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Composition and Activity of N_2-Fixing Microorganisms in Mangrove Forest Soils

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Abstract: Mangrove forests are considered to be a highly productive ecosystem, but they are also generally nitrogen (N)-limited. Thus, soil N_2 fixation can be important for the stability of both mangrove ecosystem functions and upland N supply. This study evaluates the N_2 fixation activity and composition of relevant microorganisms in two coastal mangrove forests—the Guandu mangrove in an upstream estuary and the Bali mangrove in a downstream estuary—using the acetylene reduction method, real-time polymerase chain reaction, and next-generation sequencing. The results demonstrated that ambient nitrogenase activity was higher in downstream mangrove forests (13.2–15.6 nmol h^{-1} g^{-1} soil) than in upstream mangrove forests (0.2–1.4 nmol h^{-1} g^{-1} soil). However, both the maximum potential nitrogenase activity and nitrogenase gene (*nifH* gene) copy number were found to be higher in the upstream than in the downstream mangrove forests, implying that the nitrogenase activity and diazotrophic abundance may not necessarily be positively correlated. In addition, amended MoO_4 (which inhibits the activity of sulfate-reducing bacteria in N_2-fixation) yielded low nitrogenase activity, and sulfate-reducing bacteria made up 20–50% of the relative diazotrophic abundance in the mangrove forests, indicating that these bacteria might be the major active diazotrophs in this environment.

Keywords: mangrove; N_2 fixation; diazotrophs; sulfate reducing bacteria; *nifH* gene; acetylene reduction

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1. Introduction

Coastal ecosystems have long been considered to have high primary production and biodiversity, but they are generally limited in nutrients such as nitrogen (N) and phosphorus (P) [1,2]. Thus, microbial N_2 fixation is an important means to sustain the N supply in coastal ecosystems. Indeed, studies have demonstrated that the microbial N_2 fixation rate can be high in coastal ecosystems. Bertics et al. [3], for example, used the acetylene reduction method and reported a N_2 fixation of 0.42–8.05 mmol N m^{-2} d^{-1} in marine sediment.

The mangrove forest is an important coastal ecosystems in tropical and sub-tropical regions and occupies more than 16.4 million hectares worldwide [4]. It provides many ecosystem services, such as wildlife habitats, blue carbon (C) storage, and storm water dissipation [5]. Mangrove plants grow quickly, so the demand for N and P can be large in this ecosystem. Studies have estimated that microbial N_2 fixation may support 40%–60% of the N demand of the mangrove ecosystems [6]. Moreover, studies are increasingly focusing on evaluating how microbial N_2 fixation contributes to mangrove forests. Microbial N_2 fixation rates can vary widely from 0 to 10.1 mmol N m^{-2} d^{-1}, depending on the mangrove forest's geographic location [7–10].

Microbial N_2 fixation rates generally depend on the availability of soil C and N [11,12]; for example, N_2 fixation is regulated by the availability of organic C from plant roots, while low NH_4^+ concentration in the vicinity of the plant roots during the plant growth season can maintain N_2 fixation activity [10]. However, there may be other environmental factors regulating the microbial N_2 fixation processes that are yet to be understood. N_2 fixation also corresponds to soil P availability [13]. Using MoO_4, a chemical that inhibits sulfate-reducing bacteria (SRB) activity, as an inhibitor, several studies have further indicated that SRB can contribute over 50% of the total N_2 fixation in mangrove forests [3,9].

Next-generation sequencing has recently been applied to elucidate the soil bacterial [14,15] and diazotrophic communities [16] in mangrove forests. The functional *nifH* gene in diazotrophs was also used to examine diazotrophic communities in mangrove soils [15,17]. The results of these analyses demonstrated that diazotrophs such as *Azotobacter*, *Azospirillum*, and *Vibrio* were dominant in mangrove soils [18], while others have found genera such as *Desulfuromonas* and *Pseudomonas* to be the most important N_2-fixing microorganisms in this system [19]. In addition, environmental factors such as soil C content [20] and plant species [21] may affect total bacterial and diazotroph communities in mangrove ecosystems.

Seawater contains high sulfate concentrations, and thus, sulfate reduction occurs when estuarine water salinity is higher than 1 psu [22]. Moreover, NaCl in saline water is toxic to cellular metabolism and is considered to cause physiological stress in most microorganisms [23]. Previous studies commonly observed a high relative abundance of diazotrophs belonging to SRB in mangrove soils [15] and thereby suggested that diazotrophs contribute to the N and S biogeochemical cycles in mangrove soils [24].

In our previous microcosm study, which aimed to determine the N_2 fixation processes in mangrove forest soils, we found that the nitrogenase activity was not affected by changing salinity [9]. Furthermore, a MoO_4 inhibition essay demonstrated that SRB may contribute more than 51% of the total nitrogenase activity in the studied mangrove forests [9]. However, whether the diazotrophic communities remain the same under different salinity conditions, and whether diazotrophs contribute to N_2 fixation to the same extent, is still unclear. Thus, the present study aimed to determine the diazotrophic community structures in tropical mangrove forest soils, using high-throughput sequencing, and evaluate how their abundances change with the various physiochemical factors that affect them.

