Effect of 20 years of mineral and organic fertilization on CO2 and N2 fixation bacteria in Moso bamboo plantation in southern China

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May 18, 2020

Abstract

The fixation of atmospheric CO2 and N2 by soil bacteria is important to the terrestrial carbon (C) and nitrogen (N) cycles and can be greatly affected by anthropogenic disturbance. The application of mineral fertilizer combined with manure (MCM) instead of MF have been used to mitigate soil degradation caused by long term mineral fertilizer (MF) practiced in widely distributed Moso bamboo. We conducted an investigation, employing the statistical approach of space-for-time substitution, to evaluate the effect of MCM on soil CO2 and N2 fixation bacteria in subtropical region of China. Intensively managed Moso bamboo plantations receiving MCM for 0, 6, 10, 15, and 20 years were examined. MCM management enhanced the content of soil organic C and available soil N, P, K, although some fluctuating values were observed. Soil pH values were generally stable, ranging between 5.0 and 5.5. The abundance of the genes cbbL and nifH decreased significantlyafter 6 years of IM and then gradually recovered. Both CO2 and N2 fixation bacteria have their similar dominant species, such as Bradyrhizobium, but these differed in their relative abundances among treatments. The diversity of both bacterial groups either decreased or increased at the 10-year sampling and later returned to their original levels. The alterations of abundances and measures of community diversity for both CO2 and N2 fixation bacteria were not driven by unique factor. In conclusion, IM practice with MCM in Moso bamboo plantation had an overall positive effect on soil CO2- and N2-fixing bacteria as well as soil properties.

Introduction

To meet increasing population demands, agricultural and forest ecosystems have been more intensively managed during the last five decades (Sengupta et al., 2015). However, the problem of soil degradation caused by long-term intensive management has become increasingly prominent (Guo, et al., 2010). Fertilization is the main practice employed in intensive management. For decades, many studies have reported that the increased application of single mineral fertilizers improved soil fertility and crop yields (Zeng et al., 2016; Wang et al.,2017b). However, this practice also accompanied various negative effects such as soil acidification, greenhouse gas emissions, nutrient losses, and deterioration of soil structure (Dalal, Wang, Robertson, & Parton. 2003; Zeng et al.,2016). Soil microbes responsible for most of the soil biochemical processes are also affected by changes in nutrient availability, pH and organic matter content resulting from heavy fertilization (Wang et al.,2017). It was reported that the input of mineral fertilizer reduced bacterial richness and disturbed soil microflora communities (Sun, Zhang, Guo, Wang, & Chu,2015). Reductions in microbial biomass was closely related to the duration and amount of N input in both field and lab-based

studies (Treseder, 2008). Thus, long-term and large-scale application of mineral fertilizers is considered to be a key reason for reduced microbial biodiversity associated with intensive management (Wang et al.,2017). Therefore, land conservation and soil fertility recovery are very important to intensive agricultural system.

Bacteria-mediated fixation of C and N play an important role in sustaining soil fertility in agricultural ecosystems (Fan,2019; Kekulandara, Sirisena, Bandaranayake, Samarasinghe, & Suriyagoda, 2019). Autotrophic microorganisms along with algae contributed about 40% and between 4-10% of CO_2 fixation in oceans and wetlands ecosystem, respectively (Cannon et al., 2001; Stanley, Johnson, & Ward, 2003). Many studies have focused on the assessing the importance of autotrophic bacteria in fixing atmospheric CO_2 into soil OC(Ge et al., 2012;Wu et al., 2014;Yuan et al., 2015). Autotrophic bacteria in soil annually capture about 0.6–4.9 Gt C, which represents 0.5–4.1 % of total terrestrial C fixation (Falkowski et al., 2000). The C fixed is first imported into unstable OC pools as microbial biomass carbon (MBC) and dissolved organic carbon (DOC) (Ge et al., 2012). Biological N fixation (BNF) process has been considered, both economically and environmentally, as a source of N for plant growth. They play an important role on N supply for most ecosystems, especially in low-fertility soils (Norman & Friesen,2017). Globally, current estimates suggest that N fixed by BNF (~300Tg nitrogen yr^{-1}) is much higher than that produced industrially (~125 TG nitrogen yr^{-1}) (Kuypers , Marchant, & Kartal, 2018). It was estimated that soil N input via BNF accounted for 16% of global N_2 input annually (Ollivier et al., 2011). Soil OC and total nitrogen (TN) contents were shown to increase significantly following inoculation with diazotrophic *Azotobacter* and *Bacillus* (Kheirfam, Sadeghi, Homaee, & Zarei, 2017). Introduction of one *Azotobacter* sp. strain, a free-living bacterium with excellent ability of N fixing, was estimated to save 50-75% of the mineral N and P fertilizer in 2 year field experiment (Dadrasan, Chaichi, Pourbabaee, Yazdani, & Keshavarz-Afshar, 2015). Besides enhancing N, C and P levels in agricultural systems, N-fixation bacteria could indirectly improve soil physical properties (Zhao, Qin, Weber, & Xu,2014), including declined soil density and increased water holding capacity, hydraulic conductivity and mean weight diameter(MWD)(Nisha, Kaushik, & Kaushik, 2007).

