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Temporal Response of Bacterial Community Associated Fe(III) Reduction to Initial pH Shift of Paddy Soils

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Abstract: The temporal response of bacterial community, especially that of bacteria with Fe(III) reducing ability, in flooded paddy soils to initial pH changes, is not well-documented. This work demonstrated variations in concentration of Fe species, bacterial activity and community succession in paddy soils with initial pH shift to acidic or alkaline level. The causal links of pH shift-induced bacterial community succession with Fe(III) reduction was also assessed. Results showed that soil initial pH shifts greatly influenced bacterial community and Fe(III) reduction. A soil pH shift from acidic to alkaline level enhanced bacterial abundance and dehydrogenase activity (DHA), which accordingly caused an increase in Fe(III) reducing ratio by 22.26% on day One of flooding. The stimulated putative Fe(III) reducing species, *Bacillus* and *Solibacillus*, caused stimulation of Fe(III) reduction with pH increase. However, there was continuous inhibition of Fe(III) reduction with a pH shift from alkaline to acidic, with Fe(III) reducing ratios decreased by 11.98–40.04%. The inhibited DHA and Fe(III) reducing bacteria were amenable for the suspension of Fe(III) reduction. This study suggests that bacterial activity and Fe(III) concentration, in responses to initial soil pH shift, are primarily responsible for pH shift-induced Fe(III) reduction in paddy soils.

Keywords: bacterial abundance and activity; bacterial community structure; iron(III) reduction; paddy soil; soil initial pH shift

Citation: Jia, R.; Fan, F.; Li, L.; Qu, D. Temporal Response of Bacterial Community Associated Fe(III) Reduction to Initial pH Shift of Paddy Soils. *Agronomy* **2022**, *12*, 1304. https://doi.org/10.3390/agronomy12061304

Academic Editor: Essaid Ait Barka

Received: 13 April 2022
Accepted: 26 May 2022
Published: 29 May 2022

1. Introduction

Paddy fields with intermittent, but cyclical, submergence and drainage are characterized by both terrestrial and aquatic ecosystems, which favor oxidation-reduction of multivalent metals and cycling of nutrient elements under alternating oxic and anoxic conditions [1]. The soil microbial community is often recognized as a critical factor in regulating biogeochemical cycles of carbon, nitrogen, sulfur, phosphorous, iron, and arsenic, as well as other metals, in paddy soils [2–4]. Microorganisms involved in such soil redox cycles are functionally diverse and interact closely [5–8]. Thus, it is essential to understand the diversity and composition of microbial communities in paddy fields so as to predict their ecological functions in global biogeochemical cycles.

Fe cycling is of significant environmental importance in bioavailability of nutrient elements, degradation of organic pollutants and remediation of heavy metals in paddy fields [5,9,10]. Microbial-mediated Fe(III) reduction is attributed to microbial respiration or fermentation, coupled with the oxidation of hydrogen (H_2) and carbon sources [11,12]. In submerged paddy fields, the obligative Fe(III) reducers generate energy during Fe(III) respiration, while the fermentative reducers mainly co-metabolize with bacteria decomposing soil organic matter [13]. Bacteria participating in organic matter fermentation are also crucial to Fe(III) reduction, because the intermediate metabolites can provide electron

donors or shuttles for Fe(III) reduction [14]. Therefore, a comprehensive investigation of the bacteria associated with Fe(III) reduction is needed to better understand Fe cycling in paddy fields.

In paddy soil, not only exogenous input by agronomic practices, but also endogenous microbial activity under seasonal submergence and drainage alternations might result in a wide range of pH changes. To achieve efficient saline-sodic amelioration, contaminant remediation and fertility improvement, exogenous amendments (like rice straw, aluminum sulfate, lime, biochar and inorganic fertilizer) are often applied to soils [15–17]. It is noteworthy that such amendments are generally thought to influence soil pH, either directly or indirectly. Soil endogenous microbial fermentation can result in temporary pH decrease, due to the accumulation of organic acid and carbon dioxide in flooded neutral or alkaline paddy fields [18,19]. On the other hand, in submerged acidic paddy soil more bio-available Fe minerals are beneficial for biotic Fe(III)/Mn(V) reduction, which causes the consumption of H^+ and, thereby, increases soil pH [18,20].

It has been universally acknowledged that soil pH is the key factor shaping microbial community composition and, further to this, influencing their associated biological functions [21–23]. A study of Whittleston et al. indicated that enhanced Fe(III) reduction in hyperalkaline soil, by pH amendment with bicarbonate to strongly alkaline, was probably associated with an improved contribution of bacteria in phylum *Firmicutes* [24]. It has also been suggested that the well-characterized model FeRB *Geobacteraceae* is more abundant at pH 5.3 in acid mining sediment than at pH 3 [25]. Our previous study confirmed that initial pH adjustment induced variation in dehydrogenase and *Clostridium*-associated hydrogen production during the flooding of paddy soil and accounted for pH-modulated Fe(III) reduction [26,27]. However, temporal responses of the abundance and composition of the bacterial community to pH changes with the duration of flooding have not been well-documented, especially as regards the specific bacterial genera having putative Fe(III) reducing abilities. Although a few studies have reported the relative abundance of dominant species associated with greenhouse gas emission responding to soil acidification or alkalization [28], this may not be sufficient without information on the absolute abundance of the microbial community when comparing across multiple pH levels [29,30].

