Free-living Bacterial Community, Abundance and Edaphic Factors Response to Soil Depth Gradient under Contrasting Fertilization in Sugarcane Continuous Cropping Field

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Abstract

Although the effects of fertilization on soil N-fixing bacterial community and abundance have been investigated extensively in the surface soil (0–20 cm), the response of free-living bacterial community and abundance under contrasting fertilizations in a consecutive sugarcane monoculture field and their relationship with edaphic factors in the dipper (20–60 cm) soil layer remain elusive. In the current study, nifH gene amplicon sequencing was used to investigate N2-fixers bacterial community and abundance by leveraging high-throughput sequencing (HTS). Moreover, edaphic factors in three soil depths (0–20, 20–40 and 40–60 cm) under control (CK), organic matter (OM), biochar (BC) and filter mud (FM) amended soils were investigated. Our analysis revealed that β-glucosidase and phosphatase activities, and ammonium (NH_4^+-N), nitrate (NO_3^--N), total carbon (TC), total nitrogen (TN) and available potassium (AK) were considerably higher in 0–20 cm in all treatments. Proteobacteria and *Geobacter* were dominant in all the samples, while 0–20 cm was overwhelmingly occupied by *Anabaena*, *Enterobacter* and *Desulfovibrio* in BC and FM amended soils, followed by *Methylomonas* in 20–40 cm under BC treatment. We also noticed that soil depth was the main environmental gradient that influenced soil diazotrophs and edaphic factors rather than fertilization. Together, these results suggest that soil depth had a profound impact on N-fixing bacteria, edaphic factors than fertilization regimes. Moreover, these findings were more pronounced in 0–20 cm soil depth under organic amendments compared with CK.

Introduction

Globally, sugarcane is one of the main economic crops and is regarded for its high sugar content and bioenergy [1,2]. China is the third-largest sugarcane-producing country worldwide with Guangxi province accounting for approximately 60% of the total sugar production in China [3]. Sugarcane consecutive monoculture farming pattern is widely practiced in China due to insufficient land and inadequate judicious planting concepts [4]. However, long-term sugarcane continuous cropping can have a deteriorating effect on essential soil nutrients in sugarcane rhizosphere zones as well as induce the proliferation of soil-borne diseases [5], which may eventually impede the overall productivity of cane [6]. These phenomena have been observed in crops, such as soybeans [7] and bananas [8]. In a recent study, Pang et al. [6] demonstrated continuous sugarcane cultivation had profound negative impacts on sugarcane agronomic parameters, soil fertility and soil microbial community.

Fertilization is generally carried out to improve crop productivity. For instance, sugarcane growers in Guangxi province apply nitrogen (N) fertilizer at the rate of 600-800kg N ha^{-1}, which is 6–8 times more than the average N application rate in Brazil [3]. On the other hand, the utilization of high-dose of N fertilizer in cane continuous cropping field may exhibit the disadvantage of negatively affecting soil fertility and health and the environment in general [9,10], thus negatively affecting soil microbial and crop growth [11]. Hence, there is mounting pressure on how to safely enhance agricultural sustainability. Organic fertilization has an obvious positive effect on soil microbial biomass, functional diversity [12], and soil enzyme activities [13], compared with mineral fertilizer [14]. Francioli et al. [15] reported bacterial diversity under organic fertilization significantly improved. In our previous study, biochar amended soil significantly increased the stalk weight and height of sugarcane, improved soil environmental parameters, and had a profound impact on the abundance of diazotrophic genera [16].

Additionally, environmental concerns and the desire for producing food using an eco-friendly approach [17] have led farmers to seek more suitable N management strategies [18]. Interestingly, it is worth noting that opting for biological nitrogen fixation (BNF) is an ameliorative strategy because it can provide nutrients for crops [19], thus boosting crops production capacity [20], and also maintains a sustainable terrestrial ecosystem [21]. BNF is the major biological mechanism by which N is available to plants, which is performed by prokaryotic bacteria called diazotrophs (N_2-fixers) [19]. Free-living N-fixing bacteria inhabiting soils contribute considerably to the N budgets of many ecosystems, which are vital for the growth and development of crops. However, soil N cycle has unprecedentedly been disturbed by the excessive use of synthetic fertilizers [22], thus shifting

a diverse range of microbial activities and communities [23]. For instance, Tan et al. [24] and Berthrong et al. [25] mentioned that the utilization of N fertilizers diminished the N_2-fixers community.

Soil depth is one of the main environmental gradients that play a major role in shifting soil biochemical properties [26], and soil microorganisms [27]. Soil microbial abundance and community as well as microbial diversity decrease with increasing soil depth [28,29]. Certainly, Lamontagne et al. [30] and Hartmann et al. [31] reported that the changes observed in bacterial diversity and composition were depth-dependent. In another study, it was also revealed that *nifH* gene abundance was considerably higher in surface soil (0–10 cm) compared with the subsoil (10–20 cm) [32]. Moreover, fertilization influences both the near-surface horizon and sub-surface horizon microbial biomass [33]. However, under consecutive monoculture farming patterns of sugarcane, little is known about N_2-fixers bacteria and edaphic characteristics response to contrasting organic amendments as well as their distribution patterns in dipper soil horizon (20–60 cm). To address these questions, we leveraged high-throughput sequencing (HTS) to: (i) investigate the response of diazotrophic community composition and abundance in 0–20, 20–40 and 40–60 cm soil profiles using functional *nifH* gene, (ii) examined the variations of soil physicochemical properties and soil enzyme activities in the different soil layers as well as assessed the relationship between diazotrophic community composition and soil biochemical properties, and (iii) measure the response of cane agronomical parameters to the different amendments in the long-term sugarcane monoculture field.

Result

Response of Sugarcane Parameters to Different Fertilization

The response of sugarcane parameters to different fertilizations: biochar (BC), organic matter (OM), filter mud (FM) and control (CK) were assessed. Compared with CK treatment, BC, OM and FM treatments did not improve the sucrose content, stem diameter and stalk height (Figure 1A,B,D). On the other hand, BC and FM significantly increased ($p < 0.05$) sugarcane stalk weight and ratoon weight compared to CK and OM (Figure 1C,E), while chlorophyll content peaked significantly ($p < 0.05$) under BC, FM and OM treatments compared with CK treatment (Figure 1F).

