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Article

Phylogenetic Diversity of Diazotrophs along an Experimental Nutrient Gradient in Mangrove Sediments

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Abstract: The diversity of diazotrophs was studied in the sediment of mangrove forests (Twin Cays, Belize) subjected to a long-term fertilization with nitrogen and phosphorus. Terminal Restriction Fragment Length Polymorphism (TRFLP) and cloning of PCR-amplified *nifH* genes were combined via *in silico* analysis to assign clones to TRFLP-*nifH* phylotypes, as well as to characterize the occurrence of phylotypes in response to environmental conditions. Results indicated that mangrove sediments from Belize harbor a unique diazotrophic community with a low metabolic diversity dominated by sulfate reducers. The variability of potential nitrogen-fixing sulfate reducers was explained by several environmental parameters, primarily by the abundance of dead roots in the sediments, and the concentration of H_2S in the pore-waters. This study describes the complexity of microbial communities within the mangrove sediments with specific functional groups varying along environmental gradients.

Keywords: diazotrophs; mangrove sediments; phylogenetic analysis; generalized additive models

1. Introduction

Mangrove forests are among the most productive ecosystems playing a significant role in nutrient sequestration in coastal zones in tropical and subtropical regions [1,2]. Their high productivity is based principally on leaf litter production and on standing biomass above- and below-ground [1,3,4]. Despite their high productivity, mangroves are often nutrient limited, particularly for nitrogen [5–8]. Mangroves ability to conserve nutrients as a whole ecosystem relies largely on the mineralization of organic matter by active microorganisms, an important component for the productivity of the ecosystem [9–11]. Diazotrophs (nitrogen fixing bacteria) are a physiologically and phylogenetically diverse microbial group responsible for most nitrogen input in the mangrove ecosystem [12–14]. Nitrogen fixation is the second major microbial processes within the mangrove ecosystem, after decomposition of organic matter by sulfate reducing bacteria [10]. Many sulfate-reducing bacteria are also able to fix nitrogen [15]. The nitrogen, sulfur, and carbon cycles are tightly coupled during organic matter mineralization, an important intersection of biogeochemical processes that has not been thoroughly studied in the mangrove ecosystem. Diazotrophic assemblages have been described in a range of other marine environments including the pelagic zone of the oligotrophic ocean, microbial mat communities, and sediments of the cordgrass *Spartina* [16–18] however, the analysis of the diversity of diazotrophs using the *nifH* gene in mangrove sediments is limited to a few studies [13,19–21]. The ecological role of diazotrophs, besides being a source of nitrogen for mangroves, has not been widely studied, although Ravikumar *et al.* [22] found that three species of *Azotobacter*, an aerobic heterotrophic diazotroph, are important producers of hormones that promote the growth of mangrove seedlings.

Most of the information on the characterization of diazotrophs in mangroves comes from isolation studies indicating diazotrophs are mostly closely affiliated to *Proteobacteria* and *Firmicutes* groups. Molecular diversity of diazotrophs has shown a similar diversity of groups as well as sequences related to the *Delta*- and *Beta-Proteobacteria* and the *Actinobacteria* group [13,19,20]. Nitrogen-fixing bacteria isolated from sediments, rhizosphere and root surfaces did not show any specificity for a mangrove tree species and were identified as *Azospirillum*, *Azotobacter*, *Rhizobium*, *Clostridium* and *Klebsiella* in India [23], and as *Vibrio campbelli*, *Listonella anguillarum*, *V. aestuarianus*, and *Phyllobacterium* sp. in Mexico [24].

Not only is there little information on the diversity of diazotrophs in mangrove sediments, but also little is known about the environmental factors that regulate their diversity. This is important especially in coastal areas where fertilizers are considered one of the most important pollutants, with deleterious effects on the nitrogen cycle [25,26]. Anthropogenic activities (e.g., eutrophication) have the potential to increase (e.g., nitrogen fertilizers) or reduce (e.g., organic amendments from agricultural practices like straw incorporation) the extent of nutrient limitation for bacterial groups in coastal and terrestrial environment [27,28], potentially affecting organic matter mineralization through changes in microbial

activity and plant-microbial interactions [24,29,30]. In mangroves, evidence suggests that anthropogenic activities (e.g., fertilization, deforestation) may negatively impact the ecosystem with a reduction of carbon burial or by the release to the atmosphere of organic carbon stored in sediments [31–35]. Persistent fertilization with nitrogen or phosphorus in mangroves has been shown to enhance tree growth, nutrient foliar content, tree mortality, and acidification in sediments, as well as affecting plant-microbe interactions [5–7,15,28,33,35]. Plant-bacterial interactions through the root exudation of recently fixed carbon are an important source of labile carbon for microorganisms [36], enhancing bacterial activity, sediment mineralization and nutrient availability for plants in terrestrial, coastal environments, and mangroves [10,37–40]. In mangroves, this interaction is important influencing the carbon and nitrogen cycles [15,41] and other microbial processes [42,43]. A weak relationship between bacterial diversity and ecosystem functions have been observed in terrestrial ecosystems where decomposer communities often exhibit high redundancy for a single function (e.g., respiration, biomass production) [44]. High functional redundancy has been observed widely in soil microorganisms [45]. The spatial niches for optimal activity of microorganisms like diazotrophs in coastal environments remain largely unknown, and their role in the biogeochemistry of marine sediments is still poorly understood.

