pm32.38\mathrm{b}$	$89.0\pm26.60\mathrm{b}$	$63.6 \pm 53.52$ a	$7.9\pm0.31$ a
S	ns	**	**	**	**	**
F	**	**	ns	ns	**	**
S  imes F	ns	ns	**	ns	**	ns

<sup>+</sup> Different characters in the same column indicate significance at the 0.05 level between different treatments (n = 12) based on the one-way ANOVA. The numbers after  $\pm$  symbol are standard errors. The results of two-way analysis of variance on the effect of fertilizer (F), season (S), and their interactions (S × F) on pH, SOC, available N, available P, available K, and *nifH* gene abundance. \*\* significant at p < 0.01; ns no significant. CK: no N fertilizer + conventional water management; CON: conventional fertilization + conventional water management; B: application of 40 t ha<sup>-1</sup> biochar + conventional fertilization + conventional water management.

#### 3.2. nifH Gene Abundance

With the same treatment, *nifH* gene abundance increased in the second year (Figure 1). Compared with the conventional water management treatment, the FCK and FCON treatments increased *nifH* abundance by 15–41% and 17–93%, respectively. The FCK treatment resulted in higher abundance than the CK treatment. Compared with the CON treatment, biochar addition increased *nifH* gene abundance by 13–74% in the four-season rice cultivation. Compared with the CK and FCK treatments, the CON and FCON treatments reduced *nifH* abundance by 11–15% and 8.0–28%, respectively. With the second year of

the early rice season being an exception, *nifH* abundance significantly increased after the biochar treatment compared with the CON treatment. However, this *nifH* abundance was higher than that after the FCON treatment only in the first year and lower than that after the FCON treatment in the second year, which was not remarkably different from that after the CK treatment.



**Figure 1.** *nifH* gene abundance in soil after planting in different rice seasons. Capital letters indicate the difference between different treatments in the same rice season (p < 0.05), and lowercase letters indicate the difference between different rice seasons in the same treatment (p < 0.05) CK: no N fertilizer + conventional water management; CON: conventional fertilization + conventional water management; FCK: no N fertilizer + flooding; FCON: conventional fertilization + flooding; B: application of 40 t ha<sup>-1</sup> biochar + conventional fertilization + conventional water management.

Under the same water management treatment, N fertilizer application decreased soil *nifH* abundance, except for the early rice season in the first year. Regarding the average *nifH* abundance in the four seasons (Tables 3 and 4), both flooding and biochar treatment significantly increased *nifH* abundance. N fertilizer application, seasonal change, and water management all significantly affected *nifH* abundance under different water management practices (p < 0.05), but only season and water management treatment, different fertilization treatments and seasonal changes significantly affected *nifH* abundance (p < 0.05), but no significant interaction was observed between them (Table 4).

According to the obtained *nifH* abundance and measured soil properties, the correlation analysis was performed (Figure 2). *nifH* abundance exhibited no significant correlation with pH and the available K content, but was negatively correlated with available N and P contents (p < 0.05). By contrast, *nifH* abundance exhibited a significant positive correlation with SOC (p < 0.05).



**Figure 2.** Correlation analysis of *nifH* gene abundance with pH, SOC, available N, available P, and available K. \* Significant correlation (p < 0.05).

## 3.3. Effects of Different Treatments on Community Structure of nifH Gene-Related Microorganisms

According to the OTU classification, alpha diversity analysis was conducted. Table 5 presents each index. With rice planting, the ACE and Chao1 indices exhibited an increasing trend. Under the same fertilization condition, the ACE, Chao1, Shannon, and Simpson indices increased in the FCK and FCON treatments. Flooding increased the richness of the microbial community and species, but decreased community uniformity. Except for the early rice season in the first year, although the ACE and Chao1 indices increased after the biochar treatment compared with the CON treatment, the impact in the second year was significantly reduced (p < 0.05) compared with that in the first year. Biochar treatment only reduced the Simpson index in the first year, whereas it increased the same index in the second year, but not as much as the flooding treatment. Thus, the effects of biochar application on microbial diversity gradually decreased with rice planting and were not associated with flooding.