SRB can be found in environments when sulfate is present [25] and in many marine environments [26,27]; consequently, we hypothesized that SRB in mangrove soils increase in abundance under increasing salinity, which in turn increases the importance of SRB in the microbial N_2 fixation process.

2. Materials and Methods

2.1. Sampling Locations

Two mangrove forests were used as study sites, both located in the downstream estuary of Tamsui River estuary in Taipei, Taiwan. The first, Guandu, is located 8 km from the Tamsui River estuary and has an average salinity of 17 psu, while the second, Bali, is located next to the mouth of Tamsui River and has an average salinity of 21 psu (Figure 1). *Kandelia obovata* (Sheue, Liu & Yong) is the dominant mangrove species in both forests, although other plants such as *Phragmites communis* (Cav.) Trin. ex Steud and *Cyperus malaccensis* Lam. were also found. Further details on the study sites are presented in a previous study [28].

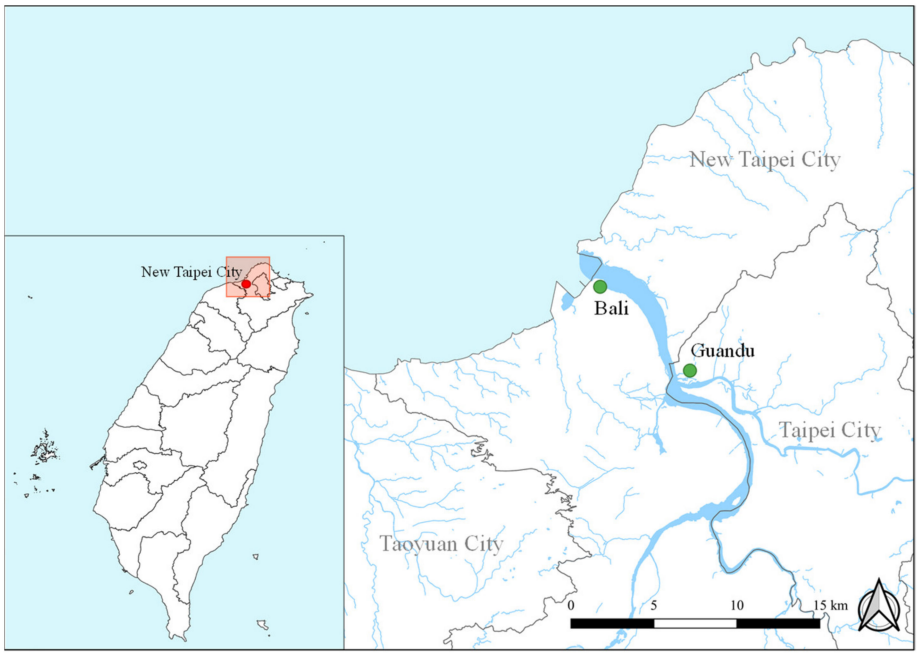

Figure 1. Two mangrove forest sites in Taiwan were sampled in the present study.

Operating on the assumption that biological activity mostly occurs on the surface soil, we only studied the surface soils. Briefly, 25 soil core samples were collected by random walk using polyvinyl chloride (PVC) tubes, 2.5 cm in diameter and 30 cm long, at each site, in August 2018. A mark that indicates 20 cm depth had been labeled on each PVC tube before the field sampling. Each soil core sample was collected by directly pushing a PVC tube into the soil over the 20 cm mark. The top of the tube was covered with a rubber stopper, after which the PVC tube was pulled out of the soil. The PVC tubes with soil samples inside were then wrapped in clean plastic bags, stored on ice, and returned to the laboratory for analysis.

The collected soils were pushed out from the top of the tubes using a silicone stopper pretreated with 70% ethanol. Then, the length of the soil core was measured with a ruler. Because the compaction factor measured was on average around 0.5, we kept only 0–9 cm depths of these apparent soil samples (originally 0–18 cm) for the subsequent experiment and discarded the remaining soil. We multiplied the compaction factor and used 0–6, 6–12, and 12–18 cm afterward to illustrate the results instead of the apparent depth.

Bulk soil samples were sliced into 3 sections (0–6, 6–12, and 12–18 cm deep) using a knife pretreated with 70% ethanol. Then, the 25 soil samples in the same depth ranges were mixed together in a plastic sealed bag to form a composite sample, and were stored at 4 °C for further analyses.

The structure of mangrove soil is highly heterogeneous due to the activities of invertebrate shredders such as crabs, so we simply combined the soil cores of different depths to form composite samples, which we believed would yield a sampling that was representative of the microbial compositions in mangrove soils. We analyzed triplicates to ensure that the composite samples were well mixed and compared the results to make sure that the composite samples were indeed representative of the mangrove forests in general.