The present study aimed to characterize the diversity of diazotrophs and their functional role in a mangrove sediment system which had been experimentally perturbed by long-term nutrient additions. To accomplish our objectives, we compared *nifH* clone phylotypes with *nifH*-TRFLP (Terminal restriction fragment length polymorphism) OTUs (operational taxonomic unit), and modeled phylotype occurrence and abundance in response to measured geochemical parameters using generalized additive models (GAM) [46]. GAM models have been applied to a wide range of natural environments due to its flexibility and effectiveness using non-linear relationships between biota and abiotic parameters [47]. Understanding how microbial communities respond to natural and disturbance conditions can reveal important relationships between community composition and their environment.

2. Material and Methods

2.1. Study Site

This study was conducted at Twin Cays (16°50′ N, 88°06′ W), a 92 ha archipelago located 12 km off-shore from the coast of Belize (Central America). Twin Cays has limited terrestrial influence and is constantly flushed by ocean water. The sediment is principally peat formed primarily by fine roots from the dominant mangrove species *Rhizophora mangle*. An ongoing fertilization experiment was established in 1997 and is maintained by I.C. Feller and coworkers of the Smithsonian Institution [48,49]. This long-term experiment was conducted to study the effects on mangrove trees physiology, growth and survival along a tidal gradient using a three-way factorial experimental design with four levels of nutrients (control: Ctrl, phosphorus: P, nitrogen: N, and both NP), two levels of tidal elevation (fringe zone and interior zone) and two levels of water depth (deep and shallow) [49]. At six-month intervals, individual trees were fertilized with nitrogen (urea) or phosphorus (P_2O_5) using dialysis tubing inserted in the sediment. At each time, 150 g of fertilizer was added in two holes cored to ~30 cm depth into the sediment on opposing sides of the tree and sealed with peat. The control

treatment included coring and plugging with no fertilizer. To accommodate replicates (n = 3) a 10 m interval was left between the experimental trees to prevent possible lateral transport of nutrients. In our study, we sampled only the interior of the mangrove forest because represents the larger area in the mangrove ecosystem of Twin Cays (about 60%) and where previous studies have shown that the long-term fertilization experiment have changed the geochemistry of the sediments [14,50].

2.2. Collection of Environmental Samples

In order to determine the main drivers of the distribution of potential N_2-fixing bacteria in the studied area, sediment samples for DNA extraction were collected parallel to environmental samples (live and dead mangrove roots, and pore-water samples). Sediment cores (n = 18) were collected at each fertilization treatment (Ctrl, P, and N) at a distance 0.6–0.8 m from the fertilizer tube located next to each mangrove tree to avoid damaging the fertilizer and main mangrove roots. A Russian Peat corer designed to avoid vertical compaction of sediment samples was used to collect sediment at three depth intervals (0–5, 5–10 and 20–30 cm) for a total of 54 samples collected (3-fertilization treatments, 3-depth intervals, and 6-replicates) for sediment characterization (live and dead mangrove roots content) and DNA extraction (*nifH* gene). Pore-water samples were collected using sippers inserted into the peat sediment at the three depth intervals (0–5, 5–10 and 20–30 cm). Temperature and pH were determined immediately after collection of pore water samples (ORION with pH and ATC electrode; calibrated with certified standards, reproducibility of ±0.02 pH units and ±1.0 °C). Salinity was measured with a refractometer (Fisher Scientific, calibrated with deionized water, with an accuracy of ±1.0 ppt). Collected pore-water samples were analyzed for hydrogen sulfide (H_2S) [51], ammonium (NH_4^+) [52], and dissolved inorganic phosphate (PO_4^{3-}) [53].

2.3. Distribution of Mangrove Roots

We measured the relative abundance (%) of live (white-brown healthy roots) and dead roots (black, decomposing roots). Sediment samples from each depth interval were washed through sieves (4 mm, 2 mm). Root types were picked manually using a dissecting microscope (10×) (Olympus, Tokyo, Japan), oven dried for 48 h at 60 °C, and weighed. Live and dead root weights were compared to the total sediment weight in each sample to calculate relative abundance (%).

2.4. DNA Extraction

Samples (~1.5 g) collected at each depth interval were stored in TE (10 mM Tris HCL plus 1 mM EDTA, pH of 7.4) and frozen at −20 °C in Belize, and later at −80 °C in the laboratory at the University of Southern California. DNA was (~500 mg of sample) with the FastDNA® SPIN Kit for Soil (MP Biomedicals Laboratories, Santa Ana, CA, USA) according to the manufacturer's protocol. DNA was eluted in 50 μL DNA free water. The quantity of the extracted DNA was analyzed by PicoGreen (Molecular Probes, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, and using a fluorometer (Stratagene, La Jolla, CA, USA). DNA elutions were stored at −20 °C.