Bamboo is an important ecological, industrial and cultural resource. The total output value of the national bamboo industry reached 117.3 billion RMB in 2010 (National Bamboo Industry Development Plan 2011-2020).*Moso* bamboo (*Phyllostachys pubescens*) covered 4.6778 million ha accounting for 73% of the total bamboo area in China by 2018 (the 9^{th} national forest investigation) is a significant component of forest ecosystems (State Forestry Administration of the P.R. China, 2018). Due to its high economic return, *Moso* bamboo has received intensive management to enhance its productivity in the past few decades (Liu et al., 2011; Li et al., 2013). The intensive management (IM) practices employed with *Moso*bamboo forests are primarily annual fertilizer application and removal of understory herbs and shrubs. Farmers usually prefer to use mineral fertilizers which are more efficient and convenient than organic fertilizers, especially in mountain and hill land where farmer are difficult to practice. However, as with agricultural systems, long-time application of mineral fertilizers has resulted in the decline of soil fertility in bamboo plantation (Qin et al., 2017). As a result of observed ecological problems due to the sole use of inorganic fertilizers, combined applications of mineral fertilizers and manure (MCM) have been introduced into *Moso* bamboo management to prevent land from negative effects of mineral fertilizer.

The application of MCM has been proved to be a potentially superior land management practice than the application of mineral or organic fertilizers alone. Some field experiments confirmed that MCM played an important role in maintaining soil health, improving soil fertility, and promoting the restoration of biotic and abiotic soil properties (Wang, Lai, Wang, Pan, & Zeng, 2015). Meta-analysis and modelling data from upland soils and paddy-upland rotation soils across the major agricultural zones in China revealed that the MCM increased the SOC content and crop yields substantially (Jiang et al., 2018). The long-term fertilization experiment showed that application of MCM improved SOC significantly (Liang, Yang, He, & Zhou, 2011). It was demonstrated that SOC was positively correlated with crop yields following more than twenty years of continuous winter wheat–summer corn rotation cultivation (Yang, Zhao, Huang, & Lv, 2015). There are several direct or indirect factors associated with MCM that contribute to improved crop yields. For example, the application of MCM has directly increased SOC and improved mineral N utilization efficiency by accelerating microbial SON mineralization activity (Pan et al., 2009). The higher SOC contents

resulting from MCM treatment led to a greater cation exchange capacity (CEC) when compared with soils receiving with no or only inorganic fertilizers in a low-productivity paddy field (Mi et al., 2018). The MCM could potentially increase and modify microbial biomass, enzyme activities, or community composition by providing an OC energy source and nutrients in organic form (Zhao et al., 2016). MCM has generally had positive effects on bacterial CO_2 and N_2fixation. Fertilization increased *cbbL* abundance, with the highest *cbbL* copy number and RubisCO enzyme activity in NPK plus rice straw soil (Yuan et al., 2012). Long-term mineral NPK fertilization decreased the diversity of diazotrophic community, whereas NPK plus rice straw and NPK plus chicken manure treatments maintained the diversity of diazotrophic community (Liao, Li, & Yao, 2017). However, positive results have not always be observed. For example, Lin et al. (2018) reported that long-term application of inorganic fertilizer plus organic material (pig manure) suppressed the abundance and diversity diazotrophs and altered community structure, while inorganic fertilizer combined with plant residue (rice straw or radish) had no effect on the community structure of diazotrophs. The inconsistent results may be due to environmental heterogeneity and the type organic materials applied.

In the past few decades, long-term intensive management of *Moso*bamboo has been reported to cause soil deterioration, including soil erosion and nutrient leaching (Shinohara & Otsuki, 2015), soil acidification (Qin et al., 2017), and reduction of soil C and N storage (Li et al., 2013). It also caused a general decrease in microbial diversity and shifts of microbial community structure (Xu, Jiang, & Xu, 2008) and more specifically reduced the abundance and altered the community structure of arbuscular mycorrhizal fungi (AMF) (Qin et al., 2017). Soil CO_2 and N_2 fixation bacteria are considered sensitive to changes in soil nutrients, pH and organic matter content caused by heavy fertilization (Wu et al., 2014; Tang et al., 2017). It was observed that the abundance of CO_2fixation bacteria in topsoil increased at the first 10 years of application of mineral fertilizer in*Moso* bamboo planation, and then decreased (Liu et al., 2018). However, the abundance and diversity of diazotrophic bacteria decreased at first and then increased (He et al.,2015). Thus, it is necessary to better understand how these bacterial groups respond to applications of MCM. We hypothesized that MCM could lead to a positive effect on CO_2 and N_2 fixation by microbial communities in *Moso* bamboo planation. The method of space-for-time substitution was used to establish a chronosequence of *Moso* bamboo stands with different durations of MCM management. We tracked changes in the genes *cbbL* and*nifH* , which respectively encode a component of ribulose-1,5-biphosphate carboxylase/oxygenase (RubisCO) and a nitrogenase reductase subunit, as these have been use previously to investigate the abundance and composition of CO_2fixation (Videmšek et al., 2009; Yuan et al.,2015) and N_2 fixation bacteria (Mmm, Marchant, & Kartal, 2018).

2. Materials and methods

2.1 study site

This study site was located in the Heping town (119°91'N, 30°79'E), changxing County, Zhejiang Province, China. This area was characterized as a subtropical monsoon climate, and the mean annual temperature and precipitation are 15.6 and 1309 mm, respectively. Soil parent material was siltstone. The stand density of the intensively managed *Moso* bamboo forests was 3000 stem ha^{-1}. Because of the rapid regeneration and growth of bamboo, plants more than 5 years old were selectively harvested every 2 years to obtain wood and achieve uniform stem density among the forest sites studied. Accordingly, a reasonable chronosequence of *Moso* bamboo forests was established with different durations (years) under intensive management even though the bamboo forests studied are uneven-aged. Inorganic fertilizers were applied at a rate of 600 Kg ha^{-1} of compound fertilizer ($N : P_2O_5 : K_2O$ = 15:15:15) every September. The annual organic manure input were about 5.25 t ha^{-1} from piggery was applied every June annually.