2.2. Determining Diazotroph Abundance and Compositions

Total genomic DNA was extracted from ~0.8 g fresh soil using the PowerSoil® DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) based on the manufacturer's instructions. The extracted total DNA was stored at −20 °C until proceeding to the sequence analysis. Subsamples of the total genomic DNA were diluted to 5 ng μL^{-1} to evaluate the abundance of *nifH* gene by real-time quantitative polymerase chain reaction (qPCR) analysis, with a primer pair (FGPH19/PolR) [19,29,30] and qPCR reagent kit

(RR420A, SYBR® Premix Ex Taq™, Takara Bio Inc., Shiga, Japan). The samples were amplified using the following cycling steps: 5 min at 95 °C, followed by 40 cycles of 92 °C for 10 s, 55 °C for 30 s, and 72 °C for 90 s.

To evaluate the composition of diazotrophs in mangrove soils, a universal primer pair (515F/907R) and reagent kit (KAPA HiFi HotStart ReadyMix, KAPA Biosystems, Wilmington, MA, USA) were used to amplify the V4 region of the 16S rRNA gene. Then, the PCR amplicons from each site were labeled with specific barcodes based on the manufacturer's instructions (Illumina Miseq, San Diego, CA, USA), pooled to form a single DNA library, and analyzed on the Illumina Miseq platform to determine microbial community structures.

The retrieved sequence data were analyzed with the Mothur package [31] and classified using the Ribosomal Database Project (RDP) pipeline (http://pyro.cme.msu.edu/, accessed on 1 June 2020), with a confidence cutoff of 80% to determine the soil microbial compositions. Then, the microbial communities associated with diazotrophs were selected based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database for analysis. The genera of diazotrophs with a relative abundance of less than 1% were pooled and reported as "Others".

To analyze the composition of diazotrophs using the *nifH* gene, a primer pair (FGPH19/PolR) was also used to perform PCR. The *nifH* gene amplicons labeled with specific barcodes based on the Illumina Miseq instructions were also sequenced on the Illumina Miseq platform. The obtained *nifH* gene sequence data were analyzed with R program (version: 3.2.2) and classified based on a *nifH* gene database (https://blogs.cornell.edu/buckley/nifh-sequence-database/, accessed on 22 June 2021)).

2.3. Soil Incubation and Nitrogenase Activity Determination

Soil nitrogenase activity was analyzed using the acetylene reduction method [32]. Three replicate samples were prepared for each site. For each replicate, 10 g of soil was weighed from a composite sample of the 2 sites and placed in a 125-mL serum bottle. The bottle was then sealed with an aluminum cap over a rubber stopper.

The headspace in the bottle was replaced with argon (Ar) gas to create an anoxic environment for diazotrophs. For this purpose, each bottle was stuck with a needle and placed in a desiccator. The air in the desiccator was pumped out and replaced with Ar gas 3 times to ensure that the oxygen concentration was almost entirely depleted. After the anoxic bottles were made, 10% of the bottle headspace was replaced with acetylene (C_2H_2) using a plastic syringe. Then, the samples were incubated at 25 °C (LM-570RD, Yihder Technology Co., Taipei, Taiwan).

After 0, 20, and 24 h of incubation, the concentrations of C_2H_4 were measured by gas chromatography with a flame ionization detector (GC-FID, GC9720, Fuli Instruments, Zhejiang, China) and fitted with a linear regression curve to calculate the C_2H_4 production rates over time (Figures S1–S3).

To determine the maximum potential activity, the soil replicates underwent the same preparation procedures as described above, except that 5 mL of the glucose solution (20 mM) was added to each replicate bottle before incubation at 25 °C. To evaluate the contribution of SRB to N_2 fixation in mangrove soils, 1 mL of an MoO_4 solution (20 mM of $Na_2MoO_4 \cdot 2H_2O$), an inhibitor that suppresses SRB activity, was added to each replicate bottle with a glucose solution (20 mM) and mixed with the soils before incubation [9].

2.4. Determining Soil Physiochemical Properties

Soil pH was measured by mixing soil with water, at a 1:1 ratio, and the solution was measured using a pH meter with a glass electrode (Jenco 6009, Jenco Instruments, San Diego, CA, USA). Soil salinity was determined with a salinity refractometer. Soil total organic C (TOC) and total N (TN) were evaluated by the combustion method with an elemental analyzer (Fisons NA1500, ThermoQuest Italia, Milan, Italy). Soil soluble organic C (S_bOC), soluble organic N (S_bON), ammonium (NH_4^+), nitrate (NO_3^-), and total

dissolved N (TDN) were extracted using a 2 M KCl extraction method [33,34]. Briefly, 5 g soil was soaked in 50 mL KCl for 1 h at 150 rpm. Then, the extracts were filtered from the slurry and analyzed with the cadmium reduction method to measure NO_3^- [35], the indophenol method for NH_4^+ [36], and the persulfate method for TDN [37]. S_bOC was analyzed with a TOC analyzer (1010, Analytical, Houston, TX, USA).

2.5. Statistical Analysis

The collected data were first tested for their normality using the Shapiro-Wilk W test. Then, the differences in nitrogenase activities across the different soil depths were tested for both study sites with a two-way analysis of variance (two-way ANOVA), using JMP 11.0 (SAS Inc., Cary, NC, USA). After differences were revealed by the two-way ANOVA, a Student's paired *t*-test was applied to further compare the means of variables between the two sites, and a Tukey's honesty significant difference (HSD) test was applied to compare the means across the three soil depths. $p < 0.05$ was considered significant for the tests.

