Cyanobacteria and black mangroves in Northwestern Mexico: colonization, and diurnal and seasonal nitrogen fixation on aerial roots

Gerardo Toledo, Yoav Bashan, and Al Soeldner

Abstract: Nitrogen fixation and colonization by associative cyanobacteria in the aerial roots (pneumatophores) of black mangrove trees was evaluated in situ at Balandra lagoon, Baja California Sur, Mexico, for 18 consecutive months. Year-round vertical zonation of cyanobacterial colonization was determined along the pneumatophores. The bottom part close to the sediment was colonized mainly by nonheterocystous, filamentous cyanobacteria resembling *Lyngbya* sp. and *Oscillatoria* sp. The central zone was colonized mainly by filaments resembling *Microcoleus* sp. and the upper part was colonized by coccoidal cyanobacteria within defined colonies resembling *Aphanothece* sp. mixed with undefined filamentous cyanobacteria. Two of the cyanobacteria (*Microcoleus* sp. and *Anabaena* sp.) isolated from the pneumatophore were diazotrophs. Massive biofilm production along the pneumatophores was evident throughout the observation period. The surrounding sediment was seasonally dominated by heterocystous *Anabaena* sp. Glass and dead-wood surfaces incubated for 18 months in the pneumatophore vicinity showed no zonation in the colonization pattern, although they were heavily colonized. In situ N_2 fixation showed seasonal and diurnal fluctuations. N_2 fixation was low during winter, increased in early summer, and reached its peak in midsummer. N_2 fixation in the summer showed diurnal peaks: one in the morning until midday and the second in the late afternoon. N_2 fixation was at its lowest levels near midnight. Light and water temperature are probably primary environmental factors governing N_2 fixation on the pneumatophores.

Key words: Avicennia germinans, black mangrove, diazotrophic cyanobacteria, nitrogen fixation, pneumatophore.

Resume : La fixation d'azote et la colonisation par cyanobactéries associatives des racines aériennes (pneumatophores) des manguiers noirs ont été evaluees in situ dans le lagon Balandra, de Baja California Sur, Mexico, durant 18 mois consécutifs. Le zonage vertical de la colonisation cyanobactérienne a été determine le long des pneumatophores durant une année entière. La partie inférieure, près du sediment, a été colonisée principalement par des cyanobactéries filamenteuses non hétérocystes ressemblant aux *Lyngbya sp.* et *Oscillatoria sp.* La zone centrale a été colonisée surtout par des filaments ressemblant à l'espèce *Microcoleus* et, la partie supérieure, par des cyanobactéries coccoïdes à l'intérieur de colonies définies ressemblant à l'espèce *Aphanothece* mélangée à des cyanobactéries filamenteuses indéterminées. Deux des cyanobactéries isolées du pneumatophore, les *Microcoleus sp.* et *Anabaena sp.,* se sont avérées diazotrophes. La production d'un biofilm massif le long des pneumatophores a été évidente tout au cours de la période d'observation. La gaine de sediment a été dominée de façot saisonniède par l'espèe hétérocyste *anabaena.* Les surfaces de bois mort et vitreuses incubées durant 18 mois dans le voisinage des pneumatophores n'ont produit aucun zonage dans le pattern de colonisation, bien qu'elles aient été fortement colonisées. La fixation d'azote in situ a présenté des fluctuation diurnes et saisonnières, savoir faible en hiver, accrue en début d'été et atteignant un pic à mi-été. Durant l'été, la fixation d'azote a montré des pics diurnes; un pic du matin jusque vers le milieu du jour et, un second, tard en après-midi.

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Les niveaux les plus faibles sont survenus près de minuit. La lumiére et la temperature de l'eau sont probablement les facteurs environnementaux principaux qui gouvernent la fixation d'azote chez les pneumatophores.

Mots clès : *Avicennia germinans,* manguier noir, cyanobact6ries diazotrophes, fixation d'azote, pneumatophores.
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Introduction

Many coastal lagoons in the tropics and subtropics support dense mangrove forests. These are some of the most productive areas in the marine and estuarine environments, supporting large plant and animal communities, many of which are economically and ecologically important (Jones 1992; Bunt 1992). Additionally, mangrove forests export large amounts of carbon and nitrogen to the coastal waters (Boto and Robertson 1990) and serve as feeding grounds and refuge for many commercial fish and shellfish (Bashan and Holguin 1995).

