



ORIGINAL ARTICLE

Long-term nitrogen and phosphorus fertilization effects on N₂ fixation rates and *nifH* gene community patterns in mangrove sediments

Isabel C. Romero¹, Myrna Jacobson¹, Jed A. Fuhrman¹, Marilyn Fogel² & Douglas G. Capone¹¹ University of Southern California, Los Angeles, CA, USA² Carnegie Institution of Washington, Geophysical Laboratory, Washington, DC, USA**Keywords**

Diazotrophs; mangroves; nitrogen fixation; sediment.

CorrespondenceIsabel C. Romero, 3616 Trousdale Parkway, AHF, Los Angeles, CA 90089, USA.
E-mail: iromero@usc.edu

Accepted: 19 May 2011

doi:10.1111/j.1439-0485.2011.00465.x

Abstract

The bioavailability of nutrients is important in controlling ecological processes and nitrogen cycling in oligotrophic mangrove forests, yet the variation of diazotrophic community structure and activity with nutrient availability in sediments remains largely unexplored. To investigate for the first time how nutrients in sediments affect spatial and temporal patterns of diazotrophic community structure and activity, the sedimentary environment of Twin Cays, Belize, was examined with respect to the effects of long-term fertilization [treatments: control (Ctrl), nitrogen (N), and phosphorus (P)] on N₂ fixation rates and *nifH* gene community structure. We found that N₂ fixation rates were significantly higher at the P-treatment, intermediate at the Ctrl-treatment and lower in the N-treatment (P: 4.2 ± 0.5, Ctrl: 0.8 ± 0.1, N: 0.4 ± 0.1 nmol·N·g⁻¹·h⁻¹; P < 0.001) with spatial (Ctrl- and P-treatments) and temporal (only P-treatment) variability positively correlated with live root abundance (r² = 0.473, P < 0.001) and PO₄³⁻ concentration (r² = 0.458, P < 0.0001). The community structure of diazotrophs showed larger spatial and temporal variability in the fertilized treatments than in the Ctrl-treatment, with the relative abundance of OTUs (*nifH* operational taxonomic units) at the fertilized treatments inversely related to live root abundance. Overall, long-term fertilization (with either N or P) affects not only nutrient levels in mangrove sediments directly, but also spatial and temporal patterns of both community structure and activity and likely plant-microbe interactions as well. Our findings suggest that the maintenance of natural nutrient conditions in mangrove sediments is important to ensure the stability of microbial functional groups like diazotrophs.

Introduction

The bioavailability of nutrients in sediments is one of the most important factors controlling ecological and physiological processes in the mangrove ecosystem (Alongi *et al.* 2002; Feller *et al.* 2002; Cheeseman & Lovelock 2004; Krauss *et al.* 2008). Although mangroves are highly productive, they are generally nutrient-deficient, relying on microbial and plant cycling processes for nutrients

(Holguin *et al.* 2001; Feller *et al.* 2002; Reef *et al.* 2010). Anthropogenic activity in coastal areas can largely affect mangroves by external inputs of nutrients, causing negative changes in forest development and sediment geochemistry (Reef *et al.* 2010).

The biological fixation of nitrogen is an important source for 'new' nitrogen in oligotrophic mangrove forests (Capone 1988; Lee & Joye 2006; Purvaja *et al.* 2008). Free-living diazotrophs are widely distributed within the

mangrove ecosystem, with N_2 fixation being detected in sediments, cyanobacterial mats, leaf litter and in association with roots (Zuberer & Silver 1978; Holguin *et al.* 1992; Toledo *et al.* 1995; Pelegri & Twilley 1998; Rejmánková & Komárková 2005; Lee & Joye 2006). Under natural conditions, mangrove trees can influence the growth and distribution of microbial groups by enriching the organic carbon pool and changing the redox conditions of the sediments (Holguin *et al.* 2001). Several studies indicate that a close root–bacterial interaction in sediments strongly influence the activity (Zuberer & Silver 1978; Ravikumar *et al.* 2004) and diversity (Flores-Mireles *et al.* 2007) of diazotrophs. Other studies claim diazotrophs are primarily driven by geochemical parameters in sediments (Zhang *et al.* 2008). To better understand the effects of external inputs of nutrients in the mangrove nitrogen cycle, the ecology of diazotrophs in mangrove sediments and the intra-forest factors that regulate its diversity and activity warrant greater study.

In this study we investigated for the first time in mangrove sediments the effects of nutrient availability on spatial and temporal patterns of diazotrophic community structure and activity by examining *nifH* gene community structure and N_2 fixation rates at an established long-term nitrogen and phosphorus fertilization experiment in Twin Cays, Belize (established 1997 and maintained by I. C. Feller and coworkers; McKee *et al.* 2007). Studies in Belizean forests have indicated that N_2 fixation is an important biogeochemical process influencing the nutrient status of the sediments (Lee & Joye 2006), and that fertilization affects growth and physiological processes of mangrove trees (Lovelock *et al.* 2006; Feller *et al.* 2007; McKee *et al.* 2007). However, it is unclear how fertilization influences spatial and temporal patterns of diazotrophs in sediments. We used a combination of geochemical and molecular tools with multivariate methods to evaluate the covariation of this microbial group with sediment environmental parameters. We tested for significant changes in spatial and temporal patterns of diazotrophic community structure and activity with sedimentary parameters at different nutrient conditions (fertilization treatments) in mangrove sediments.

Material and Methods

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. There are two seasons, a wet season from July to October with an average rainfall of 218 cm per

year and a dry season the rest of the year (Rützler & Ferraris 1982). The island substrate is principally peat formed from the fine roots of mangrove trees, primarily from the dominant species *Rhizophora mangle*. The study area is located in the interior zone of the mangrove forest, which covers about 60% of the mangrove ecosystem in Twin Cays and where dwarf trees (~1.5 m tall) are found (Rodríguez & Feller 2004). The interior mangrove zone is almost continually flooded, although during the dry season sediments can be exposed for long periods. An ongoing fertilization experiment was established in 1997 and maintained by I. C. Feller and coworkers of the Smithsonian Institution. For more details of this study see Feller *et al.* (2002) and McKee *et al.* (2007). Briefly, at 6-month intervals, individual trees were fertilized with nitrogen (urea) or phosphorus (P_2O_5) using dialysis tubing (three trees per treatment). 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. A control treatment included coring and plugging with no fertilizer.