The abundance of *nifH*-related microorganisms in each treatment was determined through high-throughput sequencing. Other unclassified bacterial genera were excluded. *Geobacter*, *Pelomonas*, and *Azospirllum* were the main dominant bacterial genera (Figure 3). The relative abundance of *Geobacter* in rice in the first year of each treatment was lower than that in the second year. In the same rice season, biochar treatment increased the relative abundance of *Pelomonas* compared with the other treatments. Compared with the conventional water management treatment, the FCK and FCON treatments increased the relative abundance of *Azospirillum*, whereas they decreased the relative abundance of *Pelomonas* and *Geobacter* in the same water management treatment, the CON and FCON treatments increased the relative abundance of *Pelomonas* and *Geobacter* in the second year. With the same water management treatment, the CON and FCON treatments increased the relative abundance of *Melones* and *Melones* abundance of *Melones* and *Melones* abundance of *Melones* and *Melones* and *Melones* and *Melones* abundance of *Melones* and *Melones* and *Melones* and *Melones* and *Melones* abundance and *Melones* abundance and *Melones* abundance and *Melones* and *Melones* abundance and *Melones* and *Melones* abundance a

Season	Treatments	ACE	Chao1	Shannon	Simpson
	СК	694.3 $\pm$ 9.12 bc <sup>+</sup>	$701.9\pm6.28~\mathrm{c}$	$0.89\pm0.01~\mathrm{ab}$	$5.4\pm0.10~{ m b}$
	CON	$674.4\pm4.82~\mathrm{c}$	$670.2 \pm 5.83 \text{ d}$	$0.88\pm0.01~{ m b}$	$5.1\pm0.02~{ m c}$
Z19	FCK	$711.7\pm0.06~\mathrm{b}$	$725.3\pm1.50\mathrm{b}$	$0.91\pm0.01~\mathrm{a}$	$5.9\pm0.03$ a
	FCON	$752.9\pm10.08~\mathrm{a}$	$754.3 \pm 9.59$ a	$0.91\pm0.01~\mathrm{a}$	$5.8\pm0.15$ a
	В	$675.7\pm18.68~\mathrm{c}$	$684.3\pm18.32~cd$	$0.81\pm0.01~{\rm c}$	$4.4\pm0.12~d$
	СК	$757.8 \pm 17.1$ a	$770.2 \pm 16.81$ a	$0.89\pm0.01~{ m bc}$	$5.1\pm0.25~\mathrm{b}$
	CON	$697.6 \pm 14.58 \text{ c}$	$730.8 \pm 29.20 \text{ a}$	$0.89\pm0.01~{ m bc}$	$5.6\pm0.21~\mathrm{ab}$
W19	FCK	$727.8 \pm 7.15 \text{ b}$	$762.7 \pm 19.69$ a	$0.92\pm0.01~\mathrm{a}$	$6.0\pm0.11~\mathrm{a}$
	FCON	$700.3\pm1.52~\mathrm{c}$	$664.9 \pm 5.29 \mathrm{b}$	$0.92\pm0.02~\mathrm{ab}$	$5.8\pm0.09~\mathrm{ab}$
	В	$773.9\pm10.58~\mathrm{a}$	$766.2\pm2.02~\mathrm{a}$	$0.87\pm0.00~{\rm c}$	$5.5\pm0.19~\mathrm{ab}$
	СК	$803.2\pm14.13~\mathrm{a}$	$822.1\pm9.35b$	$0.84\pm0.01~\text{b}$	$5.4\pm0.03~\mathrm{ab}$
	CON	$686.5 \pm 7.41 \text{ d}$	$727.8 \pm 15.85 \text{ d}$	$0.86\pm0.02\mathrm{b}$	$5.2\pm0.08~\mathrm{b}$
Z20	FCK	$832.5 \pm 23.59$ a	$864.7 \pm 16.97$ a	$0.84\pm0.02~{ m b}$	$5.4\pm0.34~\mathrm{ab}$
	FCON	$718.5 \pm 12.48 \text{ c}$	$734.1 \pm 0.95 \text{ d}$	$0.90\pm0.01~\mathrm{a}$	$5.7\pm0.04$ a
	В	$766.8\pm0.99~b$	$786.2\pm11.00~\mathrm{c}$	$0.88\pm0.01~\mathrm{ab}$	$5.7\pm0.04~\mathrm{a}$
	СК	$778.3\pm21.4~\mathrm{bc}$	$794.5\pm29.40bc$	$0.84\pm0.01~\mathrm{a}$	$5.2\pm0.07b$
	CON	$746.0 \pm 18.54 \; \mathrm{d}$	$753.5 \pm 11.63 \text{ c}$	$0.85\pm0.03~\mathrm{a}$	$4.8\pm0.03~{ m c}$
W20	FCK	$856.9 \pm 7.16$ a	$862.0 \pm 0.23$ a	$0.88\pm0.01~\mathrm{a}$	$5.6\pm0.02~\mathrm{a}$
	FCON	$817.9 \pm 47.01 \text{ ab}$	$832.8\pm43.07~\mathrm{ab}$	$0.87\pm0.01~\mathrm{a}$	$5.7\pm0.16$ a
	В	$787.2 \pm 3.65  \mathrm{bc}$	$808.7\pm4.76~\mathrm{abc}$	$0.84\pm0.01~\mathrm{a}$	$5.2\pm0.05\mathrm{b}$