In addition, a canonical correlation analysis (CCA) was used to determine the relationship among the diazotrophs and soil physiochemical factors. The CCA was analyzed with XLSTAT (Addinsoft, New York, NY, USA).

3. Results

Potential nitrogenase activity increased as the samples moved downstream from Guandu to Bali, but no difference was found among the three sampling depths (Figure 2; Table 1). With the amended glucose solution, the maximum potential activities increased 10–40 and 3–4-fold in the Guandu and Bali mangrove soils, respectively, compared to their ambient potential activities, and the rates decreased with increasing soil depth (Figure 3). In addition, the nitrogenase activities at the 0–6 and 6–12 cm soil depths were almost undetectable in the Guandu forest after being amended with MoO_4 solution. However, about one-third to one-tenth of the nitrogenase activities were still detected in all the Bali soils and in the 12–18 cm deep Guandu soils after the MoO_4 solution was added.

The qPCR results of *nifH* copies demonstrated similar patterns as the nitrogenase activities in each mangrove forest (Figures 4 and 5) with different treatments. Basically, the *nifH* genes increased about 10-fold from the ambient potential to maximum potential conditions (i.e., amended glucose solution), while the copies decreased to about one-fifth of those under potential conditions after MoO_4 was added. Interestingly, the *nifH* gene copies were found to be higher in the Guandu soils than those in the Bali soils under the potential, amended with glucose and amended with MoO_4 solution conditions at all depths.

Table 1. Differences in soil nitrogenase activities and *nifH* copies for samples collected in the two mangrove forests, and three soil depths by two-way ANOVA, based on the significant level of 0.05 ($p < 0.05$).

Factor	Site Effect	Depth Effect	Interaction Effect (Site × Depth) (Soil That Is More Sensitive to Depth Change)
Potential C_2H_4 rate	Bali > Guandu	n.d. *	n.d.
Maximum potential C_2H_4 rate (amended with glucose)	Guandu > Bali	0–6 > 6–12 >12–18	Guandu
Potential C_2H_4 rate with amended MoO_4 and glucose	Bali > Guandu	6–12 = 12–18 > 0–6	Guandu
Potential *nifH* copies	Guandu > Bali	n.d.	n.d.
Maximum potential *nifH* copies (amended with glucose)	Guandu > Bali	0–6 > 6–12 >12–18	Guandu
Potential *nifH* copies with amended MoO_4 and glucose	Guandu > Bali	0–6 > 6–12 = 12–18	n.d.

* No difference (n.d.).

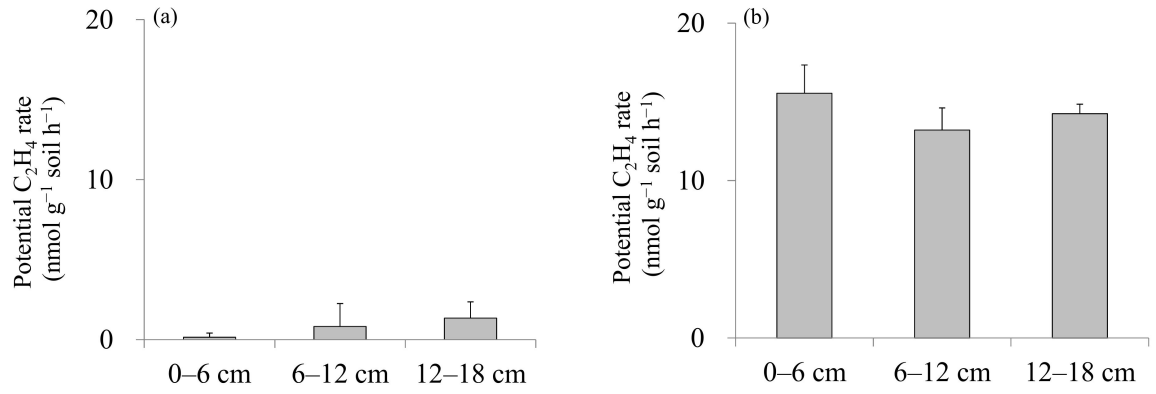

Figure 2. The soil potential nitrogenase activities in Guandu (**a**) and Bali (**b**) mangrove forest soils at the three soil depths. The height of bars and the error bars indicate the mean and standard deviation, respectively.