Collection of environmental samples

Samples were collected from an on-going fertilization experiment in the interior mangrove zone [treatments: control (Ctrl), phosphorus (P), and nitrogen (N)]. At each experimental tree (three trees per treatment) sediment cores were taken randomly at a distance >0.5 m from the fertilizer tube during two different seasons (wet and dry). We used a Russian Peat corer designed to avoid vertical compaction of sediment samples. Sediment cores were divided by depth intervals (0–5, 5–10 and 20–30 cm) for sediment characterization, N_2 fixation measurements and DNA extraction (details below). Live roots and bulk sediment samples were also collected from each depth interval ($n = 3$ per depth) for DNA extraction (*nifH* gene analysis). During the dry season only, additional sediment cores for N_2 fixation measurements were collected at a 10-day interval (at day 1 and later after at day 10) and within a day period (at 8:00 and 17:00 h). The 10-day interval allowed comparison of N_2 fixation rates with environmental parameters changing over a short period of time. Measurements within a day allowed calculation of N_2 fixation rates at different times of the daily cycle of photosynthetic activity of the mangrove trees. In Belize, the photosynthetic activity of mangrove trees occurs primarily in the morning due to rapid light saturation (at ~10:00 h) (Cheeseman & Lovelock 2004).

Porewater samples were collected randomly in each experimental treatment [control (Ctrl), phosphorus (P), and nitrogen (N)] at a distance >0.5 m from the fertilizer tube at each mangrove tree during two different seasons

(wet and dry), within the 10-day interval and within the day period. We used sippers inserted at three depth intervals (0–5, 5–10 and 20–30 cm; $n = 3$ per depth). Sippers were attached to a filter holder with pre-combusted glass-fiber filters (Whatman GF/F 0.7 μm , 4.7 cm diameter) and connected through a two-way stopcock valve to a luer-lock syringe (which serves as a reservoir for the sample). Samples were then refiltered with an Acrodisc syringe filter (Pall 0.45 μm HT Tuffryn® membrane) and divided into subsamples for each porewater measurement. All glass vials used were acid-washed, rinsed with ultrapure deionized water and combusted at 500 °C for 5 h.

Porewater analysis

Temperature and pH were determined immediately after collection of the porewater 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). Samples for hydrogen sulfide (H_2S) were analyzed following the method of Cline (1969). For ammonium (NH_4^+), analysis was done as in Solorzano (1969). Dissolved inorganic phosphate (PO_4^{3-}) was measured colorimetrically using the method of Strickland & Parsons (1972). A spectrophotometer (Shimadzu UV-1700) was used for porewater analysis at the University of Southern California.

Sediment characterization

The abundance of live and dead roots in each experimental treatment was determined by washing the sediment samples collected at each depth interval ($n = 3$) with different-sized sieves (4 mm, 2 mm). Each of these sediment components was picked manually, oven-dried for 48 h at 60 °C, and weighed. The weight of the live and dead roots was compared to the weight of the total sediment in each sample to calculate abundance of roots relative to total sediment mass (%).

N_2 fixation rates

Nitrogen fixation rates were measured by the acetylene reduction technique (Capone & Montoya 2001) on whole sediment samples collected from each experimental treatment. Intact sediment cores were divided into samples in a glove bag filled with N_2 ($n = 3$ per depth). Each sample contained 3–4 g of wet sediment. After sealing each sample bottle (volume 25 ml), 4 ml of acetylene was added. Samples were incubated in the dark at ambient water temperature (24 °C) for 24 h. For each incubation assay, gas samples were taken every 4–6 h and stored in

vacutainers for analysis in the laboratory at the University of Southern California. The ethylene concentration in the gas samples was determined by comparison with standards on an FID Shimadzu GC-9A gas chromatograph. The acetylene reduction rates were calculated and converted to N_2 fixation rates assuming the conversion factor of 4:1 ($\text{C}_2\text{H}_2:\text{N}_2$) (Postgate 1982; Lee & Joye 2006). Rates were calculated based on dry weight of sediments (samples were oven-dried at 60 °C for 48 h).

DNA extraction and PCR

Root and bulk sediment samples (~ 1.5 g) collected at each sediment depth from each experimental treatment were mixed with TE (10 mM Tris-HCl plus 1 mM EDTA, pH of 7.4) and frozen. DNA was extracted from the samples using the FastDNA SPIN kit (Biogene) according to the manufacturer's instructions. DNA concentration from the extractions was estimated using PicoGreen dsDNA Quantitation kit (Invitrogen) according to the manufacturer's instructions. Final DNA concentration from extracts was 50–100 $\text{ng}\cdot\mu\text{l}^{-1}$. The functional gene targeted for amplification was dinitrogenase reductase (*nifH*) using previously designed degenerate primers (Zehr & McReynolds 1989). Reactions for the nested PCR for amplification of the *nifH* gene consisted of 1 \times PCR Buffer, 2.5 mM MgCl_2 , 0.2 mM dNTPs, 400 $\text{ng}\cdot\mu\text{l}^{-1}$ BSA, 5.0 U Taq, and 0.8 μM for each primer: *nifH3* (5'-ATRTT-RTTNGCNGCRTA-3') and *nifH4* (5'-TTYTAYGGNAAR-GGNGG-3') for the first reaction, *nifH1* (5'-TET-TGYG AYCCNAARGCNGA-3') and *nifH2* (5'-ANDGCCATCA-TYTCNCC-3') for the second reaction. Negative controls were always included for the first and second PCR reactions. The quality and concentration of the PCR products were checked by SYBR® Gold staining on a 2% agarose gel. PCR products were run in a 2% agarose gel for 2 h and the desired product size (~ 360 to 380 bp) was excised, purified (Qiagen), and quantified (PicoGreen; Invitrogen) following the manufacturer's instructions. Products were stored at -80 °C. In addition, the purity of the *nifH* products was confirmed by cloning and sequencing (I.C. Romero, M.E. Jacobson, J.A. Fuhrman, D.G. Capone unpublished data).

Terminal restriction fragment length polymorphism (TRFLP)

TRFLP of PCR-amplified *nifH* gene was used to describe the variability of the potential N_2 -fixing bacteria. TRFLP analysis is a PCR-based method and therefore is limited by all PCR artifacts (Hewson & Fuhrman 2006). However, TRFLP analysis has been used widely in ecological studies to compare microbial populations in natural

environments due to its rapid output and high reproducibility (Blackwood *et al.* 2003; Thies 2007). In addition, TRFLP can be used to calculate the presence and relative abundance of OTUs. This is ideal for studying the variability of diazotrophic communities under different impacted conditions (Deslippe *et al.* 2005; Moseman *et al.* 2009).