Table 5.  $\alpha$  diversity of soil microorganisms in different treatments after the end of different rice seasons.

<sup>†</sup> Different letters indicate significant difference between different treatments in the same rice season (n = 3; p < 0.05). The numbers after  $\pm$  symbol are standard errors. Z19, W19, Z20, W20, respectively, denote the early rice season of 2019, the late rice season of 2019, the early rice season of 2020, and the late rice season of 2020. CK: no N fertilizer + conventional water management; CON: conventional fertilization + conventional water management; FCK: no N fertilizer + flooding; FCON: conventional fertilization + flooding; B: application of 40 t ha<sup>-1</sup> biochar + conventional fertilization + conventional mater management.



**Figure 3.** Horizontal abundance of each treatment genus after the end of different rice seasons. (**a**–**d**) represent early rice season in 2019, late rice season in 2019, early rice season in 2020, and late rice season in 2020, respectively. CK: no N fertilizer + conventional water management; CON: conventional fertilization + conventional water management; FCK: no N fertilizer + flooding; FCON: conventional fertilization + flooding; B: application of 40 t ha<sup>-1</sup> biochar + conventional fertilization + conventional water management.

According to the RDA results (Figure 4), biochar application mainly affected soil pH and the SOC content. Biochar mainly affected bacteria belonging to *Pelomonas*. *Pelomonas* was positively correlated with pH, which indicated that biochar mainly affected the relative abundance of *Pelomonas* by increasing soil pH (Figure 4a). However, *Pelomonas* was significantly and negatively correlated with pH, while *Azospirllum* exhibited a negative correlation with the available P content. Flooding affected the diazotrophic microbial composition mainly by changing soil pH and available N and P contents (Figure 4b). *Geobacter* negatively correlated with the available N content in all treatments. This indicated that the available N content was the main factor influencing *Geobacter*. *Pelomonas* was positively correlated with the available P content.



**Figure 4.** RDA analysis of population composition of diazotrophic microorganisms and soil properties factors. (**a**,**b**) represent biochar and flooded RDA analysis, respectively.