There are two types of mangrove communities (mangals): the estuarine soft bottom and the euhaline-methaline hard bottom (Por and Dor 1975). The main difference is the contribution of continental runoff. Estuarine mangroves grow on soft, thick mud substrates, which have an almost fluid consistency with little associated benthic algae. The euhaline-methaline mangroves lack continental runoff, thus the water in this ecosystem has a marine salinity. In methaline ecosystems, the water has a higher salinity than the nearby coastal waters (Por 1984). Those of Baja California Sur are methaline type with shrub-like trees (1-5 m in height) and clear water (the average depth of the lagoon is less than 1 m at high tide). The mangrove community in our study area at Balandra lagoon is composed of three plant species: the red mangrove *(Rhizophora mangle* L.), the white mangrove *(Laguncularia racemosa* Gaertn.), and the black mangrove *(Avicennia germinans* (L.) Stern), similar to Florida's mangroves (Zuberer and Silver 1978). The latter species has dense pneumatophores (aerial roots emerging from the sediment).

The subtropical mangrove forests on the Mexican Pacific Ocean side, together with Florida's forests at approximately the same latitude on the Atlantic Ocean side, represent the most northern distribution of mangroves in the hemisphere (24-25°N). Although both locations contain the same plant species, they do not share the same environmental conditions. The climate in Baja California strongly resembles that of the Sinai peninsula in Egypt, yet the mangroves there have different plant species (Potts 1979). Owing to the dry desert conditions of the Baja California peninsula (approximately 100 mm annual rainfall), the terrestrial nutrient contribution to the lagoons from runoff is nil and the level of nitrogenous compounds is low. For example, in the sediment of our subtropical study site, the total nitrogen content was 410 mg $N \cdot Kg^{-1}$. In the seawater of this site, the soluble organic nitrogen was 6.3 µg $N \cdot L^{-1}$ and the ammonia level was 3.8 and 5 $\mu g \cdot L^{-1}$ in the mangrove seawater and in the sediment, respectively (Holguin et al. 1992). These low nitrogen conditions are similar to those in tropical mangrove sediments (Alongi and Sasekumar 1992). Paradoxically, the mangrove forest in Balandra lagoon shows no chlorosis, is extremely dense, and apparently, is as self-sustaining in nitrogen as the Sinai mangroves (Pons 1984).

Nitrogen fixation in mangroves, first described by Zuberer and Silver (1974,1978), is well documented in several different mangrove communities (Pons 1984; Hicks and Silvester 1985; van der Valk and Attiwill 1984) but rarely with any particular reference to certain microorganisms (Holguin et al. 1992). With regards to cyanobacteria, the only information available is from the Sinai mangroves (Potts 1984).

Diurnal and seasonal diazotrophic activities vary in different mangrove ecosystems depending on many biotic factors (Pons 1979) and abiotic vehicles (Sheridan 1991). Light seems to be a prime factor in several mangrove ecosystems (Alongi et al. 1992; Bohlool and Wiebe 1978; Gotto and Taylor 1976; Jones 1992; Potts 1979; Potts and Whitton 1980) and salt marshes (Griffiths et al. 1987).

Various diazotrophs have been found on mangrove leaf and root litter, live roots, and on the surrounding sediments (van der Valk and Attiwill 1984). Cyanobacterial mats cover the sediment surrounding mangrove trees (Lotto and Taylor 1976; Hussain and Khoja 1993). Many cyanobacterial species like *Microcoleus sp., Lyngbya* sp., and *Oscillatoria* sp. are commonplace in different mangrove forests, while others are site specific. Dominance of heterocystous forms over nonheterocystous forms was observed for several mangrove ecosystems (Pons 1979). However, the nonheterocystous forms were the dominant diazotrophs in microbial mats from North Carolina (Bebout et al. 1993; Paerl et al. 1991) and from the North Sea (Stal et al. 1984).

The aerial roots (pneumatophores) of black mangroves are essential plant structures that exchange gases for the plant in an otherwise anaerobic sediment (Dawes 1981). During the tidal cycles, these roots are submerged and thereby are subjected to colonization by marine micro- and macro-organisms (Por 1984). Information on N_2 fixation and colonization of pneumatophores is scarce (Potts 1984). There is a vertical zonation of species along the pneumatophores of the black mangrove and on prop roots of the red mangrove in Sinai peninsula and Aldabra Islands in the tropics (although sharing the same common name, they are different plant species from those in Balandra lagoon), where they form dense crusts (Pons 1979, 1980; Potts and Whitton 1980; Sheridan 1991; Zuberer and Silver 1979), but cyanobacterial colonization might be more important in black than in red or white mangrove because of the high amount of colonized surface area if one takes all the pneumatophore surface of one tree into consideration.