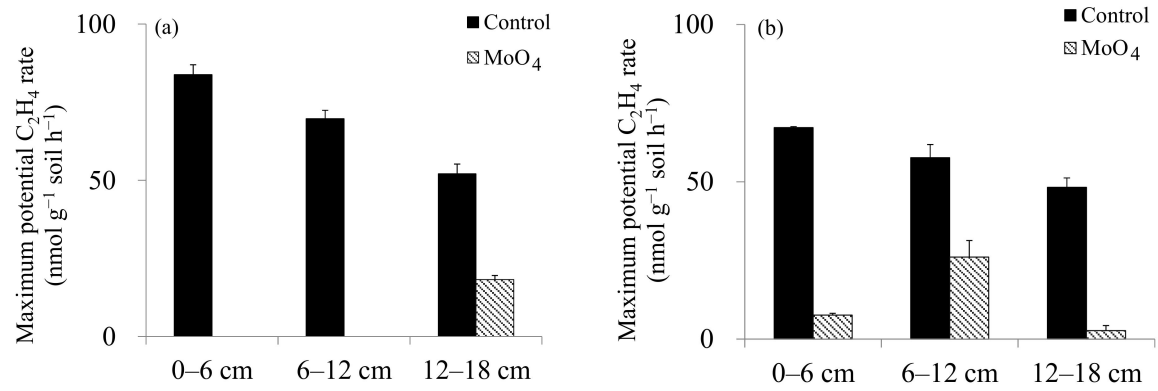

Figure 3. The soil maximum potential (amended with glucose) nitrogenase activities in Guandu (**a**) and Bali (**b**) mangrove forest soils, with and without amended MoO_4, at the three soil depths. The height of bars and the error bars indicate the mean and standard deviation, respectively.

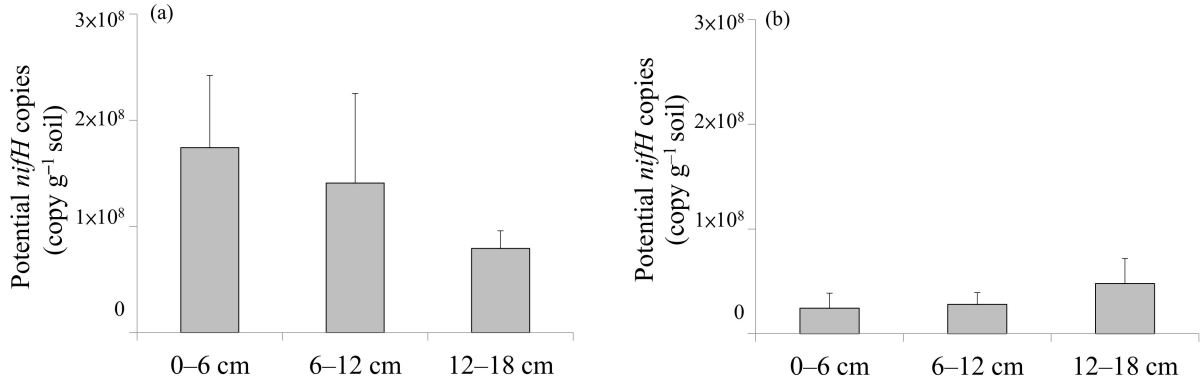

Figure 4. The number of nitrogenase gene (i.e., *nifH* gene) copies in fresh mangrove forest soils in Guandu (**a**) and Bali (**b**) at the three soil depths. The height of bars and the error bars indicate the mean and standard deviation, respectively.

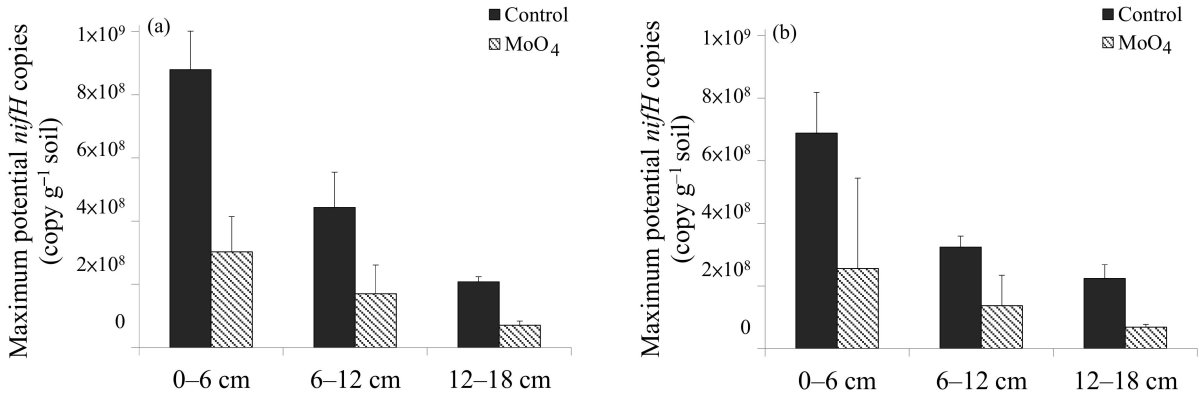

Figure 5. The number of nitrogenase gene (i.e., *nifH* gene) copies in Guandu (**a**) and Bali (**b**) mangrove forest soils amended with glucose, and with or without MoO_4 added, at the three soil depths. The height of bars and the error bars indicate the mean and standard deviation, respectively.