## 4. Discussion

# 4.1. Effects of Flooding and Biochar Amendment on nifH Gene Abundance

In topsoil, *nifH* abundance was positively proportional to the N fixation rate [25,47], so *nifH* abundance can serve as a relevant index of soil activity. In this study, *nifH* abundance in the early rice season was lower than that in the late rice season (Figure 1). *nifH* abundance may increase because of an increase in temperature and precipitation in the late rice season. BNF activity in soil changes with temperature and humidity [53]. In the present study, low temperature in the early rice season easily inhibits nitrogenase activity and microbial metabolism. This is possibly why *nifH* abundance in the early rice season, the field was prone to insufficient irrigation water, which affected the microbial community structure of rice soil [54]. Moreover, compared with conventional irrigation measures, flooding significantly increased *nifH* abundance and microbial alpha diversity. This also suggests that soil flooding in paddy fields affects the soil diazotrophic microbial activity and community structure [55].

The SOC content is among the primary factors affecting the growth of soil microorganisms. BNF in soil is often affected by the SOC content. The SOC content may affect bacterial *nifH* abundance and increase BNF in soil [56]. A significant positive correlation was also observed between SOC and *nifH* abundance in this study (Figure 2). In addition, the input of N and P affected the abundance of soil diazotrophic microorganisms and *nifH* genes. Long-term application of the chemical fertilizer in paddy soil reduced soil nitrogenase activity [57], with an increase in the N application rate and a decrease in the soil N<sub>2</sub> fixation rate [48]. This may be due to the sensitivity of *nifH*-encoded nitrogenase to N input [25]. Similar results were obtained in the present study as N fertilizer application reduced *nifH*  abundance. According to previous studies, N input reduced N fixation activity and *nifH* abundance, whereas P input exerted a positive effect [58]. P deficiency inhibited *nifH* gene expression and decreased N fixation activity in the paddy field soil [37]. In this study, the soil available P content was inversely proportional to *nifH* abundance (Figure 2), which is in disagreement with the results of other studies [37], which may due to the high fertilizer application rate and higher P residues in the tropical field. Thus, *nifH* abundance was inhibited [59]. These results suggest that reducing P application in this cropping system is beneficial for BNF.

Furthermore, as a microbial habitat, biochar boosts microbial activity [60]. However, in this study, the impact of biochar treatment on the *nifH* gene in the second year was significantly lower than that of the flooding treatment. This can be attributable to the decreased C availability after biochar aging, which made it challenging for bacteria to decompose and utilize biochar [61]. However, C availability often affects the ability of microbes to fix N [62], and so, the effect of second-year C in this study was far lower than that of the flooding treatment. Additionally, biochar aging was related to the decreased impact on the amended soil environment [63]. Consequently, the biochar treatment only reduced the available N content in the early rice season in 2019, which decreased the effect of biochar on *nifH* gene stimulation in the following year.

#### 4.2. Effects of Flooding and Biochar on the Community Structure of Diazotrophic Microorganisms

Long-term N fertilizer application affects the soil diazotrophic microbial community structure [34]. In this study, fertilization decreased the abundance of the soil microbial community and ACE, Chao1, and Shannon indices of the diazotrophic microbial community. This indicated that the N fertilizer applied decreased the diversity of the diazotrophic microbial community [64]. This was mainly because of the increase in the soil available N content caused by the applied N fertilizer, which reduced the diazotrophic microbial diversity [65].

Biochar addition previously affected the diazotrophic microbial community structure in alkaline soil [40]. Compared with the CON treatment, although the biochar treatment increased the diazotrophic microbial community diversity, the increase was less than that resulting from the flooding treatment in the second year. This may be due to certain SOC present in the first-year biochar, which induces an increase in *nifH* gene abundance [66]. After aging, the SOC in the second-year biochar decreased [67], thereby reducing the impact on the amended soil environment [63] and thus decreasing the effect in the second year.

Different water management treatments could also affect the soil microbial community structure and function [30,68]. In this study, flooding increased the diazotrophic microbial community diversity (Table 5). This may be related to flooding-induced reduction in soil pH and the available P content. In the second year [33], *nifH* abundance after the biochar treatment was lower than that after the FCON. The flooding treatment increased the abundance of diazotrophic microbial communities in all planting years. This suggests that the effect of aging biochar on microbes may be far less than that of flooding.