The aims of this study were (*i*) to locate dominant cyanobacterial communities along the black mangrove pneumatophores and (*ii*) to measure the diurnal and seasonal changes in "in situ" N_2-fixation activity occurring on these pneumatophores. Short accounts were presented elsewhere (Toledo and Bashan 1994; Toledo et al. 1994).

Materials and methods

Sampling site and pneumatophore collection

The Balandra coastal lagoon lies 25 km north of La Paz, Baja California Sur, Mexico (24°20'N, 110°20'W), and has a dense, intact mangrove forest. Its exact geographical location and our sampling sites were previously described in detail (Holguin et al. 1992; Pedrin-Avilés et al. 1990). Ten black mangrove (*Avicennia germinans* (L.) Stern) pneumatophores (aerial roots; 10-17 cm in length) were collected in November 1992, in May and June 1993, and in April 1994, by cutting them with a scalpel from their emerging sites in the sediment, 3 cm below the soil surface. Sampling site was approximately at mean high tide (e.g., the pneumatophores were covered nearly every day by seawater). They were immediately transferred to the laboratory in sterile screw-capped test tubes. The pneumatophore surface was analysed (from bottom to top) on the same collection day by sampling 10 pneumatophores at random. Twenty bark samples (up to 0.5 cm^2 each) were taken at random from each zone of the pneumatophore (described later); a total of 60 samplings per pneumatophore and at least 10 separate observations were made on each sample. Each pneumatophore was divided into three segments: lower, middle, and top. The lowest part (close to sediment) was up to 3 cm from the sediment surface. The middle part was up to 7 or 10 cm (according to the height of the pneumatophore). The highest part covered the area from the middle segment to the pneumatophore's tip. The size of this segment depended on the total height of a particular pneumatophore. The observations of pneumatophore colonization were made by immersing the bark samples in a drop of sterile ASN-III medium (described later) and observing them under phase contrast microscopy.

Identification and isolation of cyanobacteria

We studied pneumatophores from different trees ranging in size from 10 to 17 cm long. The cyanobacteria residing on the pneumatophores were identified according to the classification and morphotypes described by Rippka et al. (1979) and Prescott (1962) under light and phase contrast microscopy. Filamentous cyanobacteria were assigned to each morphotype group according to their cell morphology and size. The LPP group corresponds to those filamentous nonheterocystous cyanobacteria that resembled *Lyngbya* sp., *Plectonema* sp., or *Phormidium* sp. They were further classified as either type A or B according to their cell shape, either disc or cylindrical shape, respectively.

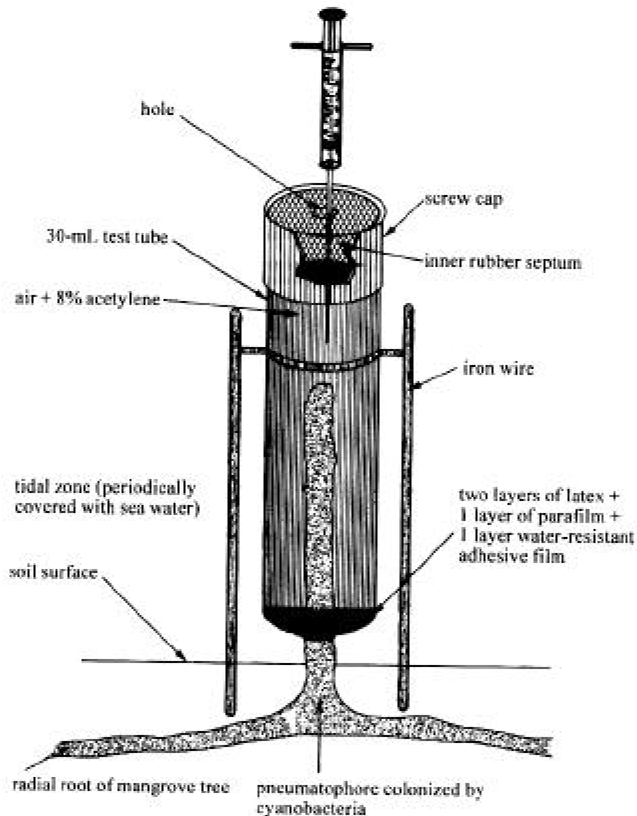
The filamentous cyanobacteria were isolated from a 1-cm^2 piece of pneumatophore bark on solid (1.2% w/v agar) ASN-III medium. The medium composition was 25 g NaCl· L^{-1}, 2 g $MgCl_2$· $6H_2O$ · L^{-1},0.5g KCl· L^{-1}, 0.75 g $NaNO_3$· L^{-1}, 0.02 g K_2HPO_4· $3H_2O$· L^{-1}, 3.5 g $MgSO_4$· $7H_2O$· L^{-1}, 0.5 $CaCl_2$· $2H_2O$· L^{-1}, 0.02 g Na_2CO_3· L^{-1}, 3 mg ferric ammonium citrate· L^{-1}, 3 mg Na_2-citrate· $2H_2O$· L^{-1}, 0.5 mg Na_2-EDTA· L^{-1}, 0.02 mg vitamin B 12· L^{-1}, 2.86 µg H_3BO_3· L^{-1}, 1.81 µg $MnCl_2$· $4H_2O$· L^{-1}, 0.222 µg $ZnSO_4$· $7H_2O$· L^{-1}, 0.39 µg Na_2MoO_4 . $2H_2O$· L^{-1}, 0.079 µg $CuSO_4$· $5H_2O$· L^{-1}, and 0.0494 µg $Co(NO_3)_2.6H_2O$· L^{-1} (Rippka et al. 1979), supplemented with 50 µg cycloheximide· mL^{-1} (Aldrich Chemical Co., Milwaukee, Wisc.) to inhibit the growth of eukaryotic organisms. The medium was sterilized by autoclaving, and the final pH was adjusted to 7.6 with 0.1 M NaOH and 0.1 M HCI.