Soil Properties and Enzyme Activities

Compared to CK treatment, BC and OM treatments significantly improved ($p < 0.05$) NH_4^+-N in 0-20 soil layer. However, FM treatment significantly decreased ($p < 0.05$) NH_4^+-N in 0-20 soil depth compared to CK treatment (Figure 2A). Compared to CK treatment, soil NO_3^--N significantly increased ($p < 0.05$) in all the treatments in 0-20 cm soil profile (Figure 2B). In 0-20 cm soil depth, soil OM significantly increased ($p < 0.05$) under BC and FM treatments compared to CK treatment. However, compared to CK treatment, soil OM was not influenced under OM amended soil across the entire soil layers (Figure 2C). It was also observed that soil TC was enhanced significantly ($p < 0.05$) under all the amended soils compared to CK treatment in soil profile 0-20 cm (Figure 2,D). Moreover, Compared to CK treatment, soil TN and TC/TN were not significantly impacted under BC, FM and OM treatments, especially in upper the soil profile (0-20 cm) (Figure 2E,F). Soil AK was significantly higher ($p < 0.05$) 0-20 and 20-40 cm soil depths compared to CK treatment, but significantly decreased ($p < 0.05$) in soil depth 0-20 cm under FM and OM treatments compared to CK treatment. We also observed that soil AK was significantly more ($p < 0.05$) under FM and OM treatments in 20-40 and 40-60 cm soil profiles compared to CK treatment (Figure 2,G). Soil AP revealed no significant change in the entire soil layers in BC treatment compared to CK, however, FM treatment significantly increased ($p < 0.05$) AP in 0-20 and 20-40 cm soil depths compared to CK treatment, while in OM treatment, AP significantly increased ($p < 0.05$) in 0-20 cm soil layer, but was not significantly impacted in 20-40 and 40-60 cm soil depths compared to CK (Figure 2H). Compared to CK treatment, soil pH was not affected under BC treatment across the entire soil profiles. However, soil pH significantly reduced ($p < 0.05$) under FM treatment in soil 20-40 and 40-60 cm soil profiles. Whereas in OM treatment, the soil pH in 0-20 and 20-40 cm soil layers significantly reduced ($p < 0.05$), but was remarkably high ($p < 0.05$ in) in 40-60 cm

soil depth compared to CK (Figure 2I). We also observed that soil EC increased significantly ($p < 0.05$) under BC and FM treatments in soil depths 0-20 cm and 0-20 and 20-40 cm compared to CK treatment, respectively. While OM treatment significantly diminished soil EC in soil depth 0-20 cm compared to CK treatment (Figure 2,J). While soil SWC significantly increased under OM treatment compared with the other treatments specifically in 20-40 cm (Figure 3K).

Meanwhile, β-glucosidase was significantly low ($p < 0.05$) in BC, and OM treatments, and enhanced under OM treatment in 0-20 cm soil depths compared with CK treatment, (Figure 3L). Under both FM and OM treatments, phosphatase was considerably improved ($p < 0.05$) in 0-20 cm soil profile, while in BC amended soil revealed no difference was observed compared to CK (Figure 3M). Moreover, urease activity under BC, FM and OM treatments was significantly higher ($p < 0.05$) in 20-40 cm soil profile compared to CK (Figure 3N). The analysis also showed that cellulose activity decrease with soil profile under BC and FM treatments compared to CK treatment. However, cellulose activity significantly reduced ($p < 0.05$) in 0-20 cm soil depth in OM treatment compared to CK treatment (Figure 3O).

nifH Gene Copies and Alpha Diversity

Compared with CK treatment, BC and OM amended soils significantly diminished the *nifH* gene in 0-20 cm soil profile. On the other hand, FM treatment significantly increased the *nifH* gene in 20-40 and 40-60 cm soil layers compared with CK treatment. Regarding different soil depths, we observed that the *nifH* gene was stable in all the three soil depths in BC amended soil, but higher in 0-20 cm soil layer compared with 20-40 and 40-60 cm soil profile in CK treatment. Furthermore, our analysis indicated that the *nifH* gene was significantly high ($p < 0.05$) in 20-40 cm soil layer compared with 0-20 cm soil depth under FM treatment, and also decreased with soil depth in OM treatment (Figure S1A). Diazotrophic community diversity was analyzed accordingly in every sample along with various soil depths using diversity estimator (Shannon and Simpson) and richness (Ace and Chao1). The observed diversity and richness under various soil amendments exhibited little or no significant change in the entire soil profiles compared with CK (Table S2).

Relative Abundance of *nifH* Genes and Dominant Diazotrophic Phyla and Genera

Compared to control, the different amendments showed little or no significant difference in the various soil depths and treatments (Figure S1A). The dominant diazotrophic relative abundance distribution pattern was examined in different soil depths (0-20, 20-40 and 40-60 cm) at the phyla and genera levels. In soil depth 0-20 cm, we detected Proteobacteria (71.1-80.2%) and Cyanobacteria (8.6-15.3%) as the most dominant diazotrophic phyla. Moreover, in 20-40 cm soil profile, Proteobacteria (88.6-94.4%) and Cyanobacteria (0.0-2.8) were the dominant diazotrophic phyla. Whereas in 40-60 cm soil layer, Proteobacteria (82.9-88.4%) was the dominant diazotrophic phyla (Figure 3A). However, we observed that FM, OM and BC amended soils revealed little impact on diazotrophic phyla compared with the CK in the entire soil profiles (Figure S1B-I). At diazotrophic genera level, *Geobacter* (89.8-94.3%), *Anaeromyxobacter* (3.2-5.1%), *Burkholderia* (0.8-2.2%), *Azotobacter* (0.1-1.7%), *Desulfovibrio* (0.3-1.5%), *Anabaena* (0.4-1.0%) and *Enterobacter* (0.1-0.5%) were the dominant bacterial genera in 0-20 cm soil depth. Furthermore, *Geobacter* (90.6-94.0) and *Anaeromyxobacter* (4.7-6.6%) were the dominant diazotrophic genera in soil depth 20-40 cm. In 40-60 cm soil profile, *Geobacter* (83.7-89.5%) and *Anaeromyxobacter* (10.0-16.1%) were highly abundant (Figure 3B). Further analysis showed that a vast majority of diazotrophic genera were significantly altered in the different soil profiles under the different soil amendments (Figure S1J-S). Noticeably, *Anabaena* was significantly ($p < 0.05$) higher in 0-20 cm in BC amended soil than the other treatments (Figure S1J). *Burkholderia, Desulfovibrio* and *Enterobacter* in FM and BC amended soils at 0-20 and 20-40 cm soil layers improved significantly ($p < 0.05$), respectively, followed by *Methylomonas* in 20-40 cm under BC treatment ($p < 0.05$) relative to that under CK, OM, and FM (Figure S1M-O,Q). Venn diagram analysis further revealed the unique and overlap enriched genera among various treatments and soil profiles. It was observed that 1 genus was enriched in both CK and BC, 3 in FM and none in OM (Figure 3C). Meanwhile, 8 genera were enriched in 0-20 cm and 1 in both 20-40 and 40-60 cm soil depths (Figure 3D).

Multivariate ANOVA Analysis for the Impact of Soil Depth and Fertilizations on Diazotrophic Parameters and Edaphic Factors

Multivariate ANOVA analysis was leveraged to test the effects of soil depth gradient and fertilization and their association with different soil parameters relating to N_2-fixers, namely, OTUs, Shannon, chao1, coverage, *nifH* gene copies and edaphic factors, such as urease, cellulase, glucosidase and phosphatase (Table 1). Multivariate ANOVA analysis revealed that soil depth significantly ($p < 0.05$) impacted diazotrophic species richness indice (Chao1) and N_2-fixers alpha diversity index (Shannon), followed by diazotrophic coverage. However, soil depth had no impact on *nifH* gene copy number. The analysis also revealed that both soil depth and treatment had a significant impact on bacteria OTUs, while treatment had little impact on coverage. Moreover, we observed that soil enzyme activities, namely, urease, glucosidase and acid phosphatase, followed by cellulase were affected to a greater extent by the different soil depths compared with fertilization (Table 1). Regarding soil biochemical properties, namely, soil pH, available phosphorus (AP), available potassium (AK), total carbon (TC), total nitrogen (TN) and organic matter (OM), ammonium (NH4+N) and nitrate (NO3-N). It was observed that soil depth was a potential environment gradient affecting the edaphic factors than fertilization. Both treatment and soil depth had little impact on soil TC/TN. However, both treatment and soil depth revealed no impact on soil OM content (Table 2).