2.5. Nested PCR Amplification

All nested PCR reactions using degenerate primers for amplification of the dinitrogenase reductase gene, *nifH* [54] were conducted in 50 μL reactions containing 1 ng template DNA, 1× PCR Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 400 ng/μL BSA, 5.0 U Taq (New England Biolabs, Ipswich, MA, USA), and 0.8 μM of each primer: *nifH*3 (5′-ATRTTRTTNGCNGCRTA-3′) and *nifH*4 (5′-TTYTAYGGNAAR GGNGG-3′) for the first reaction, *nifH*1 (5′-TET-TGYGAYCCNAARGCNGA-3′) and *nifH*2 (5′-ANDGCCATCATYTCNCC-3′) for the second reaction. The PCR reactions were carried out with a denaturation step of 3 min at 95 °C, followed by 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 55 °C, and 45 s extension at 72 °C, followed by a final extension step of 7 min at 72 °C. All amplification reactions were performed in a Thermocycler (Stratagene RoboCycler 96 PCR, La Jolla, CA, USA). Extreme care was taken to avoid any DNA contamination and controls (PCR reaction free of DNA template) were run always for the first and second PCR reactions. The quality of the PCR products were evaluated by gel electrophoresis (SYBR®Green staining on a 2% agarose gel) (Applied Biosystems™, Foster City, CA, USA). The quantity of the extracted DNA was analyzed by PicoGreen (Molecular Probes, Eugene, OR, USA) following the manufacturer's instructions, and using a fluorometer (Statagene, La Jolla, CA, USA).

2.6. Terminal Restriction Fragment Length Polymorphism Analysis (TRFLP)

We used TRFLP of PCR amplified *nifH* gene to study the composition of potential N_2-fixing bacteria in the collected sediment samples. Although TRFLP analysis is limited by all PCR artifacts [55], its application in environmental studies has shown to be adequate in studying diazotrophic communities under different perturbed conditions [14,56]. Extracted and cleaned PCR products (MinElute Gel Extraction Kit; QIAGEN Laboratories, Valencia, CA, USA) were digested with the restriction enzyme *Hae*III [14] following the manufacture's instructions (New England Biolabs, Ipswich, MA, USA). For each reaction, 10 μL of PCR product was digested with 20U enzyme and 10× buffer at 37 °C overnight. Reactions were terminated at 80 °C for 15 min. DNA clean and concentrator kit (Zymo Research, Irvine, CA, USA) was used on the digested products following the manufacturer's instructions. Clean digested products were run in duplicates in an automated sequencer (Applied Biosystems 96 capillary 3730 DNA Analyzer, Foster City, CA, USA), and the outputs were aligned against all possible fragment lengths (100–360 bp) and binned to analyze the bacteria community containing the *nifH* gene. The area under each peak was used to calculate the relative abundance (%) of each OTU in each sediment sample [14].

2.7. DNA Cloning and Sequencing

A total of 3 clone libraries were constructed using surface sediment samples (0–5 cm) from each fertilization treatment (Ctrl, N, P). *NifH* PCR products were ligated into the pCR®4-TOPO cloning vector (Thermo Fisher Scientific Inc., Waltham, MA, USA) and transformed into *Escherichia coli* (Mach1™-T1®) (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Tranformed cells were selected on LB agar plates containing kanamicyn (50 μg mL^{-1}) and ampicillin (50 μg mL^{-1}) after overnight growth at 37 °C. White colonies (40 to 50) were randomly picked from

each library. Picked colonies were transferred to deep-well culture blocks containing 1 mL of LB medium, kanamicyn (50 μg mL^{-1}) and ampicillin (50 μg mL^{-1}), and grown for 24 h at 37 °C. Overnight cultures were stored in glycerol, and clones were isolated and sequenced with vector-specific primers (M13 reverse, CAGGAAACAGCTATGAC) by MCLAB (San Francisco, CA, USA). All *nifH* sequences obtained were edited to remove non-coding sequences up- and down-stream from the primers, and primers were removed. The sequences obtained were compared with *nifH* sequences from GenBank (October 2012) using blastx. Sequences were translated into amino acids (Geneious Pro 4.0 software), and aligned together with the reference sequences from Genbank using ClustalW. A neighbor-joining phylogenetic tree was constructed (MEGA 4.0) using the Poisson distance correction, pairwise deletion of gaps, and 1000 bootstrapping replicates to estimate the reliability of the tree. Sequences with similarity >98% were not included in the phylogenetic tree. The TRFLP patterns of cloned *nifH* fragments were confirmed by using sequence-based theoretical restriction fragmentation with Geneious Pro, version 4.0. Theoretical fragmentation patterns were calculated and sequenced clones were assigned to TRFLP-OTUs.

2.8. Statistical Comparisons

The diversity of the *nifH* gene was estimated by ranking the most to least abundant clones and calculating the nonparametric richness estimator of Chao1 [57] using the software EstimateS at 92% and 98% similarity. Accumulation curves of the number of sequences observed and of Chao1 were generated by randomization and averaging from 50 to 60 runs. A cluster analysis was conducted based on the matrix of TRFLP-OTUs abundance (% relative abundance) among nutrient and sediment depth treatments using JMP11 Software (SAS Institute Inc., Cary, NC, USA). The variability among the treatments of selected TRFLP-OTUs was analyzed with a One-way ANOVA with nutrient and sediment depth as treatment factors (using JMP11). When significant differences were found, Tukey's test was used to compare the means of the treatments. The level of statistical significance was set to p values of <0.05. All data were tested to fulfill normality and equal variance assumptions, and transformed using $\log(x + 1)$. TRFLP-OTU variability in response to specific environmental variables were analyzed with the CANOCO software (Microcomputer Power, Ithaca, NY, USA). We used unimodal constrained models (redundancy analysis, RDA) [58] where each ordination axis in the plot corresponds to a direction in the multivariate scatter of the OTU data that is related to the environmental variables [59]. To test for nonlinear relationships between OTUs and specific environmental parameters we used generalized additive models (GAMs) [58]. To summarize the variability of the OTUs with all environmental parameters the scores of individual samples on the first RDA axis were used as the explanatory variable in the GAM analysis [59]. Prior to statistical analysis the %abundance of OTUs was transformed using $\log(x + 1)$, and the environmental data was centered and standardized. A Monte Carlo permutation test was ran (n = 499) to assess for the significance of statistical analyses. p values of <0.05 were considered significant.