Herein, paddy soils with initial pH adjustment to acidic or alkaline levels were anaerobically incubated, to explore the effect of soil acidification or alkalization on bacterial community abundance, activity and composition during flooding of soils, by a combination of high-throughput sequencing and quantitative polymerase chain reaction (qPCR). Additionally, the successions of putative FeRB, as well as associated Fe(III) reduction processes, in flooded paddy soils under different initial pH levels were also identified to better understand the Fe dynamics in flooded paddy fields. Results of this study are expected to aid in understanding the causal links between pH-shift-mediated bacterial communities and metabolic functional trails, and to provide an essential insight into the ecological and agricultural significance of soil pH fluctuation.

2. Materials and Methods

2.1. Soil Sampling

Two paddy soils with contrasting pH values were taken from drained post-harvest paddy fields in Nanchang County, Jiangxi Province (NC; latitude: 28°19′48″ N, longitude: 115°33′36″ E) and Baodi district, Tianjin Municipality (BD; latitude: 39°38′24″ N, longitude: 117°33′36″ E). The NC soil, with initial pH of 5.2, was an Fe-accumili-Stagnic Anthrosol, while the BD soil, with initial pH value of 7.9, was an Hapli-Stagnic Anthrosol. The basic physicochemical properties of the tested soils are listed in Table S1, and partial characteristics have been referred to in our previous study [27].

2.2. Treatments and Anaerobic Flooding

The experimental design was based on a control treatment and a soil acidification or alkalization treatment for each paddy soil. For the control treatments, 5.00 g of NC (NCCK) or

BD (BDCK) soils were saturated with 5 mL distilled water in 10-mL serum bottles, respectively. For alkalization treatments, 5 mL sodium carbonate solution (45 mmol/L) was substituted for distilled water to adjust the initial pH of NC soil to approximately 7.9 (NCNa) [26,31]. Likewise, 5 mL aluminum sulfate solution (50 mmol/L) was substituted to adjust the initial pH of BD soil to approximately 5.2 to simulate soil acidification (BDAl) [26,32]. There were 60 serum bottles conducted for each CK and pH-adjusted treatment. After being bubbled with nitrogen gas, the serum bottles were sealed with rubber stoppers and aluminum caps to make anaerobic conditions. Subsequently, the serum bottles were subjected to culture in darkness at 30 °C.

2.3. Chemical Analysis

On days 0, 1, 3, 5, 7, 10, 13, 16, 20, 25, 30, 35, and 40 of anaerobic flooding, serum bottles from each treatment were randomly selected and withdrawn in triplicate for determination of DHA by the 2,3,5-triphenyl tetrazolium chloride (TTC) colorimetric method [26]. Contents of acid-dissolved Fe(II) and total Fe were measured in triplicate on days 1, 5, 10, 20 and 40 of flooding by the o-phenanthroline colorimetric method, after extracting 0.5 mL soil slurries with 0.5 M hydrochloric acid [33]. The content of acid-dissolved Fe(III) was calculated from the difference between acid-dissolved total Fe and Fe(II). The Fe(III) reducing ratio was obtained by calculating the ratio of acid-dissolved Fe(II) to total Fe.

2.4. DNA Extraction and Bacterial Community Analysis

During anaerobic flooding, another three bottles for each treatment were also withdrawn in triplicate on days 1, 5, 10 and 20 to extract soil total DNA for analysis of bacterial community abundance and structure. The sampled soil slurries were primarily centrifuged at 2870× *g* for 15 min at 4 °C to discard the supernatant. Then the deposit soil was subjected to DNA extraction with the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA).

The absolute bacterial abundance was quantitated with primers 515F (5′-GTGCCAGC -MGCCGCGG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) using the StepOnePlusTM Real-Time PCR system (Applied Biosystems, Foster, CA, USA). Each 25-μL qPCR reaction mixture contained 0.5 μL of each primer, 1 μL of DNA template, 12.5 μL of SYBR Premix Ex TaqTM (TaKaRa Bio, Otsu, Japan), 0.5 μL of ROX Reference Dye (TaKaRa Bio, Otsu, Japan) and 10 μL of sterile ddH_2O. The qPCR consisted of an initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 45 s and a final extension at 72 °C for 45 s.

For bacterial community structure analysis, the genomic DNA was amplified with universal primers 515F and 907R targeting the hypervariable V4-V5 region. The purified amplicons were then subjected to sequencing on an Illumina MiSeq platform by Majorbio Science and Technology Ltd. (Shanghai, China). With a cutoff of 97% similarity, the valid sequences were clustered into operational taxonomic units (OTUs), whose representative sequence was selected by RDP Classifier at 80% confidence [34]. In the tested NC and BD soils in the present study, over 95% of the OTUs were classified to taxonomic group at phylum level and the rarefaction curves are shown in Supplementary Materials Figure S1. Representative OTU sequences, related to known FeRB at 97% identity, were selected and defined as putative FeRB, according to references and sequences published in NCBI (Table S2). Additionally, the absolute abundance of putative FeRB was estimated by multiplying the absolute bacterial abundance and relative abundance of putative FeRB in consideration of the same primers used in qPCR and amplicon sequencing [30,35].