Table 1

Multivariate ANOVA for the effects of soil depth, and different fertilizers on *nifH* OTUs, diversity, species richness, coverage, gene abundances and soil enzyme activities

Factor	OTUs	Shannon	Chao1	Coverage	*nifH*	Urease	Cellulase	Glucosidase	Acid phosphatase
Treatment	**	NS	NS	*	***	NS	NS	NS	NS
Depth	**	***	***	**	NS	***	***	***	***
T x D	***	***	***	***	***	***	***	***	***

Depth stands for soil depth with 0-20 cm, 20-40 cm and 40-60 cm soil layers, treatment stands for fertilizer different fertilization with CK, BC, FM and OM.

Table 2

Multivariate ANOVA for the effects of soil depth, and different fertilizers on soil biochemical properties

Factor	pH	EC	SWC	TN	TC	TC/TN	AP	OM	AK	NO_3^--N	NH_4^+-N
Treatment	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS
Depth	***	***	***	***	***	*	***	NS	***	***	***
T x D	***	***	***	***	***	*	***	**	***	***	***

Depth stands for soil depth with 0-20 cm, 20-40 cm and 40-60 cm soil layers, treatment stands for fertilizer different fertilization with CK, BC, FM and OM.

Diazotrophic Community Composition under Contrasting Fertilization in Different Soil Profiles

Principal coordinates analysis (PCoA) was adopted to assess N_2-fixers community composition in different soil profiles (0-20, 20-40 and 40-60 cm) and the different soil amendments. The analysis demonstrated that N_2-fixers community composition in the entire soil depth revealed distinct patterns compared with the different treatments (Figure 4A,B). Redundancy analysis (RDA) was then employed separately in two soil depths (0-20 and 20-60 cm) to assess the impact of soil biochemical properties on diazotrophic community composition at the phyla level. The analysis showed that soil AP (R^2 = 1.1860, p < 0.05), EC (R^2 = 1.0933, p < 0.05), NH_4^+-N (R^2 = 1.0915, p < 0.05), TN (R^2 = 1.9840, p < 0.05), OM (R^2 = 1.8575, p < 0.05), followed by pH (R^2 = 1.5793, p < 0.01) and AK (R^2 = 1.5232, p < 0.01) were the major impact factors shifting diazotrophic community composition, while TC (R^2 = 1.5702, p < 0.05) was the manor driver influencing diazotrophic community composition in 0-20 cm soil profile (Figure 4C). Whereas in 20-60 cm soil layer, soil AP (R^2 = 0.4968, p < 0.001), AK (R^2 = 0.4273, p < 0.001), NO_3^--N (R^2 = 0.7832, p < 0.001) were the major impact factors shifting diazotrophic community composition, while TC (R^2 = 0.2532, p < 0.01) and EC (R^2 = 0.2184, p < 0.01) were the manor drivers altering diazotrophic community composition in 20-60 cm soil profile (Figure 4D).

Correlation between Edaphic Characteristics and Diazotrophic Community Composition

Network correlation analysis was used to measure the possible interaction between environmental variables and specific diazotrophic community composition. The patterns in network structure demonstrated diazotrophic genera related to *Proteobacteria* demonstrated a significant and positive association with significant a vast majority of edaphic factors. Noticeably, *Azoarcus*, *Dechloromonas* had a positive relationship with soil NO_3^--N, AP, AK, EC, NH_4^+-N, TC and soil TN, but showed a negative relationship with soil pH. However, *Anaeromyxobacter* showed a negative correlation with soil with important soil variables, namely, NO_3^--N, AP, AK, EC, NH_4^+-N. Moreover, *Zoogloea* was negatively associated with soil NH_4^+-N, EC, TC, AK, TC, pH, NO_3^--N, but revealed a positive correlation with soil AP, TN, pH. Whereas genus classified as *Cyanobacteria*, such as *Microcoleus* exhibited a positive relationship with soil EC, AP, NO_3^--N, TN, TC, AK and NH_4^+-N (Figure 5A). Additionally, the taxonomic composition of *nifH* OTUs showed a significant and positive correlation with a vast majority of the edaphic factors, including NO_3^--N, TC, EC, NH_4^+-N, AK, TN and AP (Figure 5B). Later, Pearson's correlation coefficients were employed separately in the three soil profiles (0-20, 20-40 and 40-60 cm) to have a better understanding of how soil biochemical properties affected diazotrophic phyla and genera community composition. The analysis showed that soil biochemical properties were significantly associated with a vast majority of diazotrophic genera than the phyla, especially in 0-20 cm soil layer (Figure 5C,D).

Discussion

In the current study, we aimed at unraveling the impacts of different organic amendments on soil enzyme activities, soil biochemical properties as well as diazotrophic abundance and community composition along different soil depths in a sugarcane continuous monoculture field. We also investigated the effect of these contrasting fertilization on sugarcane growth parameters. Li et al. [34] and Orndorff et al. [35] revealed that organic fertilization enhanced sugarcane growth parameters compared with mineral fertilizers. Similarly, it was observed that the sugarcane stalk weight and ratoon significantly increased under BC and FM treatments, whereas sugarcane chlorophyll content under various organic amendments was profoundly enhanced compared with CK treatment. These results may be explained by the fact the accumulation of organic materials are often in surface soil, which in turn could be made available to plants [36]. Organic fertilization has been considered as an alternative to inorganic fertilization, with the benefits of enhancing soil nutrients [36,37].

Likewise, we found that soil biochemical properties such as NO_3^--N, and TC under BC, OM and FM treatments increased significantly. It has also been mentioned that soil biochemical properties decreased with increasing depths [38,39]. In the current study, soil NH_4^+-N, NO_3^--N, TC, TN, AK and EC were significantly higher in the upper soil layer compared with the subsoil layers, which is consistent with our previous study [16]. Soil enzyme activities are considered as important indicators of soil fertility due to the pivotal role they play in soil biochemical reactions, as well as maintaining and sustaining soil fertility [40]. Akhtar et al. [41] and Zhao et al. [42] documented that organic matter increased soil enzyme activities in topsoil and they tend to decrease with increasing soil depth [16,43]. Similarly, we observed that β-glucosidase and phosphatase were significantly higher in 0–20 cm soil layer in all treatments compared with the subsoil, which may, in part, suggest that surface soil could show a more significant improvement in β-glucosidase and phosphatase turnover in the topsoil than subsoil. The increase in these enzyme activities in the topsoil in all treatments could be associated with the different soil amendments used [44].