2.2 Experimental design and soil sampling

Before soil sampling, we conducted a strict investigation and evaluation of each plot to confirm it met the criteria for the chronosequence of intensive management. Ultimately, a chronosequence of *moso* bamboo plantations with different ages was established to represent 4 different stand ages: 6 year (IM6), 10 years (IM10), 15 years (IM15) and 20 years (IM20), respectively, each with three replicates. Additionally, three

extensively-managed *Moso* bamboo forests without fertilization or weeding located adjacent to the IM stands were considered as controls (CK). Due to having similar site conditions, including elevation, soil type, slope gradient and soil texture, we considered the study plots of represent a completely randomized design. Within each of the stands, a 10 m x 10 m plot was established, and thus 15 plots were established for the present study. All bamboo forests in the study were randomly selected within an area of about 6 km^2.

The soil samples used in this study were collected from five soil cores (0-20 cm and 20-40 cm depth) of each plot taken approximately 30 cm away from the plants; these were mixed to provide one composite sample for each layer for a total of 30 samples. Any mulch layer present was avoided during soil sampling. All the soil samples were placed on ice for transporting to the laboratory where they were sieved (2 mm) to homogenize the sample and remove visible roots and plant fragments. After sieving, a portion of each soil sample was used to analyze for physicochemical properties and another portion was stored immediately at -80 for soil DNA extraction.

2.3 Soil properties

Soil subsamples were air-dried and analyzed for chemical and physical parameters. Soil pH was determined in deionized water (soil : water, 1 : 2.5) using a pH meter (Mettler-Toledo, Switzerland)). Total SOC was measured by wet digestion with 133 mmol $L^{-1}K_2Cr_2O_7$ and concentrated H_2SO_4 at 170–180 Total N (TN) was determined using a semi-micro-Kjeldahl method. Available N (AN) was analyzed using a diffusion method. available P(AP) was extracted using a mixed solution of 0.03 mol L^{-1} NH_4F and 0.025 mol L^{-1} HCl. Available potassium (AK) was determined by flame photometric method. Soil inorganic N (NH_4^+ and NO_3^-) was extracted from soil samples with 1 M KCl and determined by colorimetrically using a segmented flow auto analyzer (Skalar, Breda, Netherlands) (Lu, 1999). The values ofδ ^{13}C and δ ^{13}N were determined using an isotope ratio mass spectrometer (IsoPrime 100,Germany) coupled with an auto elemental analyzer (vario MICRO cube,Germany).

2.4 DNA extraction and real-time PCR

For each soil sample, total microbial genomic DNA was extracted from 0.5 g frozen soil using a MoBio PowerSoilTM DNA isolation kit (MoBio Laboratories, CA, USA) according to the manufacturer's protocol. The extracted DNA was evaluated on 1% agarose gel. The quantity and quality of DNA extracts then was determined by NanoDrop ND-1000 (Thermo Scientific, USA), and stored at -20. The *cbbL* and *nifH*gene copy number were detected using gene-specific primers K2f / V2R (Yuan et al., 2012) and PolF / PolR (Poly et al.,2001; Simonet, Grosjean, Misra, Nazaret, & Normand,1991), respectively. The reactions were performed in 20 uL containing 10 uL of SYBR® Premix Ex Taq (Takara Bio Inc, Shiga, Japan), 0.2 uM of each primer, 1 uL of ROX Reference Dye, and 1 uL diluted DNA (1-10 ng) template in a final volume of 20 uL. Real-time PCR was conducted using an ABI7100 (Applied Biosystem, CA, USA) in the following conditions: 3 min at 95 for initial denaturing, followed by 40 cycles of 95 for 10 s, 62 (*cbbL*) (Yuan et al., 2012) and 55 (*nifH*) (Poly et al.,2001; Simonet, Grosjean, Misra, Nazaret, & Normand,1991) for 40 s, and 72 for 30 s, followed by a final extension for 5 min at 72 . The concentration of plasmids was measured by a Nanodrop(r) NanoDrop ND-1000 and the standard copy numbers were calculated. The plasmids containing each target gene were diluted by 10 times successively with the spanning of 10^1–$10^8$and ten-fold serial dilutions were used as standard curves. The amplification efficiency ranged from 92% to 95% with the R^2 values ranging between 0.998 and 0.999.

2.5 Terminal restriction fragment length polymorphism (T-RFLP) analysis

The microbial community structures of*cbbL*-and *nifH* -carrying populations were estimated by terminal restriction fragment length polymorphism (T-RFLP) analysis. The PCR amplification was performed using primer pairs K2f / V2r for *cbbL*and PolF / PolR for *nifH* , labeled with 6-carboxy-fluorescein (6-FAM) at the 5'end. The labeled PCR amplicons were cleaned using a SanPrepPCR Purification Kit (Sangon Biotech, Shanghai, China), and then digested digested at 37degC for 4 h using the following restriction enzymes: *Msp* I for *cbbL* and *Hae* III for *nifH* . The fluorescently labeled PCR products were cleaned as described above and then digested with *Taq* I (Takara Bio Inc, Shiga, Japan) by incubating at 65 degC for 6 h. Subsequently,

DNA fragments were analyzed using capillary electrophoresis (3730 Genetic Analyzer; Applied Biosystems, CA), using a GeneScan ROX-labeled GS500 internal size standard. Peaks were manually edited, imported into the T-REX online software (Culman et al., 2009), filtered with one standard deviation height, and clustered at 1.0 bp. Only T-RFs with the relative abundance (RA) $>$ 1 % in all three replicates were included for further analysis, and fragments with RA $>$ 10 % were regarded as dominant T-RFs (Yuan et al., 2012).