In Baja California lagoons, as in other marine microbial mats, cyanobacterial colonization of surfaces is always associated with colonization by diverse heterotrophic bacteria as well (Bebout et al. 1993; López-Cortés 1990). To isolate single filaments, we used the method of Stal and Krumbein (1985) as follows: 7 days after placing the piece of bark on the solid medium, we collected single filaments that had developed. This was done under a microscope using a sterile Pasteur pipette to excise these filaments from the edges of the developing colonies. The filaments were placed in the centre of a fresh ASN-III agar plate. Then, they were incubated at 22 ± 1 °C under a continuous light intensity of 50 µmol. M^{-2} s^{-1} for an additional 10 days. This method is based on the assumption that the growth of colonies of filamentous cyanobacteria on solid medium is faster than the swarming of the heterotrophic bacteria on this medium. By picking filaments from the colony edge after serial transfers (5-10 repetitions of the procedure), it is possible to obtain cyanobacterial morphotypes (strains) not contaminated with other bacteria. Light and scanning electron microscopy preparations of the isolated morphotypes, as well as plating the cyanobacteria culture on common medium for heterotrophic bacteria (nutrient agar), did not reveal any presence of accompanied heterotrophic bacteria. The isolated strains were further maintained on ASN-III agar slants under the same incubation conditions for 3 months. Next, each strain was transferred separately to 30 mL N-free ASN-III in 100-mL Erlenmeyer flasks for 2 days before evaluating their N_2-fixation capacity by common acetylene reduction assay in serum bottles (described later).

In situ measurements of diazotrophic activity in Balandra lagoon

N_2 fixation in situ was evaluated by the acetylene reduction assay (ARA) (described later) in assemblies that were a modification of those designed to measure N_2 fixation in intact roots of Douglas-fir trees (Li et al.1992). A drawing of this assembly is shown in Fig. 1. We cut off the bottom of glass, screw-cap test tubes (30 mL). Then, the upper part was sealed with a rubber septum under the original plastic screw cap. The screw caps were prepunctured in the centre to allow air sampling through the rubber septum with a 1-mL syringe. The test tube was then placed over the pneumatophore and sealed to its surface by two layers of latex film, several layers of parafilm, and finally, by a water-resistant adhesive film (Scotch No. 23). Two thick wires were stuck in the mud close to each pneumatophore and used to keep the assembly upright and to prevent its bending by wave action. Additional test assemblies were checked for possible gas leaks by cutting the pneumatophore base (outside of the test tube), submerging the assembly in a bucket of water, and injecting 30 mL of air with a syringe to detect any release of bubbles. This method, however, cannot exclude the possibility of translocation of acetylene through the aerenchyma. To estimate N_2-fixation activity in the field, 1 mL of air from each assembly was replaced by acetylene. Later, 1-mL head-space gas samples (i.e., air plus other gases) were taken at 2 to 4-h intervals during 24 h from the air surrounding the pneumatophore in the assembly and stored in 10-mL sealed serum bottles, from which 1 mL of air was preevacuated. The samples were kept in an ice chest in the field.