Environmental gradients such as soil management practices and soil depths are major factors influencing the density of soil microorganisms [38,45]. For instance, Seuradge et al. [46] reported that soil depth was the primary environmental gradient that affected the bacterial community. In a related study, it was revealed that bacteria abundance was profoundly altered in the different soil horizons under straw retention farming systems [43]. Likewise, the majority of diazotrophic genera under the various treatments in the surface were profoundly altered than diazotrophic phyla. However, Proteobacteria accounted for a substantial number of bacteria phyla, especially in the upper soil layers (0–20 and 20–40 cm). Proteobacteria are Gram-negative, with outer membranes largely consisting of lipopolysaccharides, and are widely known as a plant growth promoter. The significantly high number of Proteobacteria identified in this study also agree with the trends observed in a study conducted by Zhang et al. [43]. *Geobacter* bacteria accounted for a substantial portion of the total bacteria genera in the entire soil profile, which is roughly in consonant with previous reports documented by Liu et al. [47] and Liao et al. [48], in which it was established that *Geobacter* was one of the abundant soil microbial detected in soil amended with biochar. Zhang et al. [43] also documented that *Geobacter* relative abundance in the subsoil was improved than the in topsoil, thus confirming that an anaerobic environment is conducive for the growth of *Geobacter*, which improves *Deltaproteobacteria* in the subsoil sequentially. Genera *Anabaena*, *Enterobacter* and *Desulfovibrio* were significantly enhanced in the 0–20 cm in BC and FM treatments. Studies have revealed that *Anabaena* is capable of fixing nitrogen, and it is a filamentous cyanobacteria genera [49,50]. Our findings corroborated with the study conducted by Chen et al. [51], wherein it was reported that the utilization of biochar improved soil microbial abundance in 0–15 cm soil profile. The significant amount of *Anabaena* detected in the surface soil (0–20 cm) may have led to the increase in soil N-related nutrients such as NH_4^+-N, NO_3^--N. *Enterobacter* is widely spread in the environment such as soil, plant, water [52], vegetation as well as human feces [53], and is considered as a nosocomial pathogenic bacteria [54], as well as a plant growth promoter [55]. The increase in *Enterobacter* in the 0–20 cm under BC and FM treatments may have led to the increase in NH_4^+-N and NO_3^--N, which in turn, can be used by sugarcane plants thus triggering the growth of various growth parameters of sugarcane [56,57]. *Desulfovibrio* genus is a sulfate-reducing bacterial that belongs to the *Desulfovibrionaceae* group [58]. A study conducted by Ding et al. [59] documented that *Desulfovibrio* genus relative abundance was improved in treatment that received 1.5 g urea/m^2 of fertilizer. Similarly, we found that *Desulfovibrio* relative abundance was enhanced in soil depth 0–20 cm under BC amendment soil relative to that under CK.

An important finding of our study was the effects of soil depth on the different parameters of diazotrophic, soil enzyme activities and soil physicochemical properties in comparison with fertilization. Multivariate ANOVA analysis revealed that soil depth played a major role in influencing *nifH* gene abundance, diazotrophic diversity and species richness. Our result is in line with previous studies [60], implying that the diversity and structure distribution and size of soil bacteria changed substantially with increasing soil profile. Moreover, we also observed that the change in soil enzyme activities and soil physicochemical properties were strongly associated with soil depth rather than the different fertilizations applied, thus further validating that enzyme activities and soil physiochemical properties are depth-dependent [16,43]. Soil microbial

communities have been widely reported to be very responsive to soil environmental variables [61,62]. Similarly, the RDA plot showed that soil AP, EC, NH_4^+-N, TN, OM, pH and AK, and AP, AK, NO_3^-N were the major impact factors shifting diazotrophic genera community composition in soil 0–20 and 20–60 cm soil layers, respectively. The patterns in network structure showed that diazotrophic genera related to Proteobacteria demonstrated a significant and positive association with significant amount edaphic factors. Additionally, the taxonomic composition of *nifH* OTUs showed a significant and positive correlation with a majority of the edaphic factors, including NO_3^--N, TC, EC, NH_4^+-N, AK, TN and AP. Pearson's correlation coefficients analysis revealed that the majority of diazotrophic genera were significantly and positively associated with soil biochemical properties than diazotrophic phyla, especially in the surface soil (0–20 cm).

Conclusion

In summary, our results demonstrated that organic soil amendments such BC and FM treatments significantly enhanced cane stalk and ratoon weight, whereas chlorophyll content substantially increased in BC, FM and OM amended soil compared with CK. Moreover, β-glucosidase and phosphatase were significantly higher in the upper soil layer (0–20 cm), and soil NH_4^+-N, NO_3^--N, OM and TC were very high in BC, FM and OM than CK in 0–20 cm soil profile. Noticeably, soil depth gradient was the main impact factor influencing the different soil parameters rather than the different soil amendments. It was also observed that the vast majority of diazotrophic genus relative abundance was significantly altered across the entire soil profiles, and these genera were significantly and positively associated with soil biochemical in the surface soil (0–20 cm). Taken together, our findings are likely to enhance our understanding of the impact of contrasting fertilization practices and soil depth gradient on soil biochemical properties and diazotrophic abundance and community composition in a sugarcane continuous monocropping field.

Materials And Methods

This experimental work was established from March 2018 to December 2020 at the Fujian Agriculture and Forestry University, Sugarcane Research Center, Fuzhou, Fujian Province, China (latitude 26˚5'0" east longitude 119˚13'47"). The site has a clay loam texture soil, with an annual temperature of 20 ˚C and rainfall of 1369 mm annually. The experiment was laid in a randomized block design consisting of four treatments replicated thrice. The experiment consisted of control (CK), organic matter (OM), biochar (BC) and filter mud (FM). On March 20, 2018, the biochar was applied at the rate of 30 t ha^{-1}, organic matter 25.5 t ha^{-1}, and filter mud was applied at the rate of 20.5 t ha^{-1}. The filter mud and biochar utilized during the study were purchased from Nanjing Qinfeng Crop Straw Technology Company, China. It is noted that the biochar was produced from sugarcane straw at the 550-650 ˚C and the organic matter used during the research was composed of pig manure, while filter mud was obtained from precipitated impurities found in the sugarcane juice that is removed when sugarcane is being processed through filtration, as mentioned by Orndorff et al. [35] and Elsayed et al. [63]. The basic soil properties were measured before the application of various amendments (Table S1). The different soil amendments were surface applied and immediately mixed into the ploughed soil at the depth of 0-30 cm using rotary tillage before cultivating the sugarcane. Sugarcane stalks were cut at about 10-15 cm in length, with two buds on each sett [64]. Fifteen setts were planted on each row with 0.3 m between plant-to-plant spacing and 0.5 m row-to-row spacing.

Soil Sampling

In December of 2020, surface soil (0-20 cm), subsoil (20-40 cm) and dipper soil layers samples of 40-60 cm were collected. In each plot, a sample was collected at five different spots, homogenized and mixed accordingly forming one sample [45]. A portion of each soil sample was air-dried, grounded and sieved through 2 mm mesh. Sieved soil (2 mm) was be used to analyze soil biochemical properties and enzyme activities, while the other portion was stored at -20 ºC for the extraction of DNA, ammonium (NH_4^+-N) and nitrate (NO_3^--N).