2.6 Cloning, sequencing, and phylogenetic analyses

In order to further identify the main T-RFs appearing in the profiles, clone libraries were established both the *cbbL* and *nifH* genes.The DNA extracted from the three replicates of each IM treatment were mixed and served as the template for PCR. The PCR amplification was performed with the same primers as before but without the fluorescent labels. Subsequently, the PCR products were ligated into the plasmid vector pGEM-T, and transformed into Escherichia coliJM109 (Takara Bio Inc, Shiga, Japan) by the manufacturer's instructions. Randomly selected clones (approximately 200 clones for each clone library) were screened through the reamplification with the vector-specific primers M13F and M13R and sequenced by the Sangon Biotech (Shanghai) Co., Ltd. Chimeras were detected using Decipher (Wright, Yilmaz, & Noguera, 2012) , and the BLASTn program was used to identify the most similar sequences from GenBank (NCBI). Based on 97% sequence similarity, 45 and 49 operational taxonomic unit (OTU) were obtained from 95 to 246 clones in the *cbbL* and *nifH* clone library, respectively. Only those OTUs containing at least two sequences were selected for phylogenetic analysis and T-RF assignment, and one representative sequence of these OTUs were defined for T-RFLP analysis as described above. Phylogenetic trees were constructed by the neighbor-joining method using MEGA 5.0 (Tamura et al., 2012). Bootstrapping (1000 replicate reconstructions) was used to test the reliability of phylogenetic reconstructions. All of the sequences generated in this study have been deposited in the GenBank database under accession numbers MF430858 to MF430936 for *cbbL* and MF6633454 to MF6633523 for *nifH* .

2.7 Statistical analysis

The statistical analyses were performed by SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA). Differences in soil chemical properties, and relative abundances and α-diversity indices, were determined between treatment means using a one-way analysis of variance (ANOVA), and Duncan's test was performed for multiple comparisons. A two-way ANOVA was employed to evaluate the interaction effects of soil depth and plantation age on relative abundances and α-diversity indices. Correlation analyses were followed using the Pearson correlation method. Microbial community structure and diversity was quantified based on results from T-RFLP. Non-metric multidimensional scaling (NMDS) and tests of Analysis of Similarity (ANOSIM) were used to determine differences in CO_2 and N_2 fixation bacteria composition among plantation age (Clarke & Green, 1988), and the r values associated to the NMDS were calculated using ANOSIM based on 999 permutations. NMDS and ANOSIM were computed based on the Bray–Curtis distance using the 'vegan' package in R. Redundancy analysis (RDA) was carried out to determine the relationship between soil property parameters and CO_2 and N_2 fixation bacteria composition using Canoco 4.5 for Windows. The statistical significance was defined at the 5% level unless otherwise stated. All results were reported as the means standard errors (SE) for the three replications.

3 Results

3.1 Soil physicochemical properties

The selected physicochemical characteristics of investigated soil samples are presented in Fig.1. Compared with other indicators, soil pH was generally stable having values between 5.0 and 5.5, with topsoil values being slightly higher topsoil than subsoil values. In both top- and subsoil, SOC, TN, AN, and AK showed a fluctuation pattern of significantly elevated levels for IM6 and IM20. Soil NH_4^+-N contents tended to decrease with increasing duration of intensive management in the topsoil, a significant difference ($P < 0.05$) has been only observed between CK and IM6, while it was just opposite in the subsoil. In contrast to NH_4^+-N, soil NO_3^--N increased sharply after IM6 ($P < 0.05$), and then drop to the level as CK. The variation

ranges of soil δ ^{13}C in top- and subsoil are -26.875—-25.935IM15 had the highest value of δ ^{13}C compared to other treatments, and significantly higher value ($P < 0.05$) was observed in CK and IM15 compared to the rest treatments. The variation ranges of soil δ ^{13}N in top- and subsoil are 3.45-5.86^{13}N value for CK was significantly lower than those for treatments of IM in the topsoil ($P < 0.05$), while it was generally stable in subsoil (F = 2.074, $P = 0.159$).

The *cbbL* and *nifH* gene abundance and their relationship with soil properties

The abundance of *cbbL* gene along the treatments ranged from 1.33×10^9 to 2.44×10^9copies g^{-1} soil and 5.00×10^8 to 6.81×10^8 copies g^{-1} soil for topsoil and subsoil respectively (Fig.2a), and the differences observed between layers was significantly different ($P < 0.01$). The abundance of*cbbL* decreased significantly in IM6, IM10 and IM15 compared to CK in topsoil ($P < 0.05$) and then recovered to the original level in IM20. Two-way ANNOVA analysis of variance showed that the abundance of *cbbL* gene was affected significantly by both IM (F = 15.147, $P = 0.000$) and soil depth (F = 384.892, $P = 0.000$), but the latter had a much greater effect than the former. The*cbbL* gene copy numbers were positively correlated with AK (r = 0.632, $P < 0.05$) in the subsoil, and with AN (r = 0.688, $P < 0.05$) and AK (r = 0.690, $P< 0.05$) in the subsoil, respectively.

The abundance of *nifH* gene ranged from $1.54\times10^6$to 2.31×10^7copies g^{-1} soil in the topsoil and from 1.43×10^6 to 1.62×10^7copies g^{-1} soil in the subsoil (Fig.2b). As for *cbbL* , the *nifH* gene copy numbers in both layers decreased significantly ($P < 0.05$) at IM6. Copy numbers increased gradually from IM10 to IM20 but remained lower than for the CK ($P < 0.05$). Two-way ANOVA showed that the abundance of*nifH* gene was affected significantly by IM (F = 11.872, $P= 0.000$) rather than soil depth (Table.2). It was found that the*nifH* gene copy number was positively correlated with NH_4^+-N (r = 0.655, $P< 0.01$) in the topsoil and with C:N (r = 0.628, $P< 0.05$) in subsoil, while negatively with NH4$^+$-N (r = 0.773, $P < 0.01$) in subsoil.