PCR products were digested with the restriction enzyme HaeIII following the manufacturer's instructions (NEB). This enzyme was chosen because previous tests showed better consistency of peak numbers over a series of replicates than other enzymes. Briefly, for each reaction, 10 μ l of PCR product was digested with 20 U enzyme and 1 \times buffer at 37 °C overnight. Reactions were terminated at 80 °C for 15 min. Digested products were cleaned with a DNA clean and concentrator kit (Zymo Research Corp.) following the manufacturer's instructions. Clean digested products were run in duplicates in an automated sequencer (Applied Biosystems 96 capillary 3730 DNA Analyzer) and the outputs were aligned against all possible fragment lengths (100–360 bp). Fragments were binned and analyzed to determine the community structure of bacteria containing the *nifH* gene. The area under each peak was used to calculate the relative abundance (%) of each OTU in each sample (peak area of an OTU divided by the total peak area of all OTUs combined). In addition, the presence of OTUs in each sample was used to calculate ecological indices including the Shannon index of community structural diversity (H' ; Shannon & Weaver 1963), Pielou index of community evenness (J' ; Pielou 1966), and total number of OTUs for community richness.

Univariate statistical analysis

The spatial and temporal variability of all porewater parameters, N_2 fixation rate data, and community structure indices were analyzed with ANOVA with nutrient treatment, depth, season, season \times nutrient treatment interaction and depth \times nutrient treatment interaction as factors (JMP Software; SAS Institute Inc., Cary, NC, USA). 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-level <0.05. All data were tested to fulfill normality and equal variance assumptions, and transformations were carried out when necessary. Results are indicated as significant or nonsignificant followed by the P-value. All values are shown as the average \pm SE (where $n \geq 3$).

Multivariate statistical analysis

NifH community patterns based on relative abundance of OTUs were analyzed along with the environmental

gradient data using unimodal constrained models [canonical correspondence analysis (CCA)]. In CCA plots, each canonical ordination axis corresponds to a direction in the multivariate scatter of the OTU data that is maximally related to the environmental variables (Legendre & Legendre 1998). How well the data are displaced in the CCA result plot was expressed by the percentage variance accounted for the fitted OTU data and by a Monte Carlo permutation test ($n = 499$; statistical significance set to P-level <0.05). The temporal variability of the community containing the *nifH* gene was analyzed using experimental treatments (Ctrl, N, P) and seasons (wet and dry) as interaction factors, and sediment depth and substrate types were used as covariables. The spatial variability of the *nifH* community was analyzed using the experimental treatments and the depth intervals as interaction factors, and seasons as covariables. Two assumptions were made when running the temporal and spatial analyses: (i) the relative abundance of dead and live mangrove roots does not change between the wet and the dry seasons and (ii) physical and chemical parameters of the porewaters are the same for the roots and sediment samples at each depth interval. CCA and ordination plots were conducted with CANOCO software (Microcomputer Power, Ithaca, NY, USA). The relative abundance of OTUs was transformed [$\log(x + 1)$], and the environmental data was centered and standardized. Generalized additive models (GAMs; Hastie & Tibshirani 1986) were used to look at the OTU response curves to the environmental parameters.

Results

Environmental parameters

Significantly higher abundance of live roots (as % of total sediment mass) was observed at the P-treatment (with the largest abundance at the 0–1 cm depth interval) compared to the other treatments (N: 0.1–21.2%, Ctrl: 0.2–23.0%, P: 0.1–55.6%; $P < 0.001$; Fig. 1), indicating that mangrove roots growth was stimulated as P limitation was alleviated by P-fertilization. In contrast, dead root abundance (as % of total sediment mass) was higher at the N- and Ctrl-treatment but not significantly different to the P-treatment (N: 0.7–37.5%, Ctrl: 0.1–39.1%, P: 0.1–21.6%; $P > 0.05$), suggesting fertilization does not strongly promote root mortality and/or changes in decomposition rates.

PO_4^{3-} concentration was significantly lower in the Ctrl- and N-treatment at all depths (range from 0.2 to 3.0 μ M; $P < 0.01$; Fig. 1) compared to the P-treatment, where accumulation of PO_4^{3-} occurs at 20–30 cm depth (up to 390 μ M; Fig. 1). NH_4^+ concentration was higher in the

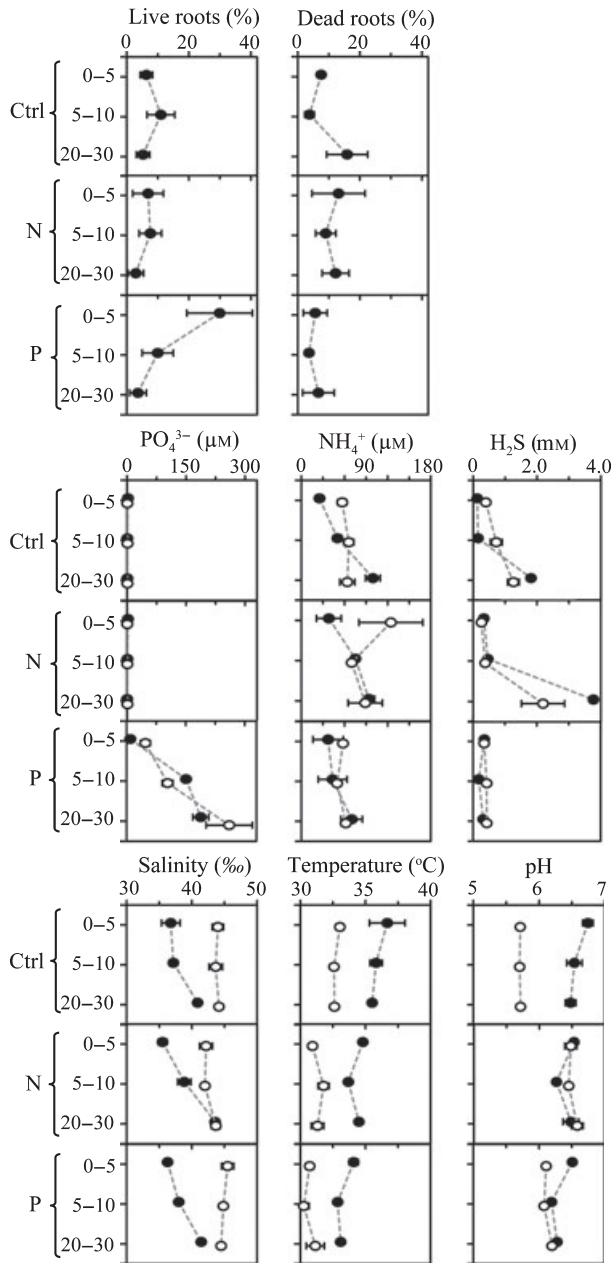


Fig. 1. Roots and porewater parameters from the wet (black circles) and dry (white circles) season in the experimental treatments (Ctrl, N, P) at each sediment depth interval (0–5, 5–10, 20–30 cm). Live and dead root abundances showed as % of total sediment mass. Data shown as average \pm SE.