Sugarcane Agronomic Parameters

Cane heights were determined in centimeters (cm) using a meter rod from the soil surface to sugarcane's top. The mean of cane heights were determined using the average of three replicates. We used Legendre [65] approach by milling and measuring the juice for pol and Brox using thirty cane stalks that randomly selected from each row. The individual weight of each cane stalk (kg stalk-^{1}) was measured using cane plant fresh weighs. Plants were harvested in December of 2020, and yield parameters were estimated for the first ratoon crops. A portable chlorophyll meter was employed to record the cane plant relative chlorophyll of ten mature and healthy leaf close to the top in each plot.

Measurement of Soil Physiochemical Properties

Soil biochemical properties, namely total nitrogen (TN) and total carbon (TC), total phosphorus (AP) and available potassium (AK) were determined as mentioned by Bao [66]. A glass electrode pH meter was used for the estimation of soil pH. Fresh soil sample was used to extract soil NH_4^+-N and NO_3^--N with 2.0 M KCl and measured using the continuous flow analyzer (San++, Skalar, Holland) [67]. Soil OM was assessed by adopting the Walkley–Black approach, which contained the soil OM oxidation by H_2SO_4 and $K_2Cr_2O_7$, and $FeSO_4$ was later used for titration [68]. Soil electrical conductivity (EC) was calculated in a 1/5 (w/v) aqueous solution using conductivimeter (Crison mod. 2001, Barcelona, Spain).

Determination of Soil Enzyme Activities

The estimation of soil enzyme activities were carried out following the methods reported by Tayyab [69] and Sun et al. [70]. In brief, after the soil was incubated, cellulose, (glucose, mg/g 24 h, 37 °C) to estimate colorimetrically by calculating a decrease in 3,5-dinitrosalicylic acid from reducing sugar using buffer sodium carboxymethylcellulose solution. Phosphatase activity was measured using a nitrophenyl phosphate disodium substrate (phenol, ug/g, 1 h, 37 °C). The β-glucosidase activity was estimated using a colorimetric p-nitrophenol assay after buffering the soil with p-nitrophenyl-β-glucopyranoside, (p-nitrophenyl, ug/g, 1 h, 37 °C). Kandeler and Gerber buffered method was employed to measured soil urea activity by using urea as a substrate.

DNA Extraction

Genomic DNA from all the samples was used to extract DNA by using the Fast DNA TM Spin kit according to the manufacturer's guideline (MP Biomedical, Santa Ana, CA, USA) which is designed for soil DNA isolation. Additionally, all isolated DNA samples were subjected to gel electrophoresis. Then, DNA purification was completed using DNA purification kits according to manufacturer instructions (Tiangen Biotech Co., Ltd., Beijing, China). Nanodrop was adopted for the quantification of all DNA samples and stored at -20 °C for further analysis.

Quantitative Real-Time PCR Assay

The abundance of the *nifH* gene was determined from the soil samples collected at different soil depths (0-20, 20-40 and 40-60 cm) under contrasting fertilization using real-time quantitative PCR according to the MIQE (Minimum Information for Population of qPCR Experiments) instructions. The SYBR Premix Ex TaxTM (Perfect Real Time) kit (TaKaRa Biotechnology Co., Dalian, China) with 7500 Fast Real-Time PCR system (Applied Biosystems, New Jersey, USA) was employed to perform qPCR. The reaction was carried out in a 25 µL volume containing 12.5 µL of SYBR Premix Ex TaqTM (2x, TaKaRa Biotechnology Co.), 0.5 µL ROX Reference dye II (50x, TaKaRa Biotechnology Co.), 10 µL dd H_2O, 1 µL (~ 10-30 ng) DNA template and 0.5 µL (5 µL) of the primer was used. The *nifH* gene primers were nifH-R (5'–

AAAGGYGGWATCGGYAARTCCACCAC-3') and nifH-R (5'-TTGTTSGCSGCRTACATSGCCATCAT-3') was used. The *nifH* gene PCR protocols consisted of an initial activation step of 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and 34 s at 60 °C. Fragments of the *nifH* gene were cloned into the pMD19-T plasmid and the correct inserted genes were chosen. The potential PCR inhibitors of the DNA samples were determined using serial dilutions, and major inhibitions were not observed in the DNA samples extracted. To develop the standard curve, serially diluting plasmid was adopted to the final concentrations of 10^8-10^2 gene copies number μL^{-1}. The qPCR efficiencies were 98% for *nifH* and the R^2 of the standard was higher than 0.99.

nifH Sequencing

High throughput sequencing was leveraged to characterize diazotrophic community composition and was later amplified with primer set PolF and PolR [71]. Amplicon was modied by adopting both Illumina adaptor sequences and barcode sequences [72]. To obtain sample libraries, we used purified PCR products. Paired-end sequencing on a Miseq benchtop sequencer (Illumina, San Diego, CA, United States) was conducted by employing Miseq 300 cycle Kit. *nifH* gene separated based on its barcodes, allowing up to one mismatch. Subsequently, Btrim was adopted to conduct quality trimming [73]. FLASH was then used to merge both the forward and reverse reads into full-length sequences [74]. We eliminated sequences comprising of short bases or ambiguous, and *nifH* gene sequences were assessed using FRAMEBOT program [75]. We also eliminated sequences with frameshift errors, and sequences with no error were then transformed into conceptual proteins sequences. Then, *nifH* gene protein sequences clustered into OTUs with a 0.05 sequence distance cutoff by employing DOTUR [76,77]. For each sample, *nifH* sequences were filtered to 10,000, and we alter discarded the singletons. We probed the representative sequences against reference *nifH* that contained taxonomic information to assign *nifH* OTUs taxonomic [16]. Finally, the raw data were submitted to the NCBI Sequence Read Archive (accession no. PRJNA815949).

Statistical Analysis

We adopted DPS software (version 7.05, www.dpssoftware.co.uk) to examine the differences between the mean values of each treatment and soil depth using ANOVA, and compared using Tukey's procedure at a 5% level [45]. Venn diagram was employed to visualize unique and overlap N_2-fixers genera in the various treatments and soil profiles (http://bioinfogp.cnb.csic.es/tools/venny/index.html). The effects of soil depth gradient and fertilization regime and their association with different soil parameters relating to N_2-fixers, soil enzyme activities and soil physicochemical properties were performed with multivariate ANOVA in the 0-20 cm, 20-40 cm and 40-60 cm soil profiles. Principal coordinate analysis (PCoA) and an analysis of similarities (ANOSIM) were conducted to test if there was a significant difference in N_2-fixers community composition in the different soil depths and treatments. We also tested the association between N_2-fixers community composition and soil physiochemical properties by adopting redundancy analysis (RDA) in 0-20 and 20-60 cm. The patterns in the network structure of N_2-fixers community composition and PERMANOVA (with permutations = 999) analyses were tested using vegan R-package and we later generated the plots using ggplot [78]. Mantel tests were adopted to examine the relationship among diazotrophic taxonomic composition and edaphic factors using "vegan" package [61]. The correlation between N_2-fixers community composition and soil physiochemical properties in the three soil depths was further conducted using N_2-fixers genera and phyla by adopting R-software [79].