Community analysis of the *cbbL*-and*nifH*-containing bacteria

A total of 17 T-RFs was identified from all samples and used to compare*cbbL* -containing bacteria numbers among the treatments (Fig.3a). The six predominant T-RFs, having lengths of 40, 44, 168, 175, 177, and 360 bp, varied in the relative abundance among soils. The T-RF lengths of 40 bp (9.7-24.1%) and 177 bp (22.1-54.4%) were among the most dominant for all treatments. In topsoil, the relative abundance of T-RFs having 44bp and 360bp decreased sharply after six years of intensive management, whereas the T-RF of 360bp decreased sharply in subsoils after 10 years of IM. The T-RF having 168bp was observed only after after 15 years of IM in both topsoil and subsoils. Two T-RFs, 439bp and 488bp, were unique to subsoils. The most dominant T-RF 177bpwas closely related to several species of α -Proteobacteria including*Bradyrhizobium sp.* (CP013949.1), *Rhodospirillum centenum*(CP000613.2), *Mesorhizobium cicero* (CP015064.1), and*Starkeya novella* (CP002026.1), one species ofβ -Proteobacteria named *Stappia meyerae* (EF101506.1), and one γ-Proteobacteria named *Thioflavicoccus mobilis* (CP003051.1). The T-RF 44 bp most closely matched *Starkeya novella*(CP002026.1). The 360bp T-RF was especially abundant in soil from the CK and IM6 treatments and most closely matched the three species*Actinopolymorpha singaporensis* (LT629732.1), *Mesorhizobium ciceri* (CP015064.1), and *Rhodospirillum centenum* (CP000613.2). The minor species*Starkeya novella* (CP002026.1) and *Bradyrhizobium sp.*(CP013949.1) were represented by the T-RF 364bp, whereas*Thermomonospora curvata* (CP001738.1) and *Stappia meyerae*(EF101506.1) were represented by T-RFs 129 and 439bp, respectively.

A total of 17 T-RFs was identified from all samples and used to analyze communities shift of *nifH* -containing bacteria (Fig.3b). The relative abundance of the five dominant T-RFs of 68, 154, 177, 180, and 332 bp varied between soils, with the 180bp T-RF (22.1-54.4%) being most dominant in all treatments. The relative abundance of T-RFs 154 and 177 bp were higher in IM6, IM10, and IM15 than in CK and IM20 in both layer of soils, while T-RFs 68 and 332bp exhibited opposite trends. The four T-RFs of 75, 81, 147, 160, 180, and 187bp were most closely related to two groups - *Rhizobium* sp. (M16710.1) and *Azorhizobium doebereinerae* (FJ223129.1). The T-RF 47 bp closely matched*Desulfovibrio vulgaris* (CP002298.1) (Fig.4b).

The relationship between soil properties and *cbbL*- and*nifH*-containing bacterial community

The difference in T-RFs profile of CO_2- and N_2-fixing bacteria between treatments was confirmed by ANOSIM ($P < 0.01$). For the former, the two-dimensional NMDS plot revealed that the treatments of all IM clustered closely together and separately from CK in the topsoils. (Fig.5a). However, the treatments were divided into three groups in subsoils, with CK and IM6 comprising the first group, IM10 the second (both groups located in the same side of NMSD1), and IM15 and IM20 the third group (located on the opposite side of NMSD1) (Fig.5b). In contrast, diazotrophic bacteria from the CK and IM20 treatments formed tight clusters separated from IM6, IM10 and IM15 (Fig.5c;5d).

Redundancy analyses (RDA) by Monte Carlo permutation test revealed that AP ($P = 0.020$), $\delta\ ^{13}$C ($P = 0.044$) and NH_4^+-N ($P = 0.042$) significantly explained the community shift of *cbbL* -containing bacteria in topsoils in response to IM duration (Fig.6a). In subsoils, only $\delta\ ^{13}$C (P =0.039) correlated well with community variations of this bacteria (Fig.6b). There was no correlation observed between soil pH and *cbbL* -containing bacterial community in either topsoils or subsoils. It is interesting that $\delta\ ^{13}$C in both layers of soil was positively correlated with the treatment IM15 and IM20, suggesting increasing $\delta\ ^{13}$C may responsible the special composition of *cbbL* -containing bacteria. The samples were divergent along the first two axis, which explains 59.7 % of the variation. For *cbbL* -containing bacteria, the first two axes explained 79.5% and 84.0% of the total variation in topsoil and subsoils, respectively. As for *nifH* -containing bacterial community, they were significantly affected soil AK ($P = 0.037$), SOC ($P = 0.024$), C:N ($P = 0.033$), and AN (P =0.038) content in topsoils (Fig.6c), and with AP ($P = 0.009$) and C:N ($P = 0.001$) in the subsoils (Fig.6d). The first two axes of the RDA accounted for 59.7% of the variance of the diazotrophic community composition, with the first axes accounting for 56.4% of the variance.

3.5 The *cbbL*- and *nifH*-containing bacterial diversity and their relationship with soil properties

Soil microbial diversity was employed as a third indicator of microbial response to IM. The diversity of CO_2 and N_2 fixation bacteria responded similarly to IM (Table 2). It was observed that IM10 was unique in both topsoils and subsoils with respect to diversity indices of CO_2 fixation bacteria, with both Shannon and Evenness values being lower level compared with the remaining treatments ($P < 0.05$), while the Simpson index displayed an opposite trend. Similar trends were observed for diversity indices of diazotrophic bacteria in topsoils, while no differences were observed in subsoils. Two-way ANOVA analysis of variance revealed that the diversity of *cbbL* - and *nifH* -containing bacteria was significantly affected by IM time and even more so by soil depth (Table 2). Correlation analysis between diversity index and soil properties based on same layer revealed that evenness and Shannon indices of *cbbL* -containing bacterial were positively correlated only with AK ($P = 0.016$) in the topsoils and with AP ($P = 0.015$) in the subsoils (Table 3), respectively. However, more soil factors were positively correlated with diversity index of *nifH* -containing bacterial, including evenness index (E) with SOC ($P = 0.021$), TN ($P = 0.033$) and AN ($P = 0.040$) in topsoils, and with SOC ($P = 0.020$), TN ($P = 0.014$) and AN ($P = 0.023$) in subsoils. Soil TN and AN were also positively correlated with Shannon index in subsoils.