N-treatment but was not significantly different from the other treatments (N: 3.73–296.5 μM , P: 3.5–152.5 μM , Ctrl: 9.4–99.5 μM ; $P > 0.05$; Fig. 1). H_2S concentration was significantly lower in the P-treatment than in the other treatments (P: 0.1–0.7 mM, Ctrl: 0.1–1.8 mM, N: 0.1–3.8 mM; $P < 0.01$; Fig. 1). Porewater salinity was

found to increase with depth in all treatments during the wet season (Fig. 1), indicating poor drainage in the study area. A porewater temperature gradient was observed from high to low in the experimental treatments (Ctrl $>$ N $>$ P; $P < 0.001$; Fig. 1) following the trend in forest canopy cover (P $>$ N $>$ Ctrl), which reduces sediment surface exposure to direct sunlight.

Seasonal differences in porewater parameters were observed (Fig. 1). Relative to the dry season, the wet season is characterized by lower salinity and higher temperature values in all treatments, higher pH values only at the Ctrl-treatment, higher H_2S values at the N-treatment, and lower NH_4^+ concentrations at the N-treatment. These results indicate that porewater parameters in each experimental treatment vary distinctly between seasons.

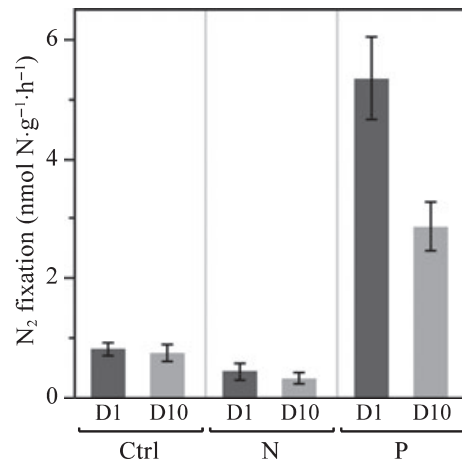


Fig. 2. N_2 fixation rates for a period of 10 days (D1 versus D10) in the experimental treatments (Ctrl, N, P). Data shown as average \pm SE.

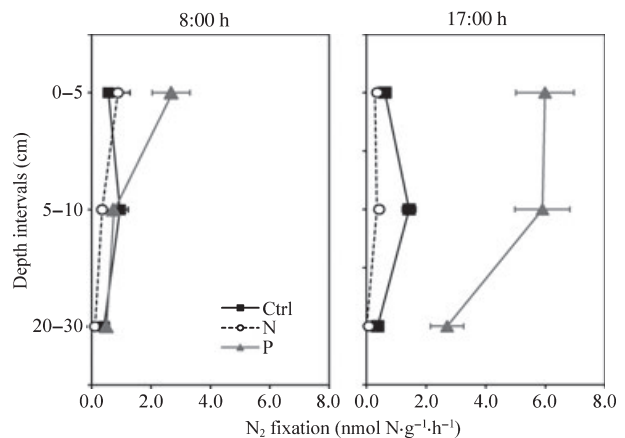


Fig. 3. N_2 fixation rates within a day (8:00 versus 17:00 h) in the experimental treatments (Ctrl, N, P) at each sediment depth interval (0–5, 5–10, 20–30 cm). Data shown as average \pm SE.

N₂ fixation rates

Significantly higher rates were found at the P-treatment compared to intermediate rates at the Ctrl-treatment and lower rates at the N-treatment (P: 4.2 ± 0.5 , Ctrl: 0.8 ± 0.1 , N: 0.4 ± 0.1 nmol·N·g⁻¹·h⁻¹; $P < 0.001$; Fig. 2). Correlation of depth distribution of nitrogen fixation rates and live root abundance was observed ($r^2 = 0.473$, $P < 0.001$). Higher rates were found at 5–10 cm depth in the Ctrl and at 0–10 cm depth in the P-treatment (Fig. 3), following live root abundance in the sediments (Fig. 1).

N₂ fixation rates were similar between seasons in all treatments (data not shown; $P > 0.05$) but large changes were found at the P-treatment over short periods of time (day 1 versus day 10: $P < 0.01$, Fig. 2; within a day period: $P < 0.001$, Fig. 3). A significant positive correlation between N₂ fixation rates and PO₄³⁻ concentration explains the observed decrease in N₂ fixation rates between day 1 and day 10 ($r^2 = 0.458$, $P < 0.0001$; PO₄³⁻ range from 130.1 ± 27.7 μM at day 1 to 66.2 ± 13.4 μM at day 10). Significant lower N₂ fixation rates were found at than at 17:00 h (8:00 h: 1.8 ± 0.4 nmol·N·g⁻¹·h⁻¹, 17:00 h: 4.9 ± 0.5 nmol·g⁻¹·h⁻¹; $P < 0.01$), indicating a potential inverse relation between N₂ fixation and the photosynthetic activity of trees within a day.

NifH community structure

nifH gene diversity (H') was similar among experimental treatments (Ctrl, N, P), root versus sediment samples, seasons and depth intervals (all $P > 0.05$). In contrast, community evenness (J') and richness (number of OTUs) indices showed seasonal variability in the N- and P-treatment from live root (except J' in the P-treatment) and bulk sediment (only for J') samples (Fig. 4). These results indicate that the diazotroph community structure is temporally variable only in the fertilized treatments (N, P).

Comparison of *nifH* communities and environmental parameters

Partial canonical correspondence analysis (pCCA) was used to assess the relative similarity of *nifH* community composition between the experimental treatments as a function of temporal and spatial variability of environmental parameters (from Fig. 1). Temporal (wet versus dry seasons) pCCA analysis showed that 42.3% ($P < 0.01$) of the seasonal variability of *nifH* communities is explained by the environmental parameters measured (24.7% on axis 1 and 11.8% on axis 2; Fig. 5). PO₄³⁻ concentration seemed to be primarily driving the temporal variability of the *nifH* community in the P-treatment.