Declarations

Conflicts of Interest:

The authors declare no conflicts of interest.

Author Contributions:

All authors contributed to this study intellectually. N.F., M.T, Z.Y., C.Z., Z.P., L.J.S., H.Z. and J.L. designed the research and conducted the experiments. N.F. and C.Z. analyzed the data. N.F. wrote the manuscript. M.T, M.S.N., W.L., A.Y.A. and H.Z. reviewed the manuscript. H.Z. supervised the work and approved the manuscript for publication.

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Figures

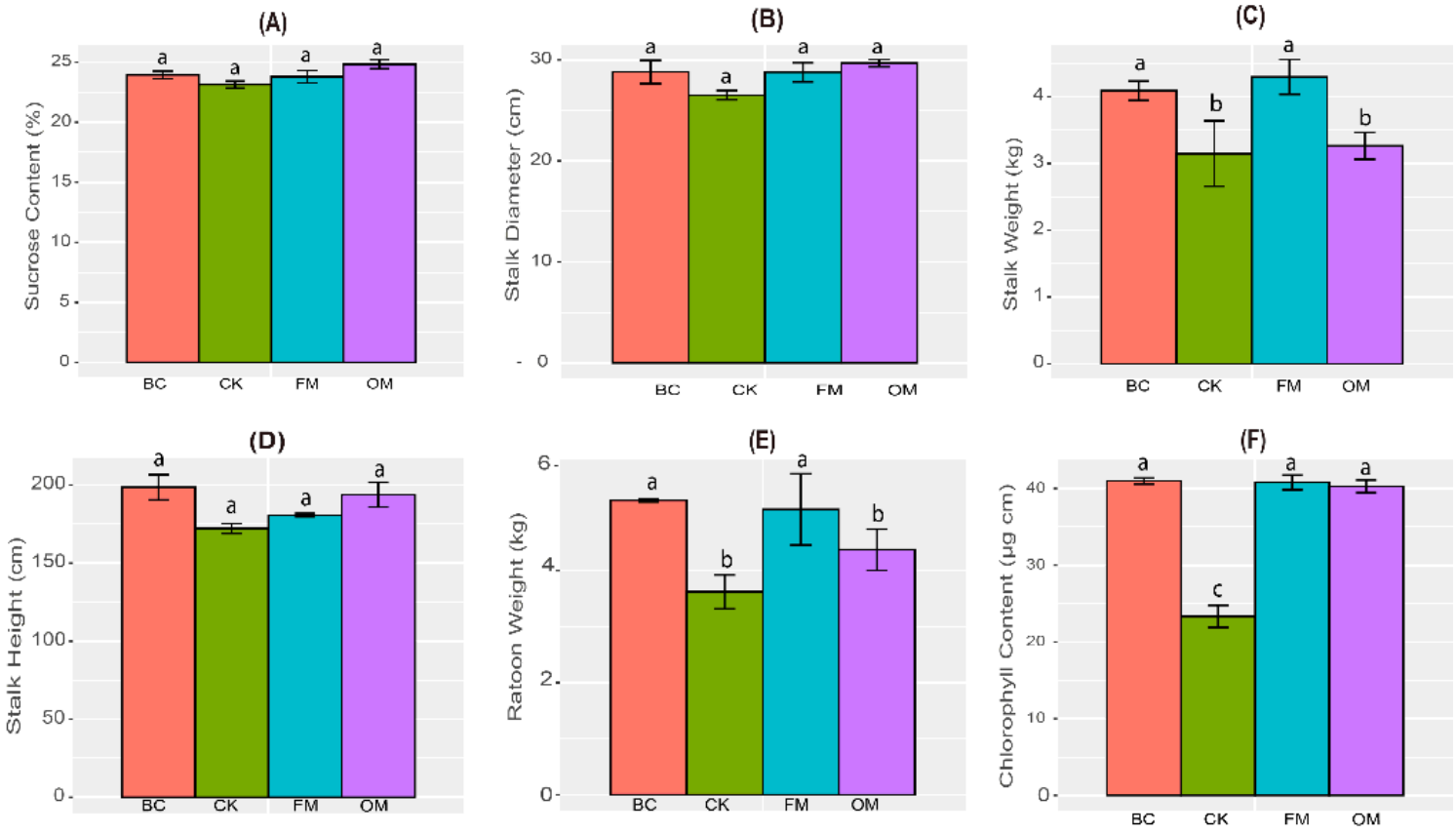

Figure 1

Sugarcane agronomical parameters response to different soil amendment practices; sucrose content (A), stem diameter (B), Stalk weight (C), Stalk height (D), ratoon weight (E) and chlorophyll content (F).