1. **Discussion**
2. **Alteration of soil properties during 20 year of IM**

Soil pH usually decreases as a result of long term fertilization with high rates of mineral fertilizer (Schroder et al.,2011). It is encouraging that soil pH was stable in response to MCM management in this study. Soil nutrients usually accumulate with increasing duration of IM because of annual fertilizer applications (Tan et al.,2013). This study demonstrated that application of MCM resulted in enhanced soil fertility with the exception of NH_4^+-N. The increased NO_3^--N and decreased NH_4^+-N over time might be the result of enhanced nitrification, a commonly observed response to intensive agriculture (Bi et al., 2017). This improved fertility is mainly attributed to direct input of mineral fertilizers and indirect supplement of organic fertilizers decomposition. Soil OC varied over time, increasing sharply at IM6, returning to a lower level similar to CK for more than 10 years, and finally recovering somewhat at IM20. It has been shown that organic fertilizer usually improves SOC in long-term studies (Han et al.,2018). We also detected an increase of SOC after 20 years of IM, although levels did fluctuate during the preceding 15 years. The dynamics of SOC depends on the balance of OC input and its mineralization. The sharp increase at the first stage was reflected the large

amount of manure applied that did not decomposed rapidly. However, mineralization accelerated with the improvement of soil microbial community during the second stage, so that SOC then dropped to the same level as CK. Soil $\delta^{13}C$ is closely related to SOC dynamics and is an important index to study the history of reconstructed plant communities, determine the SOC source, soil quality, and soil C sequestration rates (Mendez-Millan et al., 2014; Zhang et al.,2015). Soil $\delta^{13}C$ increased with duration of IM and reached the maximum at IM15 (Fig. 1). This increase in $\delta^{13}C$ may be attributed two factors. One was that the lighter isotopes of ^{12}C were easier to volatilize via organic matter decomposition compared to those containing^{13}C, which resulted in the increased relative abundance of ^{13}C (Guillaume, Muhammad, & Kuzyakov, 2015). It is possible that the chemical bonds containing ^{12}C are easier to break down than ^{13}C in enzymatic reaction (Powers & Schlesinger, 2002). Another one was that input pig manure was rich in^{13}C because pig food contain C4 plant of maize having higher $\delta^{13}C$ (ranged from-17plant (ranged from-32& Hubick, 1989).

4.2 Alteration of abundance of gene *cbbL* and *nifH* and their mediated factors during 20 year of IM

The significant difference in abundance of *cbbL* gene in both two soil layers indicted that bamboo IM practice with MCM affects CO_2 fixation bacteria. The change in *cbbL* gene copy numbers was positively related with AK in both topsoils (r = 0.63,$P < 0.05$) and subsoils (r = 0.69, $P < 0.05$), and also with AN (r = 0.69, $P < 0.05$) in subsoils. It has been reported that the abundance of *cbbL* gene could affected by several factors including pH, SOC, AN, and C:N (Guo et al., 2015; Yuan et al., 2013; Yuan et al., 2015), and the major factor responsible may have varied over time. In this study, soil AK was in short supply and may have become the limiting nutrient for CO_2 fixation bacteria, as so too may have AN in subsoils. The lower abundance during the middle years may reflect lower AK. The IM management induced a great decline in copy numbers of gene*nifH* in both soil layers ($P < 0.05$) for IM6, although they recovered gradually afterward to some extent. Diazotrophic bacteria were logically more active when N was limiting (Pereira e Silva, Semenov, van Elsas, & Salles, 2011; Reardon, Gollany, & Wuest,2014). The increased soil AN and NO_3^--N may be responsible for sharp reductions in gene *nifH* copy numbers. However, no significantly negative relationship was observed between *nifH* gene number and AN, nor with NO_3^--N. The opposite relationship between *nifH* gene copy number and NH_4^+-N was observed in the topsoil and in subsoil. The biological N_2 fixation is a high energy consuming process and need an ample of OC supply (Pfister, Meyer, & Antonopoulos,2018). The positive relationship between *nifH* gene number and C : N in topsoil was in accordance with result from long-term experienced 25 years of inorganic fertilization (Wang et al., 2017).

4.3 Community shift of CO_2 and N_2 fixation bacteria and driving factors during 20 year of IM

The CO_2 and N_2 fixation bacterial communities responded differently to IM. The CO_2fixation bacterial community changed gradually with IM duration, with the structures associated with CK and IM20 differing the most. However, a relatively similar structure for soil diazotrophs was observed between CK and IM20, indicating that community of N_2 -fixing bacteria responded to the disturbance at earlier stage of IM but returned to resemble the original structure at IM20. These results suggest that the community structure of soil diazotrophic bacteria is more resilient than that of CO_2 bacteria, despite declines in abundance. Soils from all treatments had six predominant species of CO_2 fixation bacteria but these varied in their relative abundances. The relative abundance of the most predominant T-RF 177 bp was lowest ($P < 0.05$) in CK, indicating IM improved the species represented by T-RF. RDA revealed that numbers of this T-RF in IM topsoil samples were positively related to AP and $\delta^{13}C$, indicating soil AP might favor the associated species. In addition, soil P content has also been shown to be a major regulator for *cbbL* -containing algae composition (Yuan et al., 2015). The most dominant group was composed of several groups of *a,* β ,γ -Proteobacteria - *Bradyrhizobium sp.* , *Rhodospirillum centenum* , *Thioflavicoccus mobilis* ,*Stappia meyerae* , *Mesorhizobium ciceri* , and *Starkeya novella.* In the contrast, the relative abundance of T-RFs 44bp and 360bp in topsoil decreased sharply after 6 or 10 years of IM, suggesting that the associated species may have been inhibited by rich soil nutrition. Proteobacteria are fast-growing copiotrophs that thrive in environments with high carbon availability (Fierer, Bradford, & Jackson, 2007). Soils in this studied contained five dominant types of diazotrophic bacteria, with the highest relative abundance observed (22.1-54.4% for T-RF 180bp) being