The high throughput sequencing of 16S rRNA genes revealed wide variations in diazotrophic community compositions between the two study sites and among the three depths (Figure 6). In the Guandu mangrove soils, the sulfur or sulfide-oxidizing bacteria (SOB), *Sulfurospirillum*, *Arcobacter*, and *Pelobacter*, were the dominant diazotrophs, accounting for 30–50% of the relative abundance. In addition, methanogens and SRB each shared 25–40% of the relative abundance. However, in the Bali mangrove soils, SRB, especially *Desulfosarcina* and *Desulfobulbus*, appeared to be the dominant diazotrophs (i.e., 20–50%), and their relative abundances decreased with increasing soil depths. Conversely, the diazotrophs associated with SOB increased in relative abundances from 10% to more than 50% with increasing soil depths in the Bali mangrove soils.

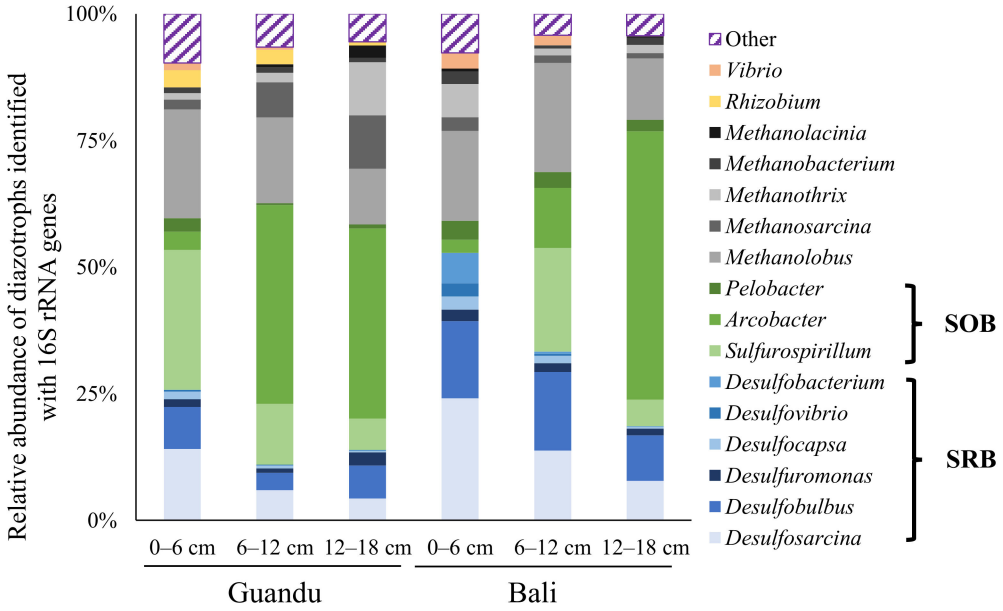

Figure 6. The relative abundances of diazotrophic genera identified using 16S rRNA genes in Guandu and Bali mangrove forest soils at the three soil depths. Sulfate-reducing bacteria (SRB) are in blue, sulfur and sulfide-oxidizing bacteria (SOB) are in green, and methane-related archaea are in grey. The diazotrophic genera selected were based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database.

The sequence data for the *nifH* gene showed some trends in diazotrophic community compositions between the two study sites and two depths. Within SRB, *Desulfuromonas* of *Desulfuromonadaceae* was dominant at 0–6 cm deep in the Guandu and Bali mangrove soils, but its relative abundance decreased at 6–12 cm deep at both sites (Figure 7). Conversely, SOB accounted for 15–45% of the diazotrophic communities. The proportion of SOB *Pelobacter* increased with increasing soil depths at both sites. Methane-producing archaea were also present, accounting for 3–9% of the abundance at the two sites.