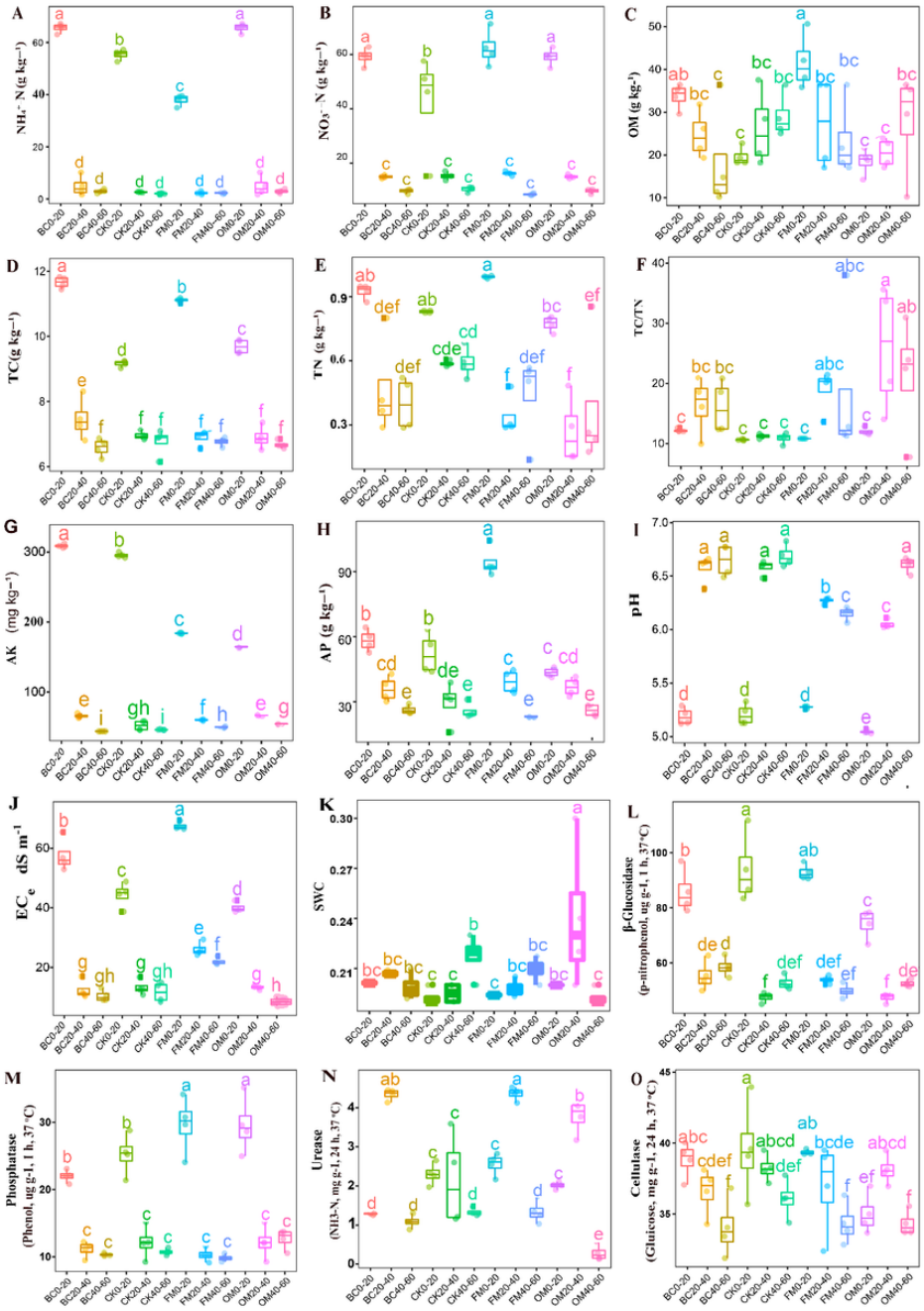

Figure 2

Soil biochemical properties (A-K) and soil enzymes activities (L-O) at three soil depths under biochar (BC), filter mud (FM), organic matter (OM) amended soil andd control (CK). Different lowercase letters depict significant differences between treatments (Tukey test, $p < 0.05$).

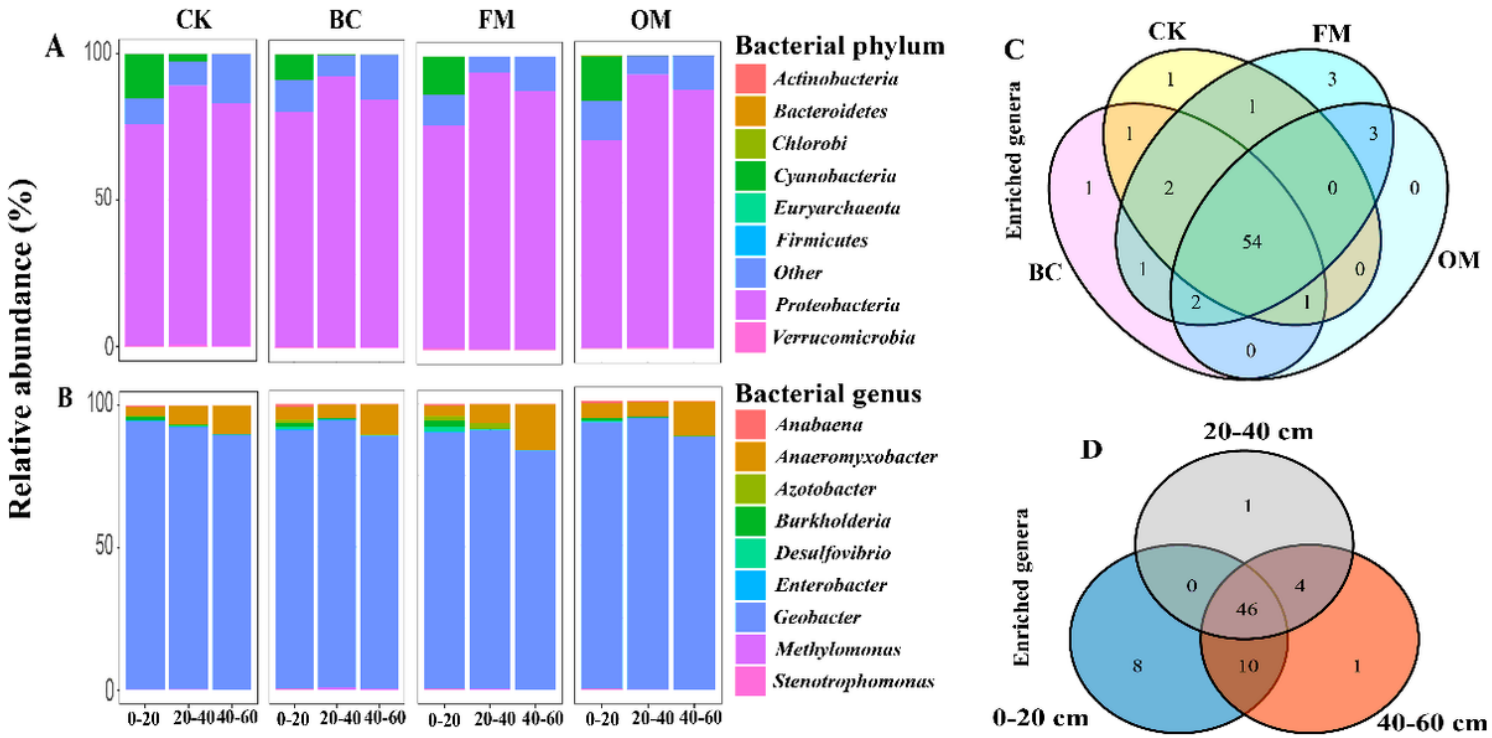

Figure 3

Distribution of diazotrophic phyla (A) and genera (B), “Other” indicates those identified phyla and genera that were beyond the top nine phyla and genera. Venn diagram illustrating unique and overlap enriched genera under (C) biochar amended soil (BC), filter mud (FM), organic matter (OM) and control (CK), and (D) soil depths (0-20, 20-40 and 40-60 cm).