2.9. Nucleotide Sequence Accession Numbers

The *nifH* sequences obtained in this study have been deposited in GenBank database under accession numbers GQ499201-GQ499272.

Table 1. Summary of the closest matches for *nifH* sequences of nitrogen-fixing bacteria from mangrove sediment samples. Letters in the phylotypes ID refer to the nutrient treatment where the clone was found (control: Ctrl, phosphorus: P, and nitrogen: N).

Clones			Closest match in GenBank			
Presumable phylogenetic association	**Phylotypes ID**	**Total**	**Match**	**Accession number**	**% Similarity**	**Reference**
Delta-Proteobacteria	N26	1	Isololate from Italian soils	AAS47809	87	
	N31, P211	6	*Paleobacter carbinolicus* DSM 2380	YP357508	96	Mussmann *et al.* 2005 [60]
	P12, P33	2	*Geobacter sulfurreducens*	NP953865	93	Methe *et al.* 2003 [61]
	P11, C13, C11, P13, N19	11	Microbial mats in the Bahamas	AAZ77144	93–97	Yannarell *et al.* 2006 [62]
	P34, N21, P210	3	Forest soils in Amazon region	AC132206	90–92	
	C33	1	Microbial mat in North Carolina	AAA65425	88	Zehr at al. 1995 [63]
	C32, P21	3	Microbial mat, dead stems of *Spartina alterniflora*	AAY85458, AAS57673	90–93	Musat *et al.* 2006 [64] Moisander *et al.* 2005 [65]
	C14	1	*Desulfovibrio vulgaris*	YP2437020	90	Heidelberg *et al.* 2004 [66]
	N17	2	Microbial mat in North Carolina	AAA65429	99	Zehr at al. 1995 [63]
	P22, N351	3	*Desulfomicrobium baculatum*	ZP04344086	92	
	N35	2	Mangrove sediments in China	ABM66820	96	Zhang *et al.* 2008 [13]
	P15	1	Chesapeake Bay	AAZ06740	96	
	C22	1	Microbial mat	AAY85430	94	Musat *et al.* 2006 [64]
	P31	1	Mangrove sediments in China	ABM67091	90	Zhang *et al.* 2008 [13]
	P25	8	Mangrove sediments in China	AAF61027	92	Zhang *et al.* 2008 [13]
	N18	8	Seagrass sediments in the Bahamas	AAL07952	89	Bagwell *et al.* 2002 [67]
	N36, N33, N314, N37, N313	8	Seagrass sediments in the Bahamas	AAL07952	90–96	Bagwell *et al.* 2002 [67]
	P26, C36	3	Microbial mat in North Carolina	AAA65422	97	Zehr *et al.* 1995 [63]
	N213, P27, N32	6	Eastern Mediterranean Sea	ABQ50824	93	
	C23, N312, P23, N112,N25, C34	20	*Desulfatibacillum alkenivorans* AK-01	YP2430688	90–97	
	N211, N13, C31, N310, N27	8	Microbial mat, mangrove sediments	AAY85423, ABM74058	90–96	Musat *et al.* 2006 [64]
	N22, C21, C35, N39, N111	6	Eastern Mediterranean Sea	ABQ50612	93–95	

Table 1. *Cont.*

	N23, N14, N110	18	Microbial mat, Eastern Mediterranean Sea	AAY85423, ABQ50807	94–95	Musat *et al.* 2006 [64]
	N38, N24	10		ABQ50691	79	
Gamma-Proteobacteria	C12, P24	2	*Vibrio natriegens* and *Klebsiella pneumoniae*	AAD55588, AAO85881	94	
	N34, N29	7	Pristine marine environment	AAY85422	99	Musat *et al.* 2006 [64]
	N16	1	*Thiocapsa roseopersicina*	ACC95826	93	
Firmicutes	P14	1	Malaysian soil	ACC95201	94	
	RP32	2	Forest soils in Amazon region	ACI32162	93	
	N311	2	Terrestrial soil	ACI26001	91	
Green sulfur bacteria	N12, P28, C15, N212	6	Rhizosphere of *Spartina alterniflora*	ABD74331	96–97	Lovell *et al.* 2008 [18]
	N28, P29	2	*Chlorobium phaeobacteroides*	YP1960150	98	

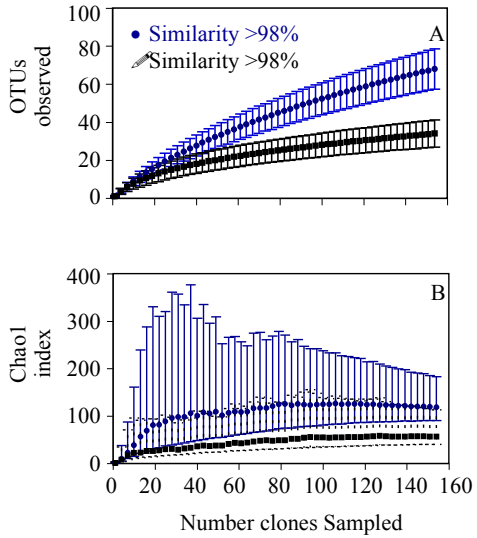

Figure 1. Rarefraction analysis of the *nifH* gene clones (**A**); and Chao1 index of richness (total number of OTUs in a community) estimated as a function of sample size (**B**). Curves are averaged over 50 simulations using the computer program EstimateS. All error bars are ±95%.