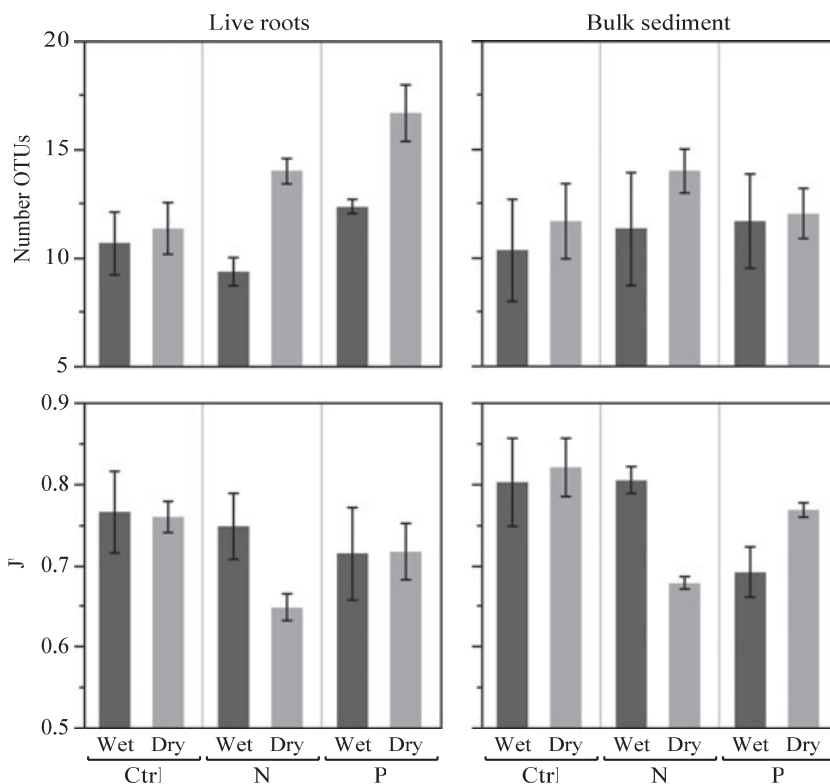


Fig. 4. Ecological indices of community structure based on *nifH* OTUs for live root and bulk sediment samples from the wet (black bars) and dry (gray bars) season in the experimental treatments (Ctrl, N, P). Data shown as average \pm SE.

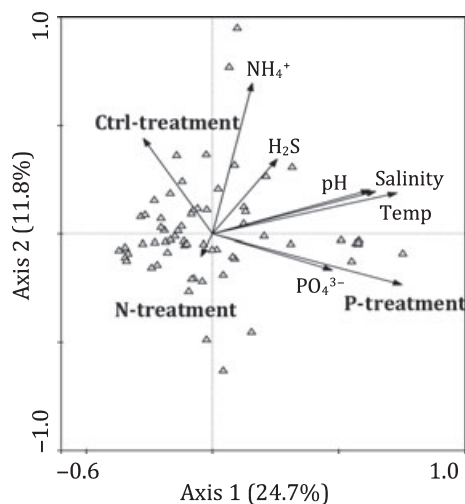


Fig. 5. Ordination plot of pCCA results for *nifH* OTUs temporal (seasonal) variability (42.3% explained variance, $P = 0.004$). Sediment depth intervals and substrate type were used as covariables. Triangles indicate OTUs at their best position in the plot relative to the treatments and environmental parameters (shown as arrows). The length of the arrows indicates the strength of environmental parameters in explaining OTU abundance variability. Arrows with same direction represents positive correlated variables while arrows at a 90° angle represents uncorrelated variables.

In contrast, NH_4^+ and H_2S concentrations showed a strong influence on the *nifH* community in the Ctrl-treatment. Parameters such as salinity and temperature contribute more in explaining the temporal variability of the *nifH* communities in the P-treatment than in the Ctrl-treatment. The *nifH* community in the N-treatment seems less variable between the seasons, although it is strongly influenced by NH_4^+ and H_2S (inversely correlated, Fig. 5).

Spatial pCCA analysis showed that 78.4% ($P < 0.01$) of the *nifH* community spatial variability is explained by sediment depth in each experimental treatment (24.7% on axis 1 and 11.8% on axis 2; Fig. 6). Larger variability in the *nifH* community composition was observed under fertilized conditions (N, P). The distribution of live root in sediments seemed to be primarily driving the spatial variability of the *nifH* community in both the N- and P-treatment. The position of the OTUs in the pCCA plot (Fig. 6) denotes that the niche optima for the majority of the OTUs was where lower abundance of live roots occurs in the N- and P-treatment (live root abundance $< 20\%$). GAM models of environmental parameters (data not shown) only showed significant OTU responses curves to live root abundance, indicating as well that community members in fertilized treatments (N, P) are preferentially only found at low abundance of live roots (Fig. 7).

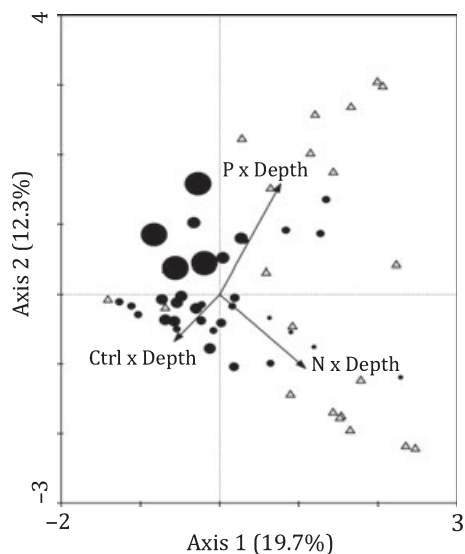


Fig. 6. Ordination plot of pCCA results for *nifH* OTUs spatial (sediment depth) variability (78.4% explained variance, $P = 0.002$). Temporal variability between the seasons was used as covariable. Triangles indicate OTUs at their best position in the plot relative to the treatments and environmental parameters (shown as arrows). 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. The size of the circles indicates the relative abundance of live roots with a range 3–50%.

Discussion

Porewater under fertilized conditions

Fertilization with N increased NH_4^+ porewater concentrations by only 35% (Fig. 1), indicating that most NH_4^+ was used or exported from the fertilized sediments. Other studies in the same interior mangrove forest showed that phosphorus and not nitrogen is the critical limiting nutrient for plants (Feller *et al.* 2002) and bacteria (I. C. Romero *et al.*, unpublished data). Accumulation of PO_4^{3-} with depth is explained by a large decrease of live roots with depth (Fig. 1). It has also been shown in the same area that N-fertilization did not increase mangrove tree biomass (Mckee *et al.* 2007) or porewater nitrate/nitrite concentration (M. Jacobson personal observation) indicating that excess nitrogen may be lost primarily through the sediment–water interface (via ammonia volatilization and denitrification) and/or during high tidal water exchange (mainly around June). In this mangrove area, ammonia emissions are high ($2.0 \mu\text{mol}\cdot\text{NH}_3\cdot\text{m}^{-2}\cdot\text{h}^{-1}$, Fogel *et al.* 2008) and denitrification rates are lower or close to N_2 fixation rates ($9.9 \text{ nmol}\cdot\text{N}\cdot\text{m}^{-2}$ per year, Lee & Joye 2006). The excess nitrogen leaving the sediment may impact other ecosystems surrounding the mangrove