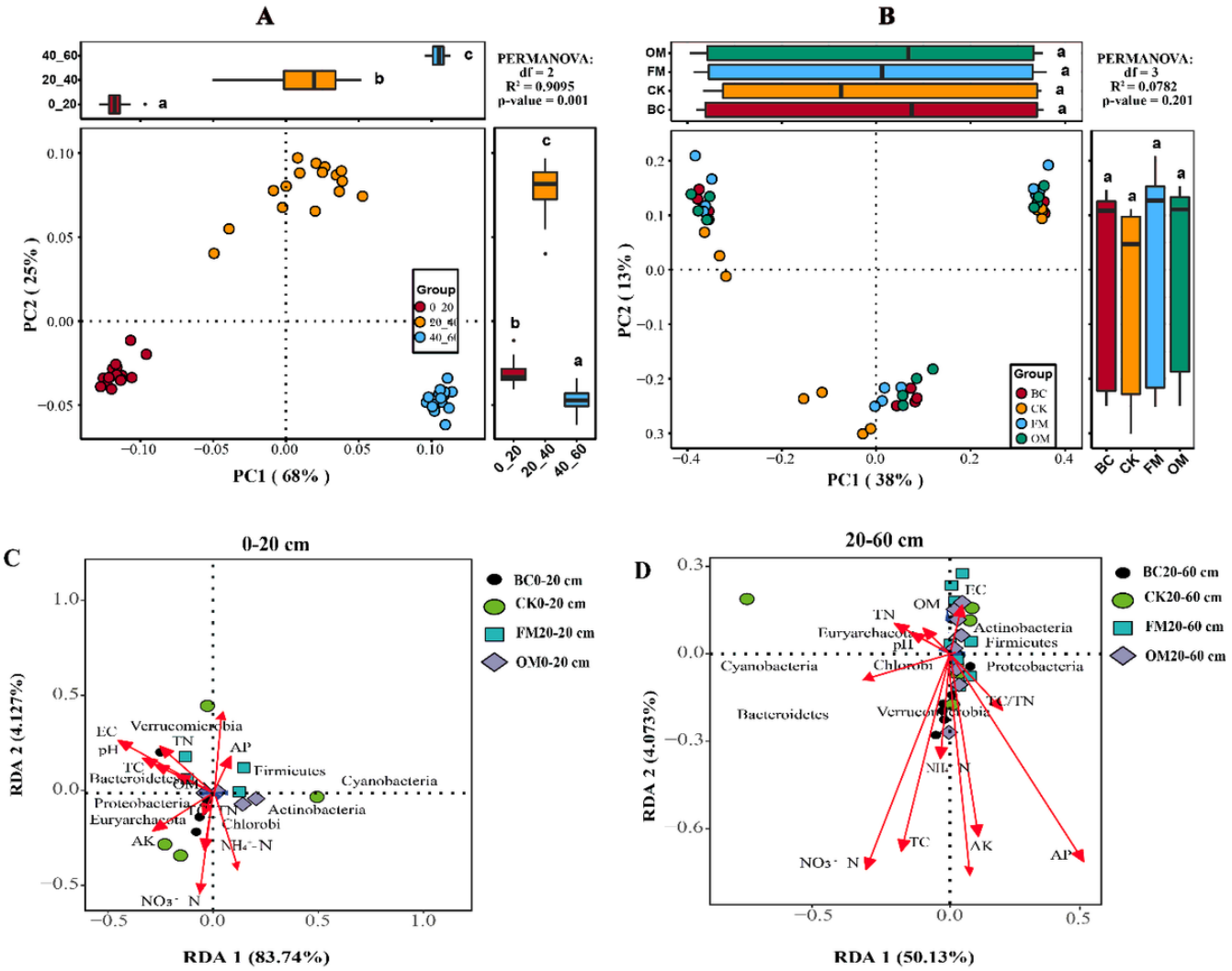

Figure 4

Principal coordinates analysis (PCoA) depicting diazotrophic bacterial community composition in different soil depths (A) and various fertilizations (B). Redundancy analysis (RDA) of soil biochemical properties and diazotrophic genera at soil depths 0-20 cm (C) and 20-60 cm (D) under biochar amended soil (BC), filter mud (FM), organic matter (OM) and control (CK).

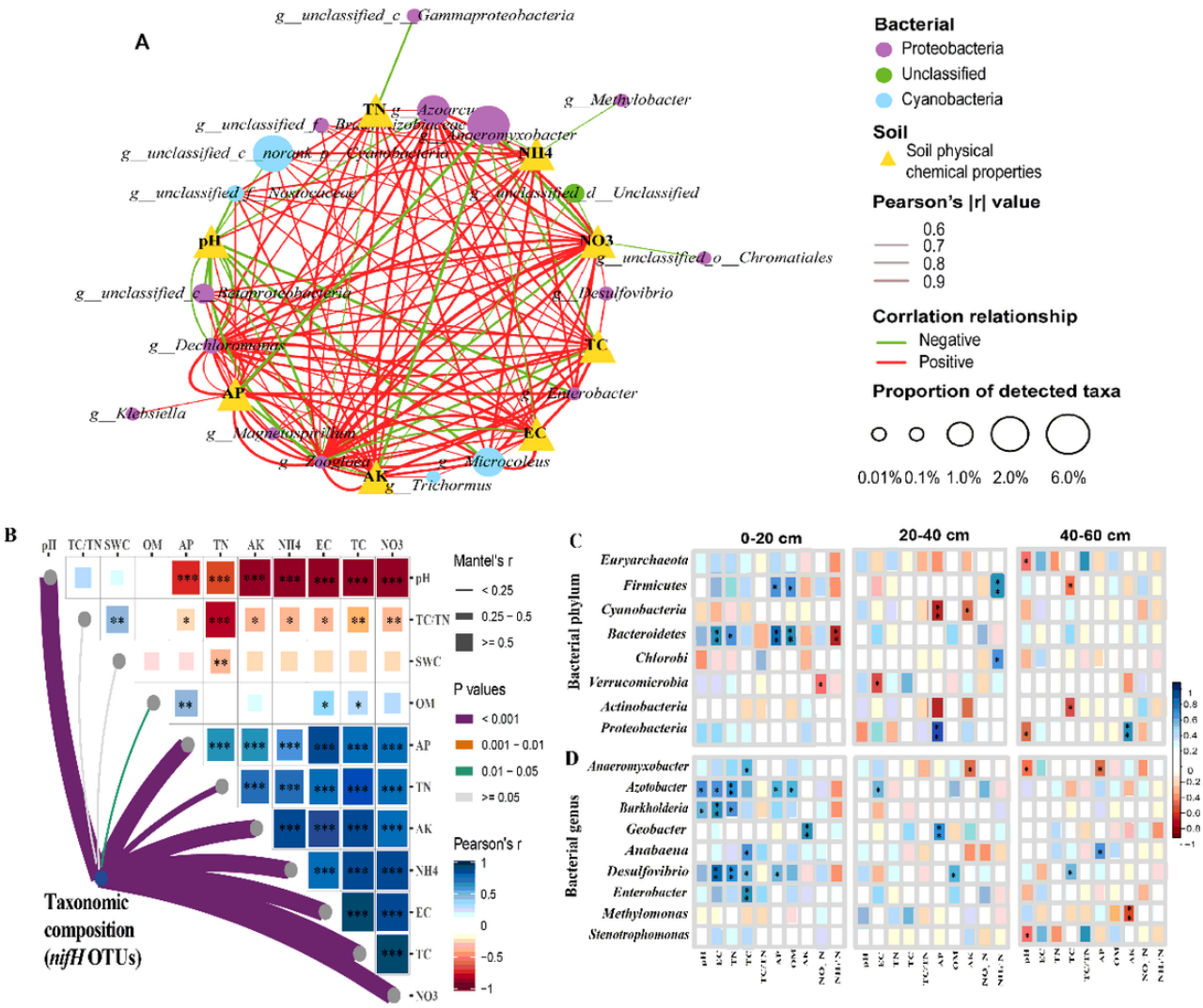

Figure 5

The correlation network analysis depicting the interaction between specific diazotrophic genera and soil environmental variables. Red and green lines indicate positive and negative associations, respectively (A). Pairwise comparisons of edaphic factors are shown with a color gradient depicting Pearson's correlation coefficients. Mantel tests depict the correlation between taxonomic composition (*nifH* OTUs) and edaphic characteristics. Each edge width correlates with Mantel's r statistic for the corresponding distance associations (B). Pearson's correlation coefficients for soil biochemical properties and the most abundant diazotrophic phyla (C); and diazotrophic genera in different soil horizons. The heatmap cells marked by "*" or "**" are statistically significant: * $p < 0.05$ and ** $p < 0.01$.

Supplementary Files

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