3. Results

The three clone libraries that were constructed from each fertilization treatment (Ctrl, N, P) showed no unique clones. A total of 154 clones were obtained, representing 71 different phylotypes (similarity >98%; Table 1). As a relative estimation of how well the sampling represented the sedimentary environment, the coverage of all clone libraries was >75%, indicating that this study was sufficient for diversity analysis and coverage of at least 76% of the diazotrophic community diversity in the Belizian mangrove forest. This is in agreement with the accumulation curves showing that the number of colonies picked yielded a sufficient fraction of the actual diazotrophic species using a similarity >98% (Figure 1A).

Representative sequences were subjected to BLAST search of the GenBank database revealing that Belizean mangrove sediments host 30% yet unidentified (at species level) *nifH* sequences (similarity >92%; Table 1). The majority of the *nifH* phylotypes (85% of the total) were found affiliated with *Delta-Proteobacteria* (Table 1, Figure S1). Other phylogenetic groups are *Gamma-Proteobacteria*, *Firmicutes*, and green sulfur bacteria (6%, 3%, 5% of the total phylotypes, respectively). Little information is available with respect to metabolic diversity in mangrove diazotrophic communities, as indicated by a high number of phylotypes with unknown metabolic function (62% of the total phylotypes; Table 1). Most of the remaining phylotypes are related to sulfate reduction (34% of the total phylotypes; Table 1). Moreover, the majority of the phylotypes found in our study are related to diazotrophs from other environments different than mangroves (81% of the total phylotypes; Table 1).

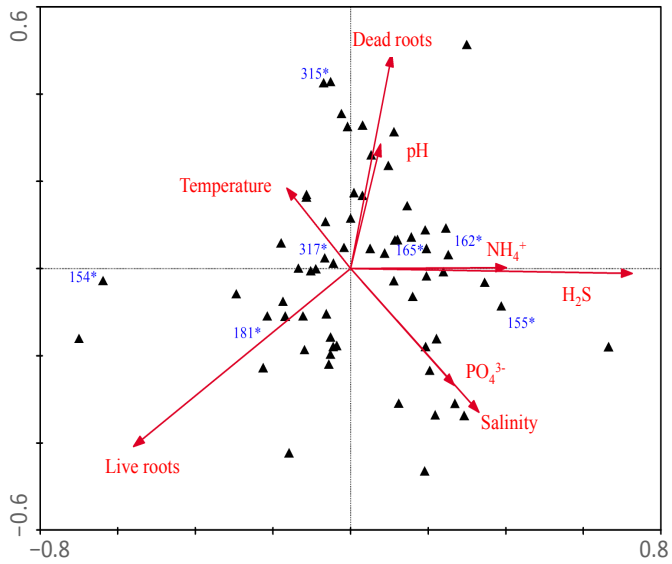

Figure 2. Ordination plot of pRDA results for *nifH* OTUs in the studied area (72% explained variance, $p < 0.05$). Triangles indicate OTUs at their best position in the plot relative to the environmental parameters (shown as arrows). The positions of seven TRFLP-OTUs (clone phylotypes that matched the TRFLP-OTUs) are shown in blue by their OTU #. The length of the arrows indicates the strength of environmental parameters in explaining OTU abundance variability. Arrows with same direction represent positive correlated variables, whereas arrows at a 90° angle represent uncorrelated variables.

Cluster analysis of the TRFLP-OTUs data (Table S1) indicated large variability among treatments without a distinct pattern between the nutrient and sediment depth treatments (Figure S2). The pRDA analysis indicated that most of this variability is explained by environmental parameters in the sediments (72%, $p < 0.05$, Figure 2, Tables 2 and S2). Parameters like dead and live roots, nutrients (NH_4^+, PO_4^{3-}), H_2S and salinity seemed to be primarily driving the % abundance of some TRFLP-OTUs.

Table 2. pRDA results for the spatial variation in the TRFLP-*nifH* OTUs explained by environmental parameters in the mangrove sediments.

	Axes	
Parameters	**1**	**2**
Eigenvalues	0.135	0.120
Species-environment Correlations	0.881	0.848
Correlation coefficients		
Dead roots	0.51	−0.23
Live roots	−0.47	0.02
Temperature	0.56	−0.46
pH	0.56	−0.48
Salinity	0.54	−0.52
NH_4^+	0.44	−0.44
H_2S	0.50	−0.40
PO_4^{3-}	0.07	−0.36
N:P (molar)	0.18	−0.16