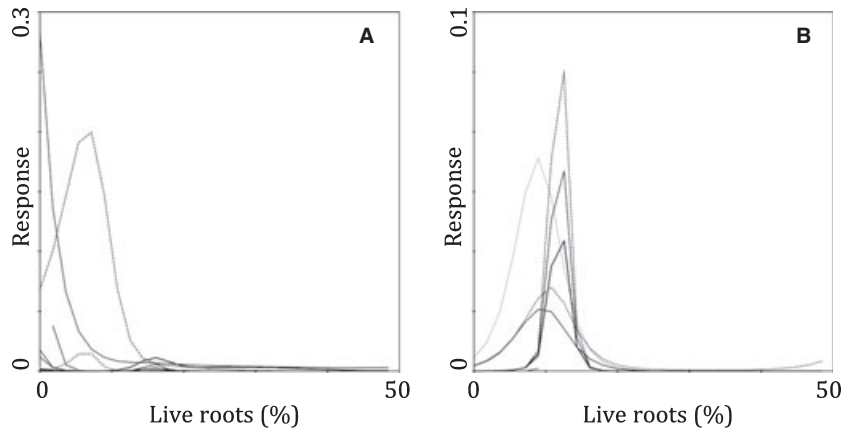


Fig. 7. Response of *nifH* OTUs to the relative abundance of live roots using generalized additive models (GAM). (A) OTU curves in the N-treatment; (B) OTU curves in the P-treatment.

forests and/or may increase the local production of gases with climatic relevance (e.g. nitrous oxide, ammonia). An increase in the emissions of N_2O in terrestrial (Lund *et al.* 2009) and mangrove (Corredor *et al.* 1999) environments from fertilized soils was observed previously. Fertilizers are considered one of most important pollutants in coastal environments, with deleterious effects on the nitrogen cycle (Vitousek *et al.* 1997; Galloway *et al.* 2004) and to mangroves (Reef *et al.* 2010). Studies evaluating the effect of fertilizers (e.g. by sewage) into the mangrove ecosystem should focus on the export pathways for nitrogen to better quantify the effect of fertilization on the nitrogen cycle in adjacent ecosystems and in the atmospheric nitrogen chemistry. Our results support the hypothesis that mangrove forests may have a limited capacity for retention of nutrients and for protecting other marine environments from land-derived nutrients (Reef *et al.* 2010).

N_2 fixation in fertilized sediments

Depth-integrated N_2 fixation rates under natural conditions (Ctrl-treatment, $54.2 \pm 4.2 \mu\text{mol}\cdot\text{N}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$; range: $0\text{--}204 \mu\text{mol}\cdot\text{N}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$) are comparable but higher in most cases than rates in other mangrove forests worldwide (Lee & Joye 2006). A similar pattern of N_2 fixation rates under N- and P-enriched conditions (lower in the N-treatment and higher in the P-treatment relative to the Ctrl-treatment, Fig. 2) has been observed in different ecosystems such as seagrasses, coral reefs and marshes (Short *et al.* 1990; Koop *et al.* 2001; Cerna *et al.* 2009).

The occurrence of N_2 fixation under N-fertilized conditions has been explained in previous studies by a mutualistic interaction between plants and diazotrophs, where bacteria are stimulated to fix nitrogen by inputs of plant photosynthate to the rhizosphere (Welsh *et al.* 1997).

However, mangrove plants in our study area are primarily P-limited (Feller *et al.* 2002) and therefore trees may not strongly promote N_2 fixation in N-fertilized areas. The NH_4^+ porewater concentration observed in the N-treatment ($81.7 \pm 1.7 \mu\text{mol}$) is lower than the concentration known to strongly inhibit sediment diazotrophs ($\sim 100\text{--}500 \mu\text{M}$, Yoch & Whiting 1986; Capone 1988), which may help explain the observed active diazotrophic community under N-fertilized conditions in Belizean mangrove sediments. Nevertheless, rates in N-fertilized areas are lower than in controls.

Plants can strongly influence diazotroph activity in coastal ecosystems (Zuberer & Silver 1978, Bagwell & Lovell 2000, Piceno & Lovell 2000, Welsh 2000; McGlathery 2008). In mangroves, trees can influence microbial activity and nutrient cycling by transferring rich labile carbon and oxygen from roots to the sediment environment (McKee *et al.* 1988; McKee 1993; Holguin *et al.* 2001). Higher rates and variability (spatial and temporal) of N_2 fixation were found in the P-treatment where high abundance of live roots occurs (Figs 2 and 3). However, the 3.5-fold increase in N_2 fixation rates from 8:00 to 17:00 h in the P-treatment (Fig. 3) indicates that the diazotrophic community is less active when the trees are photosynthetically active in the morning. This pattern in our study differs from other mangrove and seagrass ecosystems where a strong dependence of diazotroph activity on root exudates for carbon under natural conditions was observed (Zuberer & Silver 1978; Capone & Taylor 1980; Welsh *et al.* 1997; Holguin *et al.* 2001). In our study area, sediments contain large amounts of organic matter ($\sim 85\%$), with decomposing roots that may provide an important source of labile carbon for diazotrophs. The oxygen released from the mangrove roots may oxidize the sediment, potentially inhibiting the nitrogenase enzyme in non-heterocystous N_2 fixers (e.g. Gallon 1992) and imposing the temporal separation in N_2 fixation

observed in the P-treatment. Low live root abundance in the other treatments (Ctrl, N) may not strongly influence N_2 fixation rates. Our results indicate that coupling between roots and N_2 fixation in Belize largely depends on the nutrient condition of the sediments.

Diazotrophic communities in fertilized sediments

Multivariate analysis of OTU relative abundance indicated that there is a distinct diazotrophic community in each treatment (Ctrl, N, P), which may be explained by differences in the geochemical composition of the sediments and live root abundance (Fig. 5). A link between functional diversity and ecosystem geochemistry has also been shown in other mangrove forests, ecosystems and microbial groups (López *et al.* 1988, Welsh 2000; Flores-Mireles *et al.* 2007; Zhang *et al.* 2008). In mangroves, previous studies in non-peaty sediments have indicated large bacteria and diazotroph populations associated with mangrove roots compared to sediments (Sengupta & Chaudhuri 1991; Sjöling *et al.* 2005). Spatial variability of bacterial diversity in these studies was related to conditions of the mangrove forests (disturbance or development) where organic matter, oxygen and nutrients are important in favoring some microbial groups such as heterotrophic nitrogen fixers. Results from our study area with peat sediments containing large amounts of organic matter (~85%) also indicated the importance of geochemical parameters such as nutrients in structuring diazotrophic communities. However, compared to the Ctrl-treatment, we observed different patterns in the diazotrophic communities at the fertilized areas (N and P), with OTU relative abundance inversely related to live roots distribution. Results in our study indicate for the first time that long-term fertilization with either N or P not only affects nutrient levels directly, and spatial and temporal patterns of both community structure and activity, but also potentially plant-microbe interactions.