2.5. Statistical Analysis

To compare bacterial community structures across all samples, principal component analysis (PCA) was processed with ggplot2 in R 3.1.2 according to the UniFrac distances using the relative abundances of OTUs. A correlation test proceeded with the mixed effect liner modeling using the "lmerTest" package in R to elucidate the general relationship

between Fe(III) reduction and associated influencing factors. Fe(III) accumulative concentration and the Fe(III) reducing ratio were taken as response variables, while real-time soil pH and partial pressure of hydrogen (referred to in reference [27]), DHA, Fe(III) concentration, and abundance of putative FeRB were taken as explanatory variables. Structural equation modeling embedding of mixed effect liner modeling was achieved using the "piecewiseSEM" package in R to evaluate the contribution of pH shift-induced microbial hydrogen production and FeRB to Fe(II) accumulation. Redundancy analysis (RDA), using the "rdacca.hp" package in R, was employed to identity the relative supremacy of pH shift-induced bacterial community, putative FeRB abundance and Fe(III) concentration to Fe(III) reduction [36].

3. Results

3.1. Bacterial Abundance and Activity

Changes in bacterial 16s rDNA copies (Figure 1A,B) and DHA (Figure 1C,D) were determined to reflect bacterial abundance and activity. As shown in Figure 1, bacterial abundance in both the original and pH adjusted paddy soils decreased by degrees along with flooding time. Bacterial activity in terms of DHA varied significantly with flooding time ($p < 0.05$ in NC soil and $p < 0.001$ in BD soil), which increased to reach a maximum over 10–20 days of flooding, and then gradually declined or remained stable. When modulating the initial pH of acidic NC soil to alkaline, remarkable increases in bacterial abundance, by 1.27×10^7~9.86×10^7 copies g^{-1} soil, were observed during the first 10 days of flooding ($p < 0.05$). An increase in DHA was also detected throughout the flooding of NC soil with initial pH shift from acidic to alkaline ($p < 0.001$). These results indicated stimulative effects of soil initial pH increase on bacterial growth. Whereas initial pH shift of BD soil to acidic significantly decreased bacterial abundance from 5.20×10^7~1.09×10^8 copies g^{-1} soil to 2.60×10^6~4.20×10^6 copies g^{-1} soil ($p < 0.01$). Accordingly, DHA in BD soil prominently declined by 5.01~53.82 μg·(ml·min)$^{-1}$ after being incubated for 3 days ($p < 0.001$). These results suggested that bacterial growth was significantly inhibited in response to paddy soil acidification.

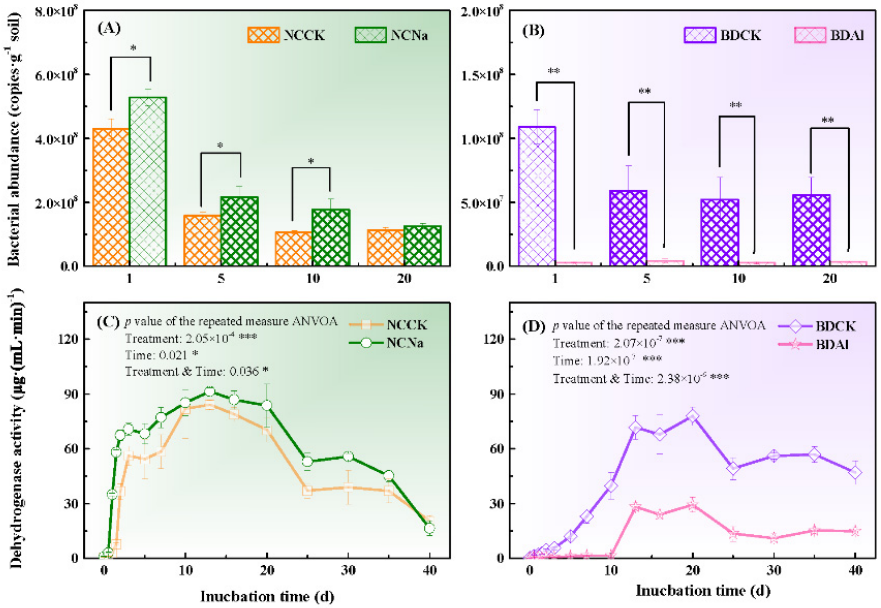

Figure 1. Variation in bacterial abundance (**A**,**B**) and dehydrogenase activity (**C**,**D**) during flooding of paddy soils with different initial pH levels (NCCK, the original NC soil without pH adjustment; NCNa, NC soil with pH adjustment to alkaline level; BDCK, the original BD soil without pH adjustment; BDAl, BD soil with pH adjustment to acidic level. Numbers following the treatments represented the flooding time. *, indicates significant difference at $p < 0.05$. **, indicates significant difference at $p < 0.01$. ***, indicates significant difference at $p < 0.001$. The same below.).

3.2. Bacterial Community Structure and Similarity

The relative abundance of the different phyla at identical flooding times varied considerably with alkalization of NC soil and acidification of BD soil (Figure 2A,B). *Firmicutes* was the most abundant phylum in both original NC and BD soils at certain flooding times, accounting for 32.6-88.1% and 25.2.7–70.0% of the total valid reads, respectively. *Proteobacteria*, *Acidobacteria*, *Chlorobi*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria* and *Amatimonadetes* were minor groups that showed successional characteristics during the flooding. However, when modulating the initial pH of NC soil from acidic to alkaline, growth of bacteria in *Firmicutes* was stimulated, while the bacteria in the minor groups were inhibited after flooding for 5 days, with increases in relative abundance of *Firmicutes* by 13.9–24.9% and decreases in the minor groups by 0.1–12.32%. In the BDAl treatment, soil acidification led to decreases in relative abundance of *Firmicutes* by 15.02% and 9.70% on days 1 and 5 of flooding, and increases of 25.17% and 15.55% on days 10 and 20, respectively. In comparison with the BDCK treatment, *Proteobacteria* increased by 3.11–25.32% with the initial pH decrease, with a relatively rapid increase on day 5 of flooding. In contrast, the relative abundances of *Bacteroidetes* and *Acidobacteria* after flooding for 5 days decreased by 4.82–14.54% and 3.65–5.47%, respectively.