We found 21 phylotypes (similarity >98%) related to 11 TRFLP-OTUs by *in silico* calculation of the TRFLP theoretical length of sequenced clones (Table 3). The phylotypes that matched the TRFLP-OTUs correspond presumably to sulfate reducers, sulfur oxidizers and phototrophic sulfur reducers. For statistical analysis, four matched TRFLP-OTUs (153, 321, 227, 117) were not included due to their low abundance ($n < 3$). The analysis of the variability of the 7-matched TRFLP-OTUs with $n > 5$ (Table 3) showed that only the most abundant OTUs (#154, #317) have a significant difference ($p < 0.05$) in % abundance between the fertilization treatments and depth intervals. Higher % abundances are found for OTU #154 at the surface of the sediments (0–10 cm depth) in the Ctrl-treatment, and for OTU #317 at 5–10 cm depth regardless of the fertilization treatment (Figure 3). Correlations between % abundance of all TRFLP-OTUs and environmental parameters indicate a strong effect of specific environmental parameters with all 7-matched TRFLP-OTUs, except OTU #317 (Figure 2). For example, the concentration of NH_4^+ and H_2S (inversely correlated), and live roots abundance (positively correlated) with OTU #154 and #181; pH, temperature, and dead roots abundance (all positively correlated) with OTU #315; and salinity and PO_4^{-3}, NH_4^+ and H_2S concentration (all positively correlated) with OTU #155. Moreover, the GAM analysis of the 7-matched TRFLP-OTUs showed a significant non-linear correlation ($p < 0.05$) with specific

environmental parameters for all matched OTUs (Figure 4), except OTU #317 (not shown). These correlations describe in detail the distribution of diazotrophic bacteria in response to the geochemical conditions of the studied area. OTU #154 presented the highest response (highest abundance) where lower dead roots abundance (10%) and H_2S concentrations (~0.2 mM) are found. In contrast, OTU #155 presented the highest response where higher dead roots abundance (30%) and H_2S concentrations (~3.5 mM) occurred. For OTU #315, the highest response was found at higher dead roots abundance (40%) and pH (~7). OTU #181 presented the highest response at lower NH_4^+ concentrations (~20 μM). The remaining OTUs have intermediate or none responses to more than one environmental parameter (Figure 4). The variability of all 7-matched TRFLP-OTUs related to all environmental parameters was summarized by fitting the values of Axis 1 from the pRDA analysis (Figure 2) in a non-linear correlation ($p < 0.05$) using GAM analysis (Figure 5). This analysis showed specific distributions for the 7-matched TRFLP-OTUs (except OTU #317) based on the geochemistry conditions of the environment indicating possible microenvironments for the diazotrophic bacteria inhabiting the mangrove sediments studied.

Table 3. Presumptive microbial processes of TRFLP-OTUs related with clone-OTUs. Similarity (%) based on amino acid sequences. Presumed microbial processes based on phylogenetic affiliation at the group level from the phylogenetic tree (Figure S1). Letters in the phylotypes ID refer to the nutrient treatment where the clone was found (control: Ctrl, phosphorus: P, and nitrogen: N).

TRFLP		Phylotypes		
OTU #	Total OTUs	ID	Total clones	Presumable microbial process
181	9	P22	2	Sulfate reducer
155	6	N12	1	Sulfur oxidizer
		N28	1	Sulfur oxidizer
		P13	2	Sulfate reducer
		C11	3	Sulfate reducer
		N211	1	Sulfate reducer
154	42	N21	1	Sulfate reducer
		C14	1	Sulfate reducer
		N111	1	Sulfate reducer
		N18	7	Sulfate reducer
		P11	2	Sulfate reducer
153	2	C35	2	Sulfate reducer
321	1	N213	2	Sulfate reducer
		N110	2	Sulfate reducer
227	2	P33	1	Sulfate reducer
117	2	N17	1	Sulfate reducer
315	6	P210	1	Sulfate reducer
317	27	C31	1	Sulfate reducer
162	8	C32	1	Sulfate reducer
		P32	2	Phototrophic sulfur reducer
165	6	N36	2	Sulfate reducer

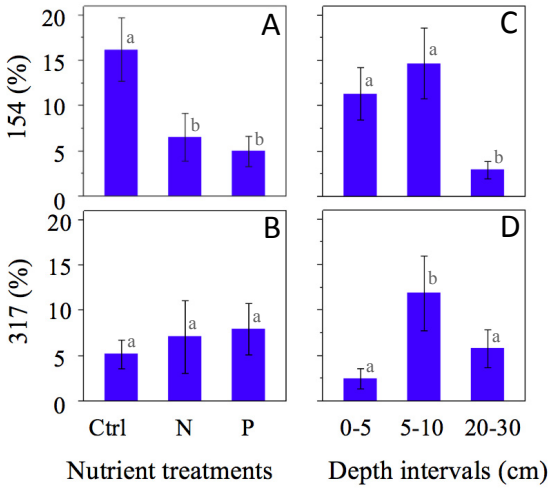

Figure 3. Relative abundance (%) of the most abundant TRFLP-OTUs among the fertilization treatments (**A**,**B**) and sediment depth intervals (**C**,**D**). Data shown as Avg. ± SE ($n \geq 3$). Bars with the same lowercase letter are not significantly different at $p < 0.05$.