reached in the middle stages of IM. These higher abundances of T-RFs exhibited an interesting relationship of 'growth and decline' between groups of CK and IM20 vs the rest group of IM treatments. This phenomenon suggested that different species of diazotrophic bacteria have unique environmental preferences. It was difficult to recognize taxa were favored under IM because several T-RFs identified to same species. The number of factors driving changes within the diazotropic community greater than for CO_2 fixation bacteria and included AK, SOC, C : N and AN content in topsoils, and AP and C:N in the subsoils. Two taxa of diazotrophic bacteria, *Rhizobium* sp. and *Azorhizobium doebereinerae* , were the most dominant of those detected in the soils sampled.

4.4 Alteration of diversity of CO_2 and N_2 fixation bacterialcommunity and driving factors during 20 year of IM

Just as the abundances of CO_2- and N_2-fixing bacteria responded differently to IM, so too did the corresponding measures of diversity used in this study. A dip in Shannon and Evenness index values detected for CO_2-fixing bacteria at IM10 in both topsoils and subsoils was opposed to the opposite trend observed for the Simpson index. Lower values for Shannon and Evenness indices after 10 years of IM may reflect a collective effect of large applications of manure. The Shannon and Evenness indices, which reflect the total numbers and evenness of microbial species, was decreased by manure input, which may have stimulated certain groups of CO_2-fixing bacteria bacteria. Then, as the effect of manure declined with further duration of IM, the indices recovered gradually. On the contrary, the Simpson index reflects the primarily the dominant species and so may have increased significantly at IM10 due to the dominance of TRF 177 bp. The recovery of Shannon and Evenness index after 10 years of IM may be attributed to an increase in the number of species, but these were quite different from those in the CK and IM6. Overall, these results indicate that the communities of CO_2 fixation bacteria were altered by IM practice. Conversely, both the Shannon and Evenness indices for the diazotrophic communities indicated a return to the original state (CK) suggesting that their communities was not impacted significantly by IM practice. The above results implied that CO_2 -fixing bacteria was more sensitive to IM practice with application of MCM than diazotrophic bacteria.

5 Conclusion

This study demonstrated that 20 years of IM practice using MCM (combined inorganic fertilizer and manure application) in *Moso* bamboo plantation enhanced the content of SOC and available soil N, P, and K, although fluctuations were observed. Soil pH was generally stable with values between 5.0 and 5.5, indicating little acidification occurred. Soil CO_2 and N_2 fixing bacteria responded strongly to IM strongly in the short term and then gradually recovered over time, indicating an inherent resilience. The abundance of CO_2-fixing bacteria eventually returned to their original levels but their communities did not. In contrast, the communities of N_2-fixing bacteria recovered to their initial state while their abundances did over after 20 years of IM. The diversity of both groups of bacteria had a turning point after 10 years of IM, as reflected by higher values for Shannon and Evenness indices but lower values for the Simpson index compared with the remaining treatments. Both CO_2 and N_2 fixation bacterial groups had similar dominant species, but these differed in their relative abundances among the treatments and also exhibited different patterns in response to IM duration. The results indicated that relationships between potential environmental drivers and measures of abundance and community composition for both CO_2- and N_2- fixing bacteria are complex. Nevertheless, IM practice using MCM in *Moso* bamboo plantation appeared to have an overall positive effect on these bacterial groups as well as on associated soil properties.

Acknowlegements

This work was financially supported by the Natural Science Foundation of Zhejiang Province (LZ16C160002) and the National Natural Science Foundation of China (31570602), and Overseas Expertise Introduction Project for Discipline Innovation (D18008).

Conflict of interest :The authors declare no conflict of interest.

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Table 1 Primers used for PCR amplification

	Primers	Sequences (5'-3')
cbbL gene	K2f	5'-ACC AYC AAG CCS AAG CTS GG-3'
	V2f	5'-GCC TTC SAG CTT GCC SAC CRC-3'
nifH gene	FGPH19	5'-TAC GGC AAR GGT GGN ATH G-3'
	PolR	5'-ATS GC ATC ATY TCR CCG GA-3'

	Primers	Sequences (5'-3')
	AQER	5'-GAC GAT GTA GAT YTC CTG-3'
	PolF	5'-TGC GAY CCS AAR GCB GAC TC-3'