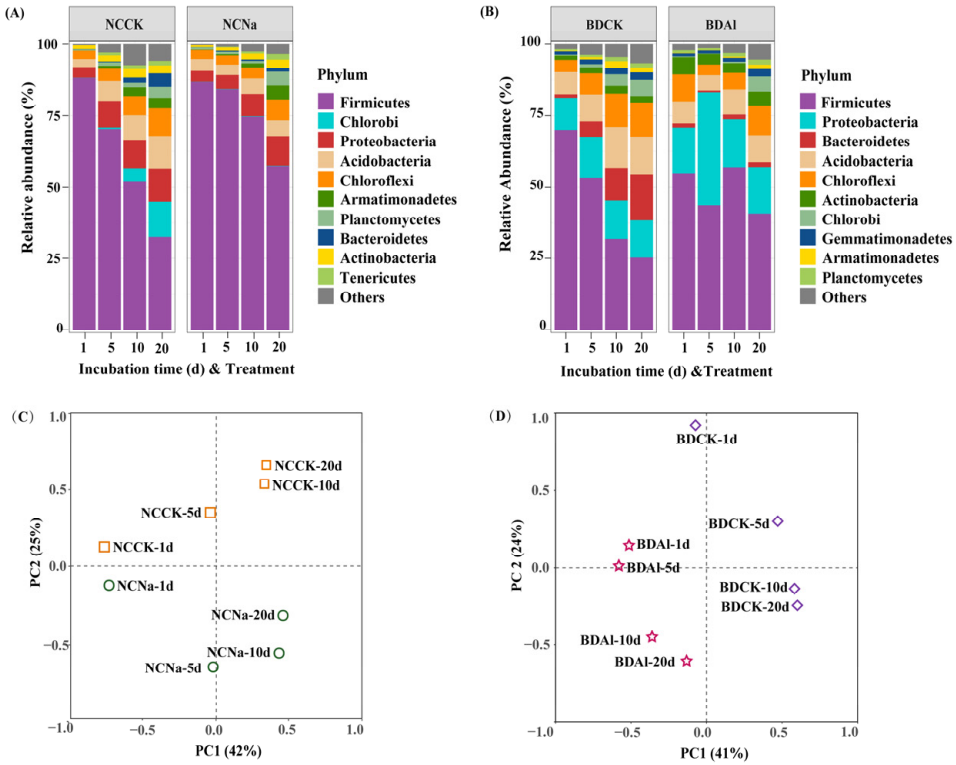

Figure 2. Relative abundance of dominant bacterial phyla (**A**,**B**) and principal component analysis plot (**C**,**D**) based on the UniFrac distances using the relative abundances of OTUs in paddy soils with acidification or alkalization.

Similarities in bacterial community composition were demonstrated by PCA, in which PCA1 and PCA2 totally explained 67% of variance in NC soil and 65% in BD soil, respectively (Figure 2C,D). In NC soil, three distinguishable groups, associated with 1 day, 5 days, and 10/20 days, were clustered, which implied that bacterial community significantly succeeds with flooding time. Samples in the NCCK treatment (except samples flooded for 1 day) clustered far away from that in the NCNa treatment at specific flooding times, revealing that initial pH shift influenced the bacterial community composition of NC soil after being flooded for 5 days. Distinct community structures were also observed between BDCK and BDAl at each sampling time because of the initial pH modulation. Samples in

BDCK clustered as two distinct groups (1/5 days, and 10/20 days), while samples in BDAl were split between day 1, which was far away from those on days 5, 10 and 20. Nonetheless, there could have been bacterial community succession over the flooding time, but the pH shift was apparently more effective in influencing the bacterial community composition in BD soil.

3.3. Fe(III) Reduction Process and Putative Fe(III) Reducing Bacteria

As Fe(II) gradually accumulated with flooding time, the Fe(III) content declined in both NC and BD soils with, or without, initial pH shift (Figure 3A). The abundance of putative FeRB also showed a decreasing trend, along with decrease in Fe(III) as electron acceptor (Figure 3B). Most of the putative FeRB were allocated to genera *Bacillus* and *Clostridium*, with estimated absolute abundances of 4.53×10^5~9.45×10^8 copies and 1.98×10^4~2.48×10^8 copies g^{-1} soil, respectively. Some Fe(III) reducing genera, like *Solibacillus*, *Pseudomonas* and *Geobacter*, were also observed. In the families of well-known Fe(III) reducers, *Anaeromyxobacter* was detected at very low abundance (relative abundance < 0.21%), while *Shewanella* and *Pelobacter* were not detected.