Acknowledgements

We thank I. C. Feller (Smithsonian Environmental Research Center) for her support in using the experimental plots in the field, and Dan Miller the Station Manager of Carrie Bow Cay Research Station (Belize) for all his help during the field trips. We also thank Matt Wooller and Quinn Roberts for their assistance during the fieldwork. We appreciate comments from Co-editor Maria Cristina Gambi, Nuria Marbà and one anonymous reviewer, whose suggestions improved the manuscript. The field trips to Belize were supported by the NSF Bio-complexity award to M.J. (DEB-9981357).

References

- Alongi D.M., Trott L.A., Wattayakorn G., Clough B.F. (2002) Below-ground nitrogen cycling in relation to net canopy production in mangrove forest of southern Thailand. *Marine Biology*, **140**, 855–864.
- Bagwell C.E., Lovell C.R. (2000) Persistence of selected *Spartina alterniflora* rhizosphere diazotrophs exposed to natural and manipulated environmental variability. *Applied and Environmental Microbiology*, **66**, 4625–4633.
- Blackwood C.B., Marsh T., Kim S.H., Paul E.A. (2003) Terminal restriction fragment length polymorphism data analysis for quantitative comparison of microbial communities. *Applied and Environmental Microbiology*, **69**, 926–932.
- Capone D.G. (1988) Benthic nitrogen fixation. In: Blackburn T.H., Sørensen J. (Ed.), *Nitrogen Cycling in Coastal Marine Environments*, SCOPE Series, J. Wiley, New York: 85–123.
- Capone D.G., Montoya J.P. (2001) Nitrogen fixation and denitrification. *Methods in Microbiology*, **30**, 501–515.
- Capone D.G., Taylor B.F. (1980) N_2 (C_2H_2) fixation in the rhizosphere of *Thalassia testudinum*. *Canadian Journal of Microbiology*, **26**, 998–1005.
- Cerna B., Rejmankova E., Snyder J.M., Santruckova H. (2009) Heterotrophic nitrogen fixation in oligotrophic tropical marshes: changes after phosphorus addition. *Hydrobiologia*, **627**, 55–65.
- Cheeseman J.M., Lovelock C.E. (2004) Photosynthetic characteristics of dwarf and fringe *Rhizophora mangle* L. in a Belizean mangrove. *Plant, Cell and Environment*, **27**, 769–780.
- Cline J.D. (1969) Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnology and Oceanography*, **14**, 454–458.
- Corredor J.C., Morell J.M., Bauza J. (1999) Atmospheric nitrous oxide fluxes from mangrove sediments. *Marine Pollution Bulletin*, **38**, 473–478.
- Deslippe J.R., Egger K.N., Henry G.H.R. (2005) Impacts of warming and fertilization on nitrogen-fixing microbial communities in the Canadian High Arctic. *FEMS Microbiology Ecology*, **53**, 41–50.
- Feller I.C., McKee K.L., Whigham D.F., O'Neil J.P. (2002) Nitrogen vs. phosphorus limitation across an ecotonal gradient in a mangrove forest. *Biogeochemistry*, **62**, 145–175.
- Feller I.C., Lovelock C.E., McKee K.L. (2007) Nutrient addition differentially affects ecological processes of *Avicennia germinans* in nitrogen versus phosphorus limited mangrove ecosystems. *Ecosystems*, **10**, 347–359.
- Flores-Mireles A., Winans S., Holguin G. (2007) Molecular characterization of diazotrophic and denitrifying bacteria associated with mangrove roots. *Applied Environmental Microbiology*, **73**, 7308–7321.
- Fogel M., Wooller M., Cheeseman J., Smallwood B., Roberts Q., Romero I., Meyers M. (2008) Unusually negative nitrogen isotopic compositions ($\delta^{15}N$) of mangroves and lichens in an oligotrophic, microbially-influenced ecosystem. *Biogeochemistry*, **5**, 1693–1704.