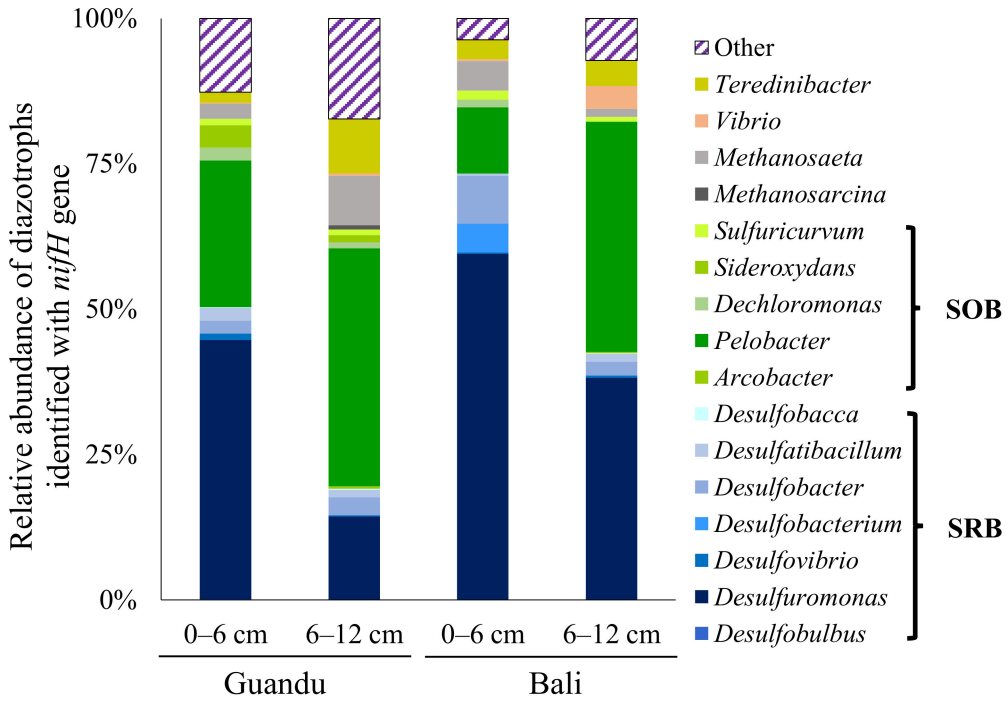

Figure 7. The relative abundances of diazotrophic genera identified with the *nifH* gene in Guandu and Bali mangrove forest soils. Sulfate-reducing bacteria (SRB) are in blue, sulfur and sulfide-oxidizing bacteria (SOB) are in green, and methane related archaea are in grey.

The results from CCA illustrated that the diazotrophs related to SRB were positively related to the increasing salinity, soil pH, and concentrations of various soluble N compounds (e.g., NH_4^+, S_bON, and TDN). In contrast, genera such as *Methanothrix*, *Methanosarcina*, and *Sulfurospirillum* were positively related to TOC and S_bOC (Figure 8; Table 2).

Table 2. Concentrations of soil total organic carbon (TOC), total nitrogen (TN), and soil soluble organic C and N in 2 M KCl extracts from the Guandu and Bali mangrove forests.

Site	TOC (%)	TN (%)	S_bOC * (mg C kg^{-1} Soil)	NH_4^+	NO_3^-	S_bON *	TDN *
				(mg N kg^{-1} Soil)			
Guandu	3.40 ± 0.0	0.32 ± 0.0	12.7 ± 2.5	7.7 ± 0.2	0.3 ± 0.0	12.8 ± 4.8	20.7 ± 4.9
Bali	2.90 ± 0.0	0.28 ± 0.0	6.4 ± 3.8	54.2 ± 0.7	0.3 ± 0.0	27.7 ± 15.3	82.2 ± 15.4

* S_bOC: soil soluble organic C; S_bON: soil soluble organic N; TDN: total dissolved N.

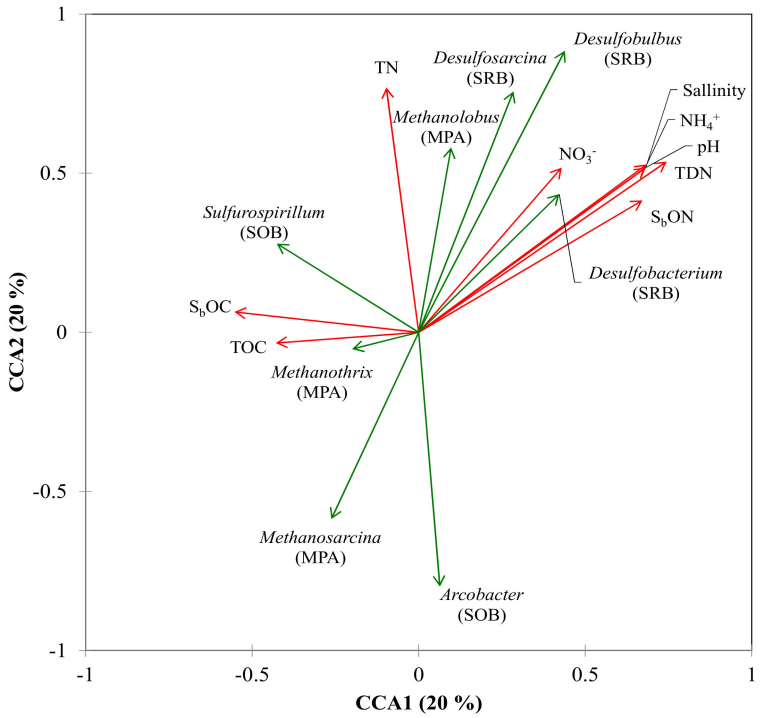

Figure 8. The relationship between diazotrophs and soil physiochemical properties in Guandu and Bali mangrove forests based on Canonical Correlation Analysis (CCA). SRB: sulfate-reducing bacteria; SOB: sulfur-oxidizing bacteria; MPA: methane-producing archaea.

4. Discussion

Nitrogen is essential for life. Microbial N_2 fixation only takes place in N-limited environments because it requires extensive energy to break the triple bond between the two N atoms [11,38,39]. Thus, potential nitrogenase activities may have been lower in the Guandu soils than the Bali ones because the former is in an upstream estuary and therefore receives more N (i.e., 3.13 mg $NH_3-N\ L^{-1}$) from the upland watershed than does the downstream forest (Bali) (i.e., 1.06 mg $NH_3-N\ L^{-1}$) [40]. Moreover, the potential nitrogenase activities (i.e., 0.1–0.4 nmol g^{-1} soil h^{-1}) measured in our previous study [9], which sampled sites located between the sites in this present study, fell between the activities determined in the present study (Guandu: 1.07 nmol g^{-1} soil h^{-1}; Bali: 15.52 nmol g^{-1} soil h^{-1}), which suggests that geographic location might be an important factor for microbial N_2 fixation.