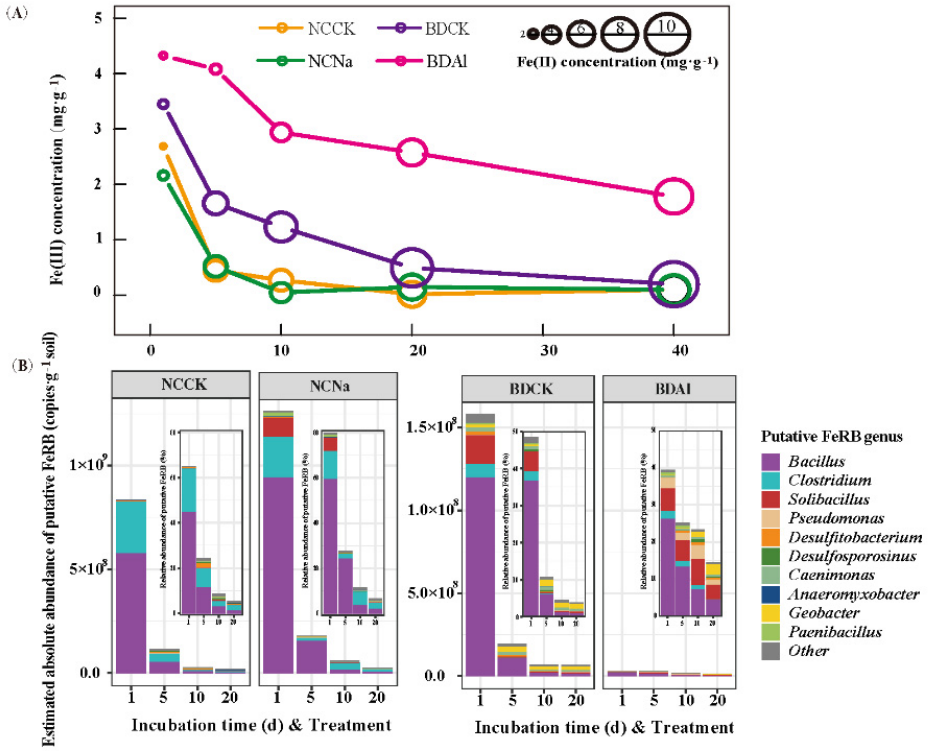

Figure 3. Variation in content of Fe(III) and Fe(II) (**A**) and the estimated abundance of putative Fe(III) reducing bacteria (**B**) during flooding of paddy soils with acidification or alkalization. The area of circles was the content of Fe(II) in soils.

When pH of NC soil was adjusted to alkaline, Fe(III) reduction was stimulated, mainly on day 1 of flooding. This was shown by the significant increase in Fe(II) by 1.02 $mg \cdot g^{-1}$ and the decrease in Fe(III) by 0.53 $mg \cdot g^{-1}$. Accordingly, there were obvious increases in the estimated absolute abundance of putative FeRB (particularly putative FeRB in genera *Bacillus* and *Solibacillus*) on day 1 of flooding. During the subsequent flooding, no significant difference was detected in the Fe(III) reducing ratio between the NCCK and NCNa treatments (Table 1). However, the pH shift of alkaline BD soil to acidic resulted in significant inhibition in Fe(III) reducing ratio throughout the 40 day flooding period. During days 1–5 of flooding, a decrease in Fe(II) accumulation by 2.49 $mg \cdot g^{-1}$ upon the initial soil pH shift was associated with a decrease in Fe(III) reducing ratio by 40%. This

was in accordance with a decrease in the estimated absolute abundance of putative FeRB by 1.60×10^7~1.55×10^8 copies·g^{-1} soil, especially putative FeRB in genera *Bacillus*.

Table 1. The Fe(III) reducing ratio during anaerobic incubation of paddy soils with acidification or alkalization.

Treatments	Day 1	Day 5	Day 10	Day 20
NCCK	27.09% ± 2.65% b	90.47% ± 0.61% a	94.54% ± 0.65% a	97.57% ± 1.90% a
NCNa	49.35% ± 1.88% a	90.66% ± 1.86% a	97.04% ± 0.45% a	97.72% ± 0.29% a
BDCK	36.92% ± 1.25% a	76.72% ± 2.96% a	85.47% ± 2.84% a	95.16% ± 2.69% a
BDAl	24.94% ± 0.73% b	36.68% ± 1.75% b	59.76% ± 1.20% b	71.56% ± 0.55% b

Note: Means followed by different letter (a, b) within the same column of a certain soil indicate significant difference at $p < 0.05$. NCCK, the original NC soil without pH adjustment; NCNa, NC soil with pH adjustment to alkaline level; BDCK, the original BD soil without pH adjustment; BDAl, BD soil with pH adjustment to acidic level.

3.4. Contribution of pH Shift-Induced Bacterial Community, Fe(III) Concentration and Biohydrogen to Fe(III) Reduction

As mentioned above, and in our previous study, variation in bacterial abundance and community, Fe concentrations, hydrogen production and soil pH [27] over the incubation time responded differently to initial pH shift in different paddy soils. The correlation test, based on mixed linear modeling (Figure 4), suggested that there were significantly positive effects of DHA and soil pH on Fe(II) accumulation and Fe(III) reducing ratio. Furthermore, partial pressure of hydrogen, Fe(III) concentration and abundance of putative FeRB, were significantly negatively related to both Fe(II) accumulation and Fe(III) reducing ratio. Pathway modeling (Figure 5A) also showed that the direct contribution of soil pH to Fe(II) accumulation was not significant. However, initial pH shift-induced bacterial activity (in terms of DHA) positively contributed to Fe(III) reduction (Figure 5A,B). Hydrogen production, which was directly influenced by soil pH and indirectly by pH shift-induced dehydrogenation, positively proceeded Fe(III) reduction. pH shift-induced Fe(III) concentration, which negatively correlated with soil pH, influenced FeRB and further positively and indirectly proceeded Fe(II) accumulation.