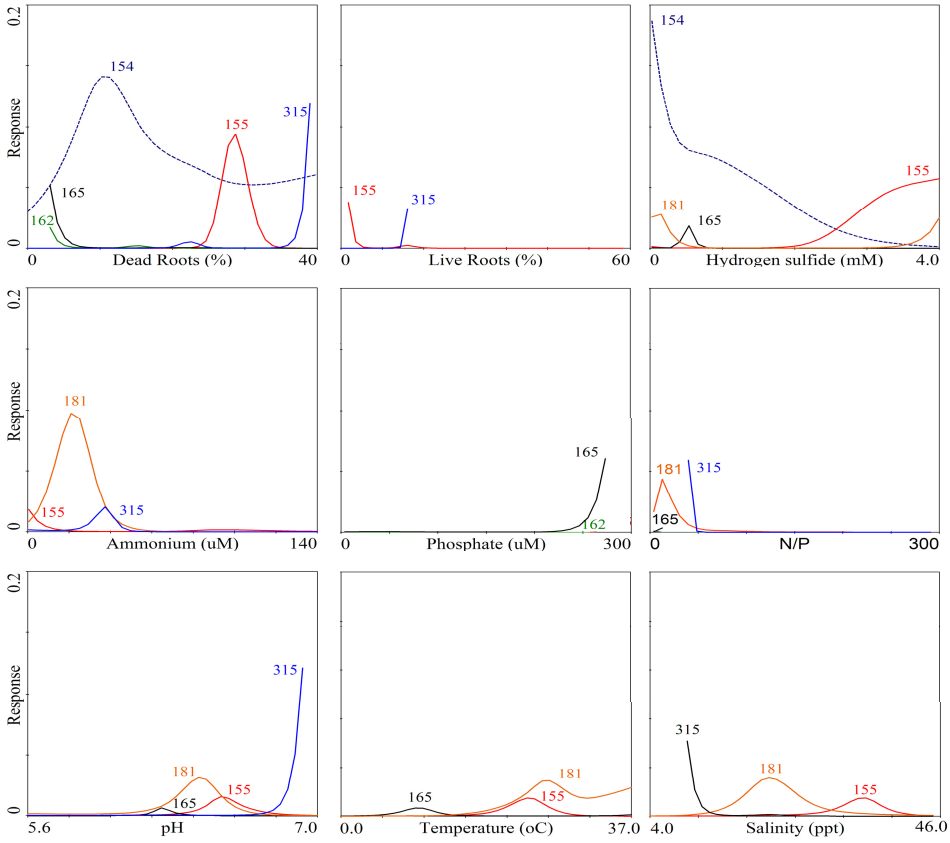

Figure 4. Response curves of seven TRFLP-OTUs to individual environmental parameters, fitted using generalized additive models (GAM) with a Poisson distribution using log link function, df = 3. The graphs summarize the functional relationship of significant non-linear relationships ($p < 0.05$) between response variables (TRFLP-OTUs fractional abundance in *y*-axis) and a covariate term (environmental parameter in *x*-axis). Each colored curve indicates a different TRFLP-OTU.

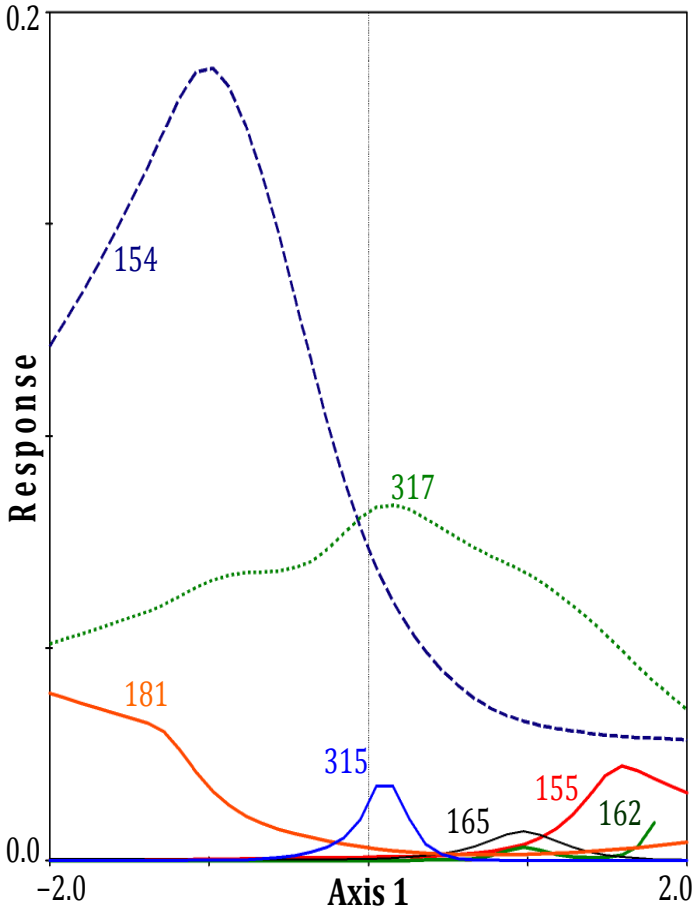

Figure 5. Response curve showing the distribution of seven TRFLP-OTUs (fractional abundance in y-axis) in response to all environmental parameters studied (as Axis 1 generated by pRDA analysis, Figure 4). All non-linear relationships are significant ($p < 0.05$), except for OTU #317. Each colored curve indicates a different TRFLP-OTU. Data fitted using generalized additive models (GAM) with a Poisson distribution using log link function, df = 3. From pRDA analysis: Axis 1 Eigenvalue = 12.3%, $p = 0.01$, model explained 56% of OTUs variability.

4. Discussion

This is the first study to report the molecular diversity of diazotrophs (*nifH* gene) and their variable distribution within an experimental nutrient gradient in an oceanic mangrove ecosystem. Mangrove sediments in our study exhibit a high diversity of the *nifH* gene with only 12% of the sequenced clones being closely related to clones from other mangrove areas [13,19]. The lack of representatives from the *Alpha-Proteobacteria* group and common cyanobacteria species found in other mangroves [12,13,19] might reflect differences in either mangrove locations or type of sediment sampled. This indicates some diazotrophs may be partially endemic to geographically distinct mangrove forests, which has important implications in restoration and conservation efforts [20,68,69].