- Gallon J.R. (1992) Reconciling the incompatible: N₂ fixation and O₂. *New Phytologist*, **122**, 571–609.
- Galloway J.N., Dentener F.J., Capone D.G., Boyer E.W., Howarth R.W., Seitzinger S.P., Asner G.P., Cleveland C.C., Green P.A., Holland E.A., Karl D.M., Michaels A.F., Porter J.H., Townsend A.R., Vöosmarty C.J. (2004) Nitrogen cycles: past, present, and future. *Biogeochemistry*, **70**, 153–226.
- Hastie T., Tibshirani R. (1986) Generalized additive models. *Statistical Science*, **1**, 297–310.
- Hewson I., Fuhrman J.A. (2006) Spatial and vertical biogeography of coral reef sediment bacterial and diazotroph communities. *Marine Ecology Progress Series*, **306**, 79–86.
- Holguin G., Guzman M.A., Bashan Y. (1992) Two new nitrogen-fixing bacteria from the rhizosphere of mangrove trees: their isolation, identification and *in vitro* interaction with rhizosphere *Staphylococcus* sp. *FEMS Microbiology Letters*, **101**, 207–216.
- Holguin G., Vazquez P., Bashan Y. (2001) The role of sediment microorganisms in the productivity, conservation, and rehabilitation of mangrove ecosystems: an overview. *Biology & Fertility of Soils*, **33**, 265–278.
- Koop K., Booth D., Broadbent A.D., Brodie J., Bucher D., Capone D., Coll J., Denison W., Erdmann M., Harrison P., Hutchings O., Jones G.B., Larkum A.W., O'Neil J., Steven A., Tentori E., Ward S., Williamson J., Yellowlees D. (2001) ENCORE: the effect of nutrient enrichment on coral reefs, synthesis of results and conclusions. *Marine Pollution Bulletin*, **42**, 91–120.
- Krauss K.W., Lovelock C., Mckee K., Lopez-Hoffman L., Ewe S.M.L., Sousa W. (2008) Environmental drivers in mangrove establishment and early development: a review. *Aquatic Botany*, **89**, 105–127.
- Lee R., Joye S. (2006) Seasonal patterns of nitrogen fixation and denitrification in oceanic mangrove habitats. *Marine Ecology Progress Series*, **307**, 127–141.
- Legendre P., Legendre L. (1998) *Numerical Ecology*, 2nd English edn. Elsevier Science, Amsterdam.
- López N.I., Duarte C.M., Vallespinós F., Romero J., Alcoverro T. (1988) The effect of nutrient additions on bacterial activity in seagrass (*Posidonia oceanica*) sediments. *Journal of Experimental Marine Biology and Ecology*, **224**, 155–166.
- Lovelock C.E., Ball M.C., Choat B., Engelbrecht B.M.J., Holbrook N., Feller I.C. (2006) Linking physiological processes with mangrove forest structure: phosphorus deficiency limits canopy development, hydraulic conductivity and photosynthetic carbon gain in dwarf *Rhizophora mangle*. *Plant, Cell and Environment*, **29**, 793–802.
- Lund M., Christensen T.R., Mastepanov M., Lindroth A., Ström L. (2009) Effects of N and P fertilization on the greenhouse gas exchange in two nutrient-poor peatlands. *Biogeosciences Discussion*, **6**, 4803–4828.
- McGlathery K.J. (2008) Nitrogen cycling in seagrass meadows. In: Capone D.E., Carpenter E., Bronk D., Mulholland M. (Eds), *Nitrogen Cycling in the Marine Environment*, 2nd edn. Elsevier Inc., Burlington: 1037–1060.
- McKee K.L. (1993) Soil physicochemical patterns and mangrove species distribution – reciprocal effects? *Journal of Ecology*, **81**, 477–487.
- McKee K.L., Mendelsohn I.A., Hester M.W. (1988) Reexamination of pore water sulfide concentrations and redox potentials near the aerial roots of *Rhizophora mangle* and *Avicennia germinans*. *American Journal of Botany*, **75**, 1352–1359.
- McKee K.L., Cahoon D.R., Feller I.C. (2007) Caribbean mangroves adjust to rising sea level through biotic controls on change in soil elevation. *Global Ecology Biogeography*, **16**, 545–556.
- Moseman S.M., Zhang R., Qian P.Y., Levin L.A. (2009) Diversity and functional responses of nitrogen-fixing microbes to three wetland invasions. *Biological Invasions*, **11**, 225–239.
- Pelegri S.P., Twilley R.R. (1998) Heterotrophic nitrogen fixation (acetylene reduction) during leaf-litter decomposition of two mangrove species from South Florida, USA. *Marine Biology*, **131**, 53–61.
- Piceno Y.M., Lovell C.R. (2000) Stability in natural bacterial communities: II. Plant resource allocation effects on rhizosphere diazotroph assemblage composition. *Microbial Ecology*, **39**, 41–48.
- Pielou E.C. (1966) Species-diversity and pattern-diversity in the study of ecological succession. *Journal of Theoretical Biology*, **10**, 370–383.
- Postgate J.R. (1982) *The Fundamentals of Nitrogen Fixation*. Cambridge University Press, Cambridge: 252 pp.
- Purvaja R., Ramesh R., Ray A., Rixen T. (2008) Nitrogen cycling: a review of the processes, transformations and fluxes in coastal ecosystems. *Current Science*, **94**, 1419–1438.
- Ravikumar S., Kathiresan K., Ignatiammal T.M., Selvam M.B., Shanthy S. (2004) Nitrogen-fixing azobacters from mangrove habitat and their utility as marine biofilters. *Journal Experimental Marine Biology and Ecology*, **312**, 5–17.
- Reef R., Feller I.C., Lovelock C.E. (2010) Nutrition of mangroves. *Tree Physiology*, **30**, 1148–1160.
- Rejmánková E., Komárková J. (2005) Response of cyanobacterial mats to nutrient and salinity changes. *Aquatic Botany*, **83**, 87–107.
- Rodríguez W., Feller I.C. (2004) Mangrove landscape characterization and change in Twin Cays, Belize using aerial photography and Ikonos satellite data. *Atoll Research Bulletin*, **513**, 1–24.
- Rützler K., Ferraris J.D. (1982) Terrestrial environment and climate, Carrie Bow Cay, Belize. In: Rützler K., Macintyre I.G. (Eds), *The Atlantic Barrier Reef Ecosystem at Carrie Bow Cay, Belize. I: Structure and Communities*. Smithsonian Institution Press, Washington, D.C.: 77–92.
- Sengupta A., Chaudhuri S. (1991) Ecology of heterotrophic dinitrogen fixation in the rhizosphere of mangrove plant community at the Ganges river estuary in India. *Oecologia*, **87**, 560–564.
- Shannon C.E., Weaver W. (1963) *The Mathematical Theory of Communication*. University of Illinois Press, Urbana, IL, 144 pp.

- Short F.T., Dennison W.C., Capone D.G. (1990) Phosphorus-limited growth of the tropical seagrass *Syringodium filiforme* in carbonate sediments. *Marine Ecology Progress Series*, **62**, 169–174.
- Sjöling S., Mohammed S.M., Lyimo T.J., Kyaruzi J.J. (2005) Benthic bacterial diversity and nutrient processes in mangroves: impact of deforestation. *Estuarine, Coastal and Shelf Science*, **63**, 397–406.
- Solorzano L. (1969) Determination of ammonia in natural waters by the phenol hypochlorite method. *Limnology and Oceanography*, **14**, 799–801.
- Strickland J.D.H., Parsons T.R. (1972) *A Practical Handbook of Sea-Water Analysis*. Fisheries Research Board of Canada, Ottawa: 311 pp.
- Thies J.E. (2007) Soil microbial community analysis using terminal restriction fragment length polymorphisms. *Soil Science Society of America Journal*, **71**, 579–591.
- Toledo G., Bashan Y., Soeldner A. (1995) Cyanobacteria and Black mangroves in Northwestern Mexico: colonization, and diurnal and seasonal nitrogen fixation on aerial roots. *Canadian Journal of Microbiology*, **41**, 999–1011.
- Vitousek P.M., Aber J.D., Howarth R.W., Likens G.E., Matson P.A., Schindler D.W., Schlesinger W.H., Tilman D.G. (1997) Human alteration of the global nitrogen cycle: sources and consequences. *Ecological Applications*, **7**, 737–750.
- Welsh D.T. (2000) Nitrogen fixation in seagrass meadows: regulation, plant–bacteria interactions and significance to primary production. *Ecology Letters*, **3**, 58–71.
- Welsh D.T., Bourgues S., de Wit R., Auby I. (1997) Effect of plant photosynthesis, carbon sources and ammonium availability on nitrogen fixation rates in the rhizosphere of *Zostera noltii*. *Aquatic Microbial Ecology*, **12**, 285–290.
- Yoch D.C., Whiting G.J. (1986) Evidence for NH_4^+ switch-off regulation of nitrogenase activity by bacteria in salt marsh sediments and roots of the grass *Spartina alterniflora*. *Applied Environmental Microbiology*, **51**, 143–149.
- Zehr J.P., McReynolds L.A. (1989) Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. *Applied Environmental Microbiology*, **55**, 2522–2526.
- Zhang Y.Y., Dong J.J., Yang Z.H., Zhang S., Wang Y.S. (2008) Phylogenetic diversity of nitrogen-fixing bacteria in mangrove sediments assessed by PCR-denaturing gradient gel electrophoresis. *Archives of Microbiology*, **190**, 19–28.
- Zuberer D.A., Silver W.S. (1978) Biological dinitrogen fixation (acetylene reduction) associated with Florida mangroves. *Applied Environmental Microbiology*, **35**, 567–575.