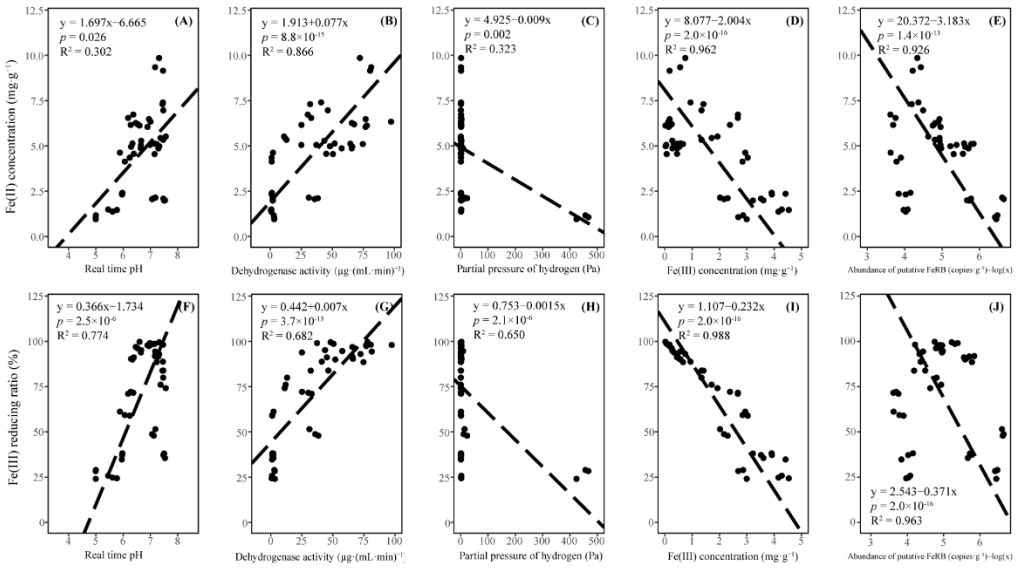

Figure 4. Correlation between real time pH, dehydrogenase activity, partial pressure of hydrogen, Fe(III) concentration and abundance of putative Fe(III) reducing bacteria (FeRB) and Fe(II) concentration (**A**–**E**), as well as Fe(III) reducing ratio (**F**–**J**).

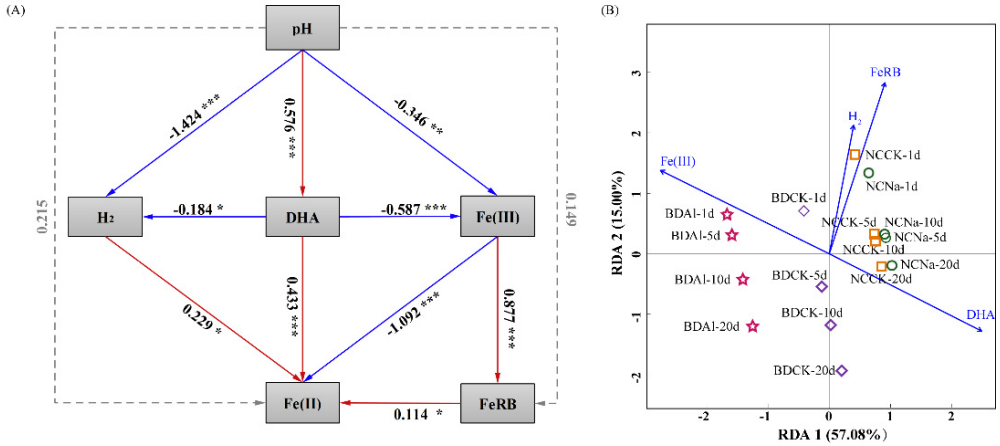

Figure 5. Structural equation modelling analysis embedding of mixed effect liner modelling (**A**) and redundancy analysis (RDA) triplot (**B**) to investigate the relationship between pH shift-mediated Fe(III) reduction and associated fermentative processes as well as succession of putative Fe(III) reducing bacteria (FeRB). (DHA, dehydrogenase activity; H_2, partial pressure of hydrogen; Putative FeRB, the estimated abundance of putative Fe(III) reducing bacteria; Fe(II) and Fe(III), concentration of Fe(II) and Fe(III) respectively).

4. Discussion

4.1. Bacterial Community Succession in Response to Soil Acidification or Alkalization

There was a consensus that microbial community assembly in soil was collectively governed by stochastic and deterministic processes. In the paddy ecosystem, the contribution of the stochastic process predominates in long-term successional eras [37], while determinism, relating to soil fertility and redox conditions, is stronger at shorter timescales [37,38]. Feng et al. also revealed that the initial state and changes of soil organic matter were the main environmental factors influencing the relative importance of stochastic and deterministic processes in microbial community assembly [39]. As paddy fields flood, the gradual decomposition of organic matter is accompanied by decrease of soil Eh and a series of reductive processes [1]. Bacteria succeeded considerably in the present study following the deterministic theory: *Firmicutes* with high growth rate for reproduction dominated in the early succession under abundant nutrient conditions; later, successional groups (including *Chlorobi, Proteobacteria, Acidobacteria, Bacteroidetes* and *Chloroflexi*) gradually replaced *Firmicutes* as consumption of nutrients, along with duration of flooding time, resulted in equilibrium species with greater adaptive capacities [38,40].