A wide range of abiotic and biotic factors can affect the distribution of diazotrophic bacteria with plants and nutrients as the main drivers influencing the activity, diversity and abundance of this microbial group [14,70–73]. Our results show that the diazotrophic community in Belize exhibits low

metabolic diversity of primarily potential sulfate reducers. Many sulfate reducers have the ability to fix N_2 and their importance in the sedimentary cycling of nitrogen, carbon and sulfur in coastal marine sediments is widely recognized [15,38]. Similarly, in mangroves it has been shown that sulfate reducers play an important role in the environment by not only degrading organic matter but also in mediating the production of soluble iron, phosphorus and nitrogen [15,41,74]. Sulfate reduction is an important process mediating the majority of the organic matter oxidation in sediments in our study area [75]. It appears that the biogeochemical signatures or microbial processes observed in Belize in previous studies are unique from other mangroves [75] with high rates of N_2 fixation and sulfate reduction [12,14,75]. This may be a reflection of sediment composition in our studied area, more typical of oceanic mangrove ecosystems [33], and may offer a unique environment for bacterial communities like nitrogen-fixing sulfate reducers. The substrate in this system is principally peat formed from the accumulation of refractory mangrove roots and calcareous algae for the last ~8000 years [48] containing large quantities of organic matter (>70%).

The distinctive community of diazotrophs observed in our study through the phylogenetic analysis of the *nifH* gene, supports the notion of a strong relationship between geochemical signatures and phylogenetics in mangrove sediments. Our study reports, for the first time, the application of GAM models to elucidate the microbial ecology of specific functional traits in mangrove sediments. These niche-based species distribution models [76] have been applied to a wide range of plants, macroorganisms and microorganisms [14,77–79] and consist of observations of species over a gradient of environmental parameters (or "predictors") that can have a direct or indirect effect on the establishment or survival of the species at a limited time period. GAM models assume pseudo-equilibrium between the environmental parameters and the species spatial pattern underlying the realized niche for the species studied [76]. In agreement with previous studies in mangroves and other environments [14,80] our statistical results indicate a strong influence of sedimentary and geochemical parameters on the variability of diazotrophs in the studied area (Figures 2 and 4). Parameters such as roots abundance (mostly dead roots) and H_2S in combination with nutrient concentrations (NH_4^+, PO_4^{3-}) influence the variability observed in the diazotrophic community. This indicates that the increase of nutrient concentrations as a direct result from fertilization was not the only factor affecting this microbial community, as only two TRFLP-OTUs showed significant differences among the nutrient treatments (Figure 3). The effect of long-term fertilization on the sedimentary environment (change in root production-decomposition processes that influence dead root abundance and H_2S concentration) [14,48] seems to have a stronger influence in the variability of this microbial group (Figures 2 and 4). GAM models supports these results as some of the potential nitrogen-fixing sulfate reducers are strongly influenced by specific environmental parameters like dead roots (% abundance) and porewater H_2S concentration in the porewater (Figure 4). Also, it appears that there is a minor direct influence of mangrove trees (live root abundance) on the community of potential nitrogen-fixing sulfate reducers (Figures 2 and 4). This is explained by the fact that sediments in our study area contain large amounts of organic matter (>70%) that may provide an important source of labile carbon to microorganisms relative to carbon from root exudates. Also, oxygen released from the mangrove roots (up to 60% of total sediment mass) may oxidize the sediment [81], potentially inhibiting the nitrogenase enzyme in non-heterocystous N_2 fixers [82].

In Belize, no correlation was observed between SO_4^{2-} depletion and dissolved organic carbon concentrations [75] supporting our findings that sulfate reducers are not only affected by the availability of organic carbon as suggested previously [83] but as well by specific pore-water parameters. The high number of microenvironments or niches observed for some potential nitrogen-fixing sulfate reducers (Figure 5) reveals the complexity of microbial communities even within specific functional groups with varied roles in sediments. The fact that even rare OTUs presented a significant response ($p < 0.05$) to specific environmental parameters (Figures 4 and 5) suggests that rare species often observed in microbial communities may play an important role when disturbed conditions change the environment to conditions favoring rare species growth [84]. Therefore, the role of rare species on the stability and performance of the community may be important, as observed previously in other ecosystems [85].

5. Conclusions

The present study describes the complexity of microbial communities within mangrove sediments in an oceanic mangrove ecosystem (Twin Cays, Belize). Results indicate the studied area harbors a unique diazotrophic community with a low diversity in metabolic traits and dominated by potential sulfate reducers. The high number of microenvironments or niches observed for the potential nitrogen-fixing sulfate reducers reveal the complexity of microbial communities even at specific functional groups with varied roles in sediments. This "species" redundancy observed at the OTUs level in Belize suggests a diverse community of microorganisms ensuring the activity of important biogeochemical processes for the well-being of the whole ecosystem (e.g., nitrogen fixation, organic matter oxidation mediated by sulfate reduction). Although further research is needed to understand the link between sedimentary geochemistry and diazotrophs, the results obtained in this study indicate the importance of identifying the diversity and the biogeochemical factors controlling microbial populations in marine environments to better understand the role of microbial diversity and multifunctionality in highly dynamic natural systems.

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Author Contributions

Conceived and designed the experiments: I.C.R. and M.E.J.-M.; Performed the experiments: I.C.R.; Analyzed the data: I.C.R., M.E.J.-M., D.G.C. and J.A.F.; Wrote the paper: I.C.R.

Conflicts of Interest

The authors declare no conflict of interest.

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