R = A / G; N = A / G /C /T; H = T / C /A; Y = C / T; S = G / C

Table 2 Shannon index, Evenness index, and Simpson index based on T-RFLP data of CO_2 and N_2 fixation bacteria in topsoil and subsoil under long-term intensively managed*Moso* bamboo forests

		cbbL gene	*cbbL* gene	*cbbL* gene	*cbbL* gene	*nifH* gene	*nifH* gene	*nifH* gene	*nifH* gene
		Shannon index (H)	Shannon index (H)	Evenness index (J)	Simpson index (D)	Shannon index (H)	Evenness index (J)	Evenness index (J)	Simpson index (D)
0-20 cm	CK	1.73±0.19 a	1.73±0.19 a	0.62±0.08 a	0.22±0.03 c	1.97±0.09a	0.81±0.01ab	0.81±0.01ab	0.18±0.01b
	IM6	1.28±0.18 a	1.28±0.18 a	0.62±0.08 a	0.39±0.07 ab	2.16±0.14a	0.91±0.02a	0.91±0.02a	0.12±0.02b
	IM10	0.83±0.03 b	0.83±0.03 b	0.54±0.05 a	0.51±0.03a	1.57±0.29b	0.71±0.11c	0.71±0.11c	0.32±0.12a
	IM15	1.16±0.24 ab	1.16±0.24 ab	0.59±0.08 a	0.42±0.09 ab	1.80±0.06ab	0.76±0.01bc	0.76±0.01bc	0.22±0.01ab
	IM20	1.40±0.20 a	1.40±0.20 a	0.72±0.07 a	0.33±0.10 bc	2.12±0.31a	0.86±0.06ab	0.86±0.06ab	0.14±0.05b
20-40 cm	CK	1.59±0.15 ab	1.59±0.15 ab	0.68±0.01 a	0.26±0.03 b	1.90±0.13a	0.80±0.05a	0.80±0.05a	0.19±0.03a
	IM6	1.43±0.03 b	1.43±0.03 b	0.69±0.02 a	0.28±0.02 b	2.06±0.22a	0.86±0.04a	0.86±0.04a	0.15±0.04a
	IM10	1.01±0.14 c	1.01±0.14 c	0.52±0.09 b	0.49±0.09 a	2.00±0.12a	0.85±0.03a	0.85±0.03a	0.15±0.02a
	IM15	1.47±0.10 b	1.47±0.10 b	0.76±0.01 a	0.27±0.04 b	2.06±0.02a	0.84±0.02a	0.84±0.02a	0.16±0.01a
	IM20	1.76±0.06 a	1.76±0.06 a	0.75±0.01 a	0.21±0.03 b	2.10±0.14a	0.87±0.04a	0.87±0.04a	0.14±0.03a
Factor	P(F) value	P(F) value	P(F) value	P(F) value	P(F) value	P(F) value	P(F) value	P(F) value	P(F) value
Depth	**0.005 (9.953)**	**0.005 (9.953)**	**0.003 (11.612)**	**0.003 (11.612)**	0.140 (2.355)	0.138 (2.392)	0.138 (2.392)	0.057 (4.071)	0.057 (4.071)
IMT	**0.000 (22.314)**	**0.000 (22.314)**	**0.000 (18.329)**	**0.000 (18.329)**	**0.009 (4.508)**	**0.020 (9.953)**	**0.020 (9.953)**	**0.006 (4.896)**	**0.006 (4.896)**
Depth*IMT	0.073 (2.529)	0.073 (2.529)	0.063 (2.656)	0.063 (2.656)	0.373 (1.125)	0.064 (2.683)	0.064 (2.683)	**0.019 (3.763)**	**0.019 (3.763)**

CK, non-managed *Moso* bamboo forest. IM6, IM10, IM15, and IM20 are*Moso* bamboo forests received 10, 15, 20, and 25 years of intensive management, respectively. Depth stands for soil depth with 0–20 cm and 20–40 cm soil layers. IMT stands for intensive management time. Shown are mean values and associated standard deviations. Different characters in a single column indicate significant difference between the treatments at $P < 0.05$.

Table 3 Correlation coefficients of environment factors with the biodiversity indices of *cbbL* and *nifH* genes

(n = 3)

	cbbL	cbbL	cbbL	cbbL	cbbL	cbbL	nifH	nifH	nifH	nifH	nifH
	0-20cm	0-20cm	0-20cm	20-40cm	20-40cm	20-40cm	0-20cm	0-20cm	0-20cm	20-40cm	20-40cm
	H	*J*	*D*	*H*	*J*	*D*	*H*	*J*	*D*	*H*	*J*
pH	0.385	0.117	-0.317	-0.149	-0.347	0.267	0.284	0.249	-0.365	0.112	0.048
SOC	0.174	0.224	-0.148	0.417	0.279	-0.395	0.451	**0.589***	-0.462	0.315	**0.423***
TN	0.094	0.148	-0.051	0.419	0.282	-0.389	0.492	**0.551***	-0.405	**0.404***	**0.445***
C:N	0.231	0.270	-0.241	-0.063	-0.031	-0.002	0.223	0.379	-0.347	0.091	0.217
AN	-0.030	0.188	0.061	0.478	0.367	-0.445	0.485	**0.534***	-0.430	**0.372***	**0.413***
AP	-0.500	-0.373	0.483	**0.612***	0.243	-0.456	-0.333	-0.366	0.296	-0.155	-0.169
AK	0.284	**0.608***	-0.391	0.315	0.190	-0.227	0.116	0.131	-0.181	0.062	0.063
NO_3^--N	-0.438	-0.253	0.404	0.084	0.089	-0.193	0.188	0.265	-0.069	0.155	0.225
NH_4^+-N	0.296	-0.245	-0.299	0.068	0.203	-0.093	-0.216	-0.192	0.145	-0.294	-0.250
$\delta^{13}C$	-0.447	-0.430	0.487	-0.167	0.213	0.129	-0.450	-0.456	0.401	0.172	0.084
$\delta^{15}N$	-0.395	-0.051	0.382	-0.332	-0.472	0.347	-0.018	0.110	0.017	-0.393	-0.208

H stands for Shannon index, *J* stands for Evenness index, and *D* stands for Simpson index. F values labeled with * are statistically significant at $P < 0.05$. SOC, TN, AN, AP, AK, NH_4^+-N, NO_3^--N indicate soil organic C, total N, available N, available P, available K, ammonium, and nitrate, respectively.

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