It is generally accepted that environmental changes (like pH, temperature, fertilizer and biochar) at multiple scales influence bacterial community dynamics in soils [21,23,27,41]. Similar studies, referring to microbial response to the stress of pH changes, have investigated in different ecosystems. Wu et al. indicated that less abundant bacteria, including Nitrospirae, γ- and δ-Proteobacteria, were closely related to pH gradient ($p < 0.001$) in vegetable soil [42]. Heděnec et al. reported that relative abundance of oligotrophs increased significantly with increasing pH gradient during a 60-day incubation, while that of copiotrophs significantly decreased with elevated pH gradient [28]. Unlike the above-mentioned studies, Firmicutes, as the abundant phylum in the present study, was stimulated throughout the flooding period in response to soil initial pH increase, which was particularly evident in the early stage of incubation, coinciding with an inhibition of later successional groups. This agreed well with a study by Jiao et al., who proposed that the abundant sub-community was predominantly mediated by the pH of agricultural soils [21]. It was indicated that soil pH exerted a stronger influence on the community assembly than successional time [43]. Especially during the early stage of soil incubation, pH changes, as deterministic factors, predominantly drive the assembly of the microbial community while stochastic processes tend to be more dominant during later incubation [28]. Grybos et al. showed that increasing

pH could improve the solubility of organic matter under reducing conditions [44]. This was why *Firmicutes* became more competitive, and contributed to increased soil carbon mineralization, with increased initial soil pH.

Moreover, studies have indicated that bacterial diversity in response to soil acidification is mainly driven by ecological filtering and evolution/dispersal [45]. Heděnec et al. further suggested that soil with more acidic pH leaned towards decreasing OTUs and phylogenetic overdispersion [28]. With the initial pH modulation from alkaline to acidic, the notable inhibition of bacterial growth in BD soil showed that bacteria could not adapt to the abrupt acidic stress. At phylum level, it is particularly visible that the bacterial community structure was disturbed in the BDAl treatment, in comparison with that in the original BD soil. This implied the disturbance of the inherent community succession while there was establishment of a new succession. Different from many previous studies, bacteria in the *Acidobacteria* phylum were not stimulated in response to such initial pH decrease, which was due to the stringent limits on survival and fitness of bacteria under such low pH levels [43]. It also highlighted the importance of both the initial properties of the community and the selection pressure after environmental changes in shaping the resulting microbial community [39].

4.2. Fe(III) Reduction Associated with the Response of H_2 Production and Oxidation to Soil Acidification or Alkalization

Dehydrogenase could not only reflect the microbial activity in paddy soil, but also play a vital role in catalyzing dehydrogenation during anaerobic fermentation of organic matter [46]. Thus, DHA was assumed to connect with the microbial respiratory process in paddy soils [47]. Given that FeRB could take organic matter and its intermediate as carbon sources to mediate the Fe(III) reducing process, dehydrogenase was considered as a parameter linking to Fe(III) reduction in anaerobic paddy soils [26]. In the present study, the elevated DHA responding to initial pH increase of NC soil coincided with stimulated Fe(III) reduction, while the inhibited DHA responding to initial pH decrease of BD soil coincided with suppressed Fe(III) reduction. These results correspond well to the previous study [26].

During the subsequent step of organic matter metabolism after dehydrogenation, proton/ion hydrogen, derived from dehydrogenase, was transferred to ferredoxin and mainly re-oxidized by hydrogenases in bacteria with biohydrogen ability, such as *Clostridium* and *Bacillus*, for further H_2 production. Li et al. reported that most of the *Clostridium* in paddy soil with/without pH shift have the function of H_2 production, which provides electrons for the reduction of Fe(III) [27]. This co-metabolic mechanism might contribute predominantly to Fe(III) reduction in paddy fields [27,48]. Based on the results of structural equation modeling and redundancy analysis (Figure 5), and the above discussions, we speculate that microbial-mediated production and oxidation of H_2, responding to soil initial pH shift, regulates Fe(III) reduction in flooded paddy soil during the early stage of community succession.

4.3. Fe(III) Reduction Associated with the Response of Putative FeRB to Soil Acidification or Alkalization

Microbes are the dominant driver for soil respiration processes, such as Fe(III) reduction, in paddy fields [3,49]. Diverse soil management practice could directly or indirectly alter bacterial communities involved in biogeochemical cycles of elements [24,28,50,51]. Due to FeRB's lack of universal functional gene, investigation regarding the FeRB community has generally been limited in enrichment cultures by different Fe oxides and carbon sources in previous studies [52,53]. Nevertheless, functionally diverse Fe(III)-reducing bacteria (FeRB), including, but not limited to, bacterial strains in genera *Geobacter, Shewanella, Anaeromyxobacter, Clostridium, Bacillus, Pseudomonas, Desulfosporosinus, Desulfuromonas, Solibacillus* and *Pelobacter*, have been isolated over the last few decades from various environments [54–58]. The increasing isolates of FeRB have built a foundation for further investigation of the diversity of FeRB in flooded paddy fields. Putative Fe(III)-reducing

Geobacter and *Desulfosporosinus* in this study were related to *G. bemidjiensis* Bem, *G.picheringii* G13 and *D. lacus* STP12 (Table S1), which were obligative Fe(III) reducers, and severally isolated from sediments and sedimentary kaolin strata [59,60]. Most putative Fe(III)-reducing *Bacillus* and *Clostridium* was closely related to facultative FeRB *Bacillus* PeC11, RPS23, RPS34 and *Clostridium* P74, RPS6 and C71 (Table S1), and putative Fe(III)-reducing *Soilibacillus* were related to *Solibacillus sp.* A20, which were all isolated from paddy soils [52,58,61].