Consistent with previous study results, Proteobacteria was the main diazotrophic bacteria family in the paddy soil [15,19]. Although diazotrophic bacteria have been extensively studied, the effects of biochar application and different water management treatments on diazotrophic bacteria in tropical regions are less known. Most current studies have believed that changes in soil properties can affect the composition of soil diazotrophic microbial communities [64,65]. However, diazotrophic microbial communities respond differently to environmental factors in different soil types [18].

In the present study, *Geobacter*, *Pelomonas*, and *Azospirllum* were the main bacterial genera involved in N fixation in paddy soil [19]. According to the RDA analysis results (Figure 4), the effects of soil properties on different diazotrophic bacterial genera varied. *Geobacter* was among the main dominant bacterial genera and was significantly negatively correlated with available N. Soil N enrichment often inhibits the growth of diazotrophic bacteria [69]. A similar conclusion was drawn in the present study, as the soil available N content decreased gradually with rice planting, whereas *Geobacter* abundance increased

gradually. However, some studies have reported that *Geobacter* grows well at a neutral pH [70]. In this study, pH had little effect on *Geobacter* growth, especially in biochar-treated soils. This indicates that pH is not a major factor influencing *Geobacter* in tropical paddy field soils.

Pelomonas is also a common diazotrophic genus [71]. It was also among the dominant bacterial genera in this study. According to previous studies, *Pelomonas* is positively correlated with pH [13], which is similar to the results of biochar treatment in our study. Biochar addition increased soil pH, which stimulated the relative abundance of Pelomonas and increased *nifH* gene abundance. However, *Pelomonas* exhibited a negative correlation with soil pH during the flooding treatment, which led to slight pH change between different water management treatments (Table 2). This indicated that diazotrophic microbial communities responded differently to environmental factors under different tillage practices [72]. In the present study, the response of *Pelomonas* to environmental factors varied between the flooding and biochar treatments (Figure 2). In the flooding treatment, Pelomonas was negatively correlated with pH. On the other hand, biochar treatment increased the relative abundance of *Pelomonas*, which was primarily due to soil pH. The contrasting results are attributable to the impact of soil changes on the symbiotic pattern of diazotrophic microorganisms, which prompt them to adapt to the altered environment [73]. The flooding treatment may have had a slight effect on soil pH, so soil pH is not a major factor influencing Pelomonas in the flooding treatment. The available P content decreased, and Pelomonas was positively correlated with the available P content in the flooding treatment. Therefore, flooding treatment can reduce Pelomonas abundance by reducing available P.

*Azospirllum* is widely found in paddy soil [74,75]. It is often believed to dissolve phosphate in soil and promote phosphorus uptake by crops [76]. In this study, *Azospirllum* was among the *Azospirllum* species affected by flooding, and a negative correlation was observed between *Azospirllum* and the available P content. This indicates that flooding stimulated *Azospirllum*, which accelerated P dissolution. The dissolved P was absorbed by crops, which reduced the soil available P content in the flooding treatment. However, the correlation between *Azospirllum* and available P was not significant, which suggested that other diazotrophic genera such as *Geobacter* also participate in the P cycle [77]. Additionally, studies have shown that *Azospirllum* promotes rice growth and increases rice yield [78]. At the same time, it also participates in the soil N cycle [79], which suggests that flooding contributes to rice growth and yield.

## 5. Conclusions

In conclusion, both flooding and biochar addition affected the diazotrophic microbial community in the paddy soil and increased *nifH* gene abundance. However, the effect of biochar decreased in the second year. These results revealed that the effects of flooding and biochar on *nifH* abundance in tropical soils were mainly related to SOC and available N and P contents. Additionally, flooding and biochar increased N fixation in the paddy soil by stimulating the relative abundance of *Azospirllum* and *Pelomonas*, respectively. Flooding treatment may promote crop yield and P absorption by stimulating *Azospirllum*. Tests specifically examining the effects of biochar were conducted under conventional water management conditions. Unfortunately, the application of the same treatment under flooded conditions caused damage, attributed to human factors.

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