In addition, the nitrogenase activities increased faster in the Guandu mangrove soils than in the Bali ones after the glucose solution was added, implying that the diazotrophic populations in Guandu may be higher than those in Bali; in turn, this indicates that the potential nitrogenase activities in both fields were limited by their available C sources and thus had lower N_2 fixing activity. Our real-time PCR results offer further evidence of this; these results suggest that there were more *nifH* copies in the Guandu mangrove soils than in the Bali ones under ambient and potential (i.e., glucose amended) conditions.

MoO_4 is typically considered an inhibitor that blocks SRB from fixing N_2 [12,41–44]. Thus, our observation that N_2 fixation rates were negligible in the MoO_4^- and glucose-amended Guandu mangrove soils implies that SRB can be the main N_2 fixers in mangrove forests with sufficient C sources. However, some diazotrophs other than SRB may also actively fix N_2 in the Bali mangrove soils. Note that because glucose was also added in the MoO_4-amended experiments, the nitrogenase activity contributed by SRB in the field soils may need to be further studied.

In addition, the Bali mangrove soil exhibited high NH_4^+ concentrations and high nitrogenase activities, implying that N_2 fixation may be regulated by a complex of various physiochemical factors that are not yet known, rather than only by bioavailable N concentrations. This concept has also been proposed in other studies [45,46]. Moreover, the

S_bOC was determined with a KCl extraction method in this present study, which may not best represent the organic C that can be utilized by soil microorganisms in the mangrove forests. Combined with the results from the glucose-amended experiments, we suspect that the nitrogenase activities in the studied mangrove forests may be more sensitive to the limitations of soil bioavailable C than to the available NH_4^+ concentrations in the soil pore space. Further studies may consider using extraction methods, such as a hot-water extraction [47] or an acid-hydrolysis method [48], to evaluate the microbial metabolizable C or bioreactive C [33,34], respectively, and obtain more details on how soil organic C may affect diazotrophic activity in mangrove forests.

Based on the sequence data from 16S rRNA and *nifH* genes, SRB had a higher relative abundance in the downstream (Bali) than the upstream (Guandu) mangrove forest soils; this may be because the former's geographic location indicates that it receives more frequent tides and had higher pore water salinity. Moreover, the high maximum potential nitrogenase activity, observed when SRB accounted for 20–50% of the relative bacterial abundance in the studied mangrove forests, also confirmed that the N_2 fixation was mainly attributed by the SRB. In addition, the CCA results showed a positive correlation among soil N sources and SRB, further confirming that the distribution of SRB was affected by bioavailable N. About 30–50% and 15–45% of the diazotrophic genera in the mangrove forest soils were SOB analyzed with 16S rRNA and *nifH* genes, respectively. Moreover, the relative abundance of SOB increased with soil depth in the Bali mangrove soils. Although we did not measure soil hydraulic conductivity in the studied mangrove forests, it is typically low in coastal ecosystems [49]. Thus, we suspect that niche differentiation occurs in our studied soils, provides aerobic conditions, and facultative SOB grows in some soil pore spaces.

In addition, considerable amounts of methane-producing archaea (i.e., methanogens) (20–30%) were found at the study sites. A previous study indicated that some methanogenic archaea can fix N_2 and synthesize NH_4^+ [50]. Moreover, the positive correlations among several methanogenic genera and soil C sources that we observed may be due to the heterotrophic growing conditions of methanogens.

5. Conclusions

Our study indicates that SRB may be important to N_2-fixation in mangrove forests when sufficient C is obtained. In addition, geographic location influences this activity, as salinity and N loads vary with location. However, other environmental factors, such as soil bioavailable C, may be important factors affecting the diazotrophic communities and activities in mangrove forest soils. In addition, based on the analyses of 16S rRNA and *nifH* genes, other diazotrophic communities, such as SOB and methane-producing archaea, are dominant in mangrove forests, although their activities may be minute compared to those of the SRB. Overall, this study reveals diazotrophic compositions in two nearby mangrove forests and demonstrates the importance of SRB in the N dynamics of mangrove forests. More direct assays such as ^{15}N-stable isotope probing could also be conducted to gain more insight into mangrove ecosystem functioning.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/f12070822/s1. All 16S rRNA and nifH gene sequences obtained from MiSeq sequencing have been deposited into the NCBI, under project number PRJNA593629 and PRJNA739810, respectively. The linear regression of the N_2-fixing rates can be found in the supplementary materials file. Figure S1. The changes in C2H4 concentrations and the linear regressions in the Guandu (a) and Bali (b) mangrove forest soils. Figure S2. The changes in C2H4 concentrations and the linear regressions in the Guandu (a) and Bali (b) mangrove forest soils with amended glucose solution (5 mL; 20 mM). Figure S3. The changes in C2H4 concentrations and the linear regressions in the Guandu (a) and Bali (b) mangrove forest soils with amended MoO4 (1 mL; 20 mM) and glucose solution (5 mL; 20 mM).

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Y.-J.S. and C.-Y.C. conducted the field experiments, and C.-Y.C. supervised and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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