Studies have indicated that the indigenous microbial community in acidic soil may be more habituated to survival in the original acidic condition [62]. Whereas, Whittleston et al. showed that microbial fermentation metabolism was more prevailing than microbial respiration under alkaline conditions [24]. *Bacillus* and *Solibacillus*, as representatives of co-metabolic FeRB, have strong ecological flexibility and could survive in spore form to resist environmental stress [63]. The two genera are highly represented in extreme ecosystems, or habitats with severe conditions, including arid mountain meadow soil and vineyard soil [28,52]. Therefore, it was not surprising that the stimulation of Fe(II) accumulation in the alkalized NC soil could be attributed to the stimulative growth of Fe(III) reducing *Bacillus* and *Solibacillus* on day 1 of incubation. Definitely, putative FeRB perform a variety of functions in ecosystems, such as *Clostridium* and *Bacillus* in decomposing soil organic carbon [26,52], *Clostridium and Geobacter* in nitrogen fixing [60,64], *Geobacter, Desulfosporosinus* and *Desulfuromonas* in sulfate reduction [59]. Thus, the competitive growth of *Bacillus* in alkalied NC soil explained the increase in organic matter mineralization under increasing pH conditions [44].

Furthermore, bacteria are sensitive to sudden soil acidification because the acidic stress decreases intracellular pH through the undissociated H^+ cross cell membrane without coupling of proton motive force [65]. Tibbett et al. (2019) found that soil acidification induced a significant decrease in soil bacterial CFUs, by 7×10^6 CFU·g^{-1} soil, and bacterial abundance in the soil was strongly positively correlated with soil pH [66]. Herein, in the acidified BD soil, most of the putative FeRB, including, but not limited to, *Bacillus, Geobacter, Desulfosporosinus* and *Caenimonas*, undoubtedly could not tolerate such strong acidic stress. This was primarily responsible for the inhibited Fe(II) accumulation. Nonetheless, the relative abundance of *Solibacillus* increased in the acidified BD soil (Figure 3B), making an impressive appearance in high-stressed environments [63]. Additionally, we observed that the absolute abundance of putative FeRB, and concentration of Fe(III) and Fe(II), decreased in the acidified BD soil compared with the original BD soil in the present study. It indicates that the inhibition of microbial Fe(III) reduction results in increased Fe(III) concentration. Therefore, Fe(III) concentration is dependent on the response of putative FeRB to pH shift. That being said, the influence of Fe(III) concentration on Fe(II) accumulation under pH-shift conditions is mainly attributed to the response of FeRB to such a pH shift.

Undoubtedly, soil pH itself not only drives microbial community and associated ecological functions regarding Fe(III) reduction, but is also related to other biogeochemical cycles. Details on the functional responses of soil microbiota to pH changes is needed to target the sequencing of functional genes associated with specific biogeochemical processes, such as carbon and nitrogen dynamics. In addition, soil acidification, coupled with pollution or drought stress, severely threatens agricultural ecosystems. In view of the relation between iron redox and in(organic) pollution, microbial community responses to co-existing soil threats need to be better understood.

5. Conclusions

Soil initial pH changes undoubtedly disturb the temporal succession of the bacterial community and greatly affect Fe(III) reduction during flooding of paddy soils. The effect was distinguished by the initial pH of paddy soil. When adjusting the initial pH of an acidic soil to alkaline level, Fe(III) reduction was stimulated, accompanied by enhanced bacterial activity and Fe(III) reducing *Bacillus* and *Solibacillus*. Bacterial fermentative hydrogen production played a vital role in the early stage of Fe(III) reduction. In contrast, with an initial soil pH shift from alkaline to acidic, the inhibition of bacterial activity and

Fe(III) reducing bacteria was related to dramatic inhibition of Fe(III) reduction. This study suggests that pH shift-induced bacterial activity, succession of Fe(III) reducing bacteria and associated Fe(III) concentrations contribute to the response of microbial Fe(III) reduction to pH shifts in paddy soils. Our results aid in understanding the mechanisms of Fe dynamics in paddy soil and provide theoretical information for Fe cycling with element cycling in agricultural systems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12061304/s1, Text 1: Illustration for selection of pH regulator [67–72]; Figure S1: The rarefaction curves of tested samples; Table S1: Soil physicochemical characteristic of tested paddy soil. Table S2: Potential Fe(III)-reducing bacteria in paddy soils with different initial pH according to references and sequences published in NCBI.

Author Contributions: Conceptualization, R.J. and D.Q.; investigation, R.J. and L.L.; validation, F.F. and L.L.; formal analysis, R.J. and F.F.; visualization, R.J. and F.F.; writing—original draft preparation, R.J.; writing—review and editing, D.Q.; supervision, D.Q.; funding acquisition, R.J. and D.Q. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China, grant number 41807138 and 41571239.

Data Availability Statement: Not applicable.

Acknowledgments: Support for this paper was provided by the National Natural Science Foundation of China (No: 41807138 and 41571239). The authors were also grateful for the anonymous reviewers for their quality comments.

Conflicts of Interest: The authors declare no conflict of interest.

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