Fig. 1. Schematic representation of an assembly used for measuring nitrogen fixation on intact pneumatophores in the field. Note that for clarity, parts of the assembly are not to the same scale.



After each sampling period of 24 h, the samples were transferred to the laboratory for gas chromatography analysis (described later).

Alternatively, N_2 fixation associated with excised pneumatophores was evaluated by removing them and incubating them in sterile test tubes with the same capping system. Negative controls (no nitrogenase activity) in tests using excised pneumatophores were obtained by rinsing the pneumatophores within the tube in 2 mL of a solution of 5% w/v trichloroacetic acid (TCA) for 5 min to determine if there was plant-derived ethylene in the assemblies. This treatment surface disinfected the pneumatophore. For intact pneumatophores (which were already placed in the assemblies), 5 mL of the same solution was applied. Additional controls included dark incubations by covering the assemblies with aluminium foil, the addition of glucose (0.1 g· L^{-1}), empty assemblies containing only ethylene, acetylene, or air, and pneumatophores incubated with air only. To eliminate the possibility of measuring "wound ethylene" created by the excision, the ethylene production (nitrogenase activity) values (of 16 replicates) of intact and excised pneumatophores was measured and the results were compared by one-way analysis of variance (ANOVA). No statistical difference ($P \leq 0.3499$) in the ethylene production between intact pneumatophores and excised pneumatophores was found, and ethylene production values were 27.6 ± 12.55 and 32.7 ± 10 nmol ethylene· µg chlorophyll a^{-1}· pneumatophore^{-1}· h^{-1} for intact and excised pneumatophores, respectively, in May 1993. By using excised pneumatophores in addition to intact pneumatophores, we were able to increase the number of field samplings and replicates from 7 to 10. We were also able to include other treatments since excised pneumatophores were easier to handle in the muddy surroundings of the mangrove swamp. Nevertheless, incubation was always under the field conditions of the mangrove forest at Balandra lagoon regardless of the assembly type used.

Results are expressed as the average of 10 replicates for each treatment and for each sampling in all field experiments.

Acetylene reduction assay

Nitrogenase activity (ARA) for axenic cultures of cyanobacteria was measured in 70-mL serum bottles, containing 20 mL of N-free ASN-III medium and sealed with a rubber septum. Acetylene reduction was assayed by replacing 1 mL of air with pure acetylene. Ethylene production was evaluated by gas chromatography using a Varian 6000 gas chromatograph (Varian Instrument Group, U.S.A.) equipped with hydrogen flame ionization detector (FID) (Stewart et al. 1967). The instrument operating conditions were as follows: a stainless-steel column 150 x 0.2 cm was packed with Porapak N 80/100; column temperature was 50°C; detector temperature was 200°C; the carrier gas was nitrogen and H_2 was used for FID, both at a flow rate of 25 mL· min^{-1}; and the air flow rate was 300 mL min^{-1}. The amount of ethylene was expressed as nmol ethylene· µg chlorophyll a^{-1}· mL culture^{-1}· h^{-1} or nmol ethylene· µg chlorophyll a^{-1} · pneumatophore^{-1}· h^{-1}. Since the size of each pneumatophore was different, the approximate volume of each pneumatophore was used in the calculation of the total atmosphere within each assembly at each sampling time. Since it is virtually impossible to measure the number of filamentous cyanobacteria colonizing a pneumatophore, chlorophyll a content was used to estimate cyanobacterial mass (Potts 1979; Stal et al. 1984). Chlorophyll *a* was measured as follows. After measuring of the nitrogen fixation, the entire bark of the pneumatophore was cut, divided at random into three pieces, and immersed in 10 mL of 90% methanol in test tubes. The solution completely covered the sample. The tubes were covered with aluminium foil and incubated for 24 h at 4 ± 1 °C. The slurry obtained was then thoroughly mixed in a vortex at the highest speed and 1-mL aliquots were centrifuged at 12 000 x g. The supernatant (chlorophyll extract) was measured in a Beckman (DU series 600, Beckman Instruments, Schiller Park, I11.) spectrophotometer at optical densities ranging from 400 to 800 nm using 90% methanol as a reference. The concentration of chlorophyll *a* was calculated according to the equations proposed by Tandeu de Marsac and Houmard (1988). We assumed the chlorophyll *a* to be from cyanobacteria on the pneumatophore surface because (*i*) by light microscopy, we always observed the presence of chlorophyll on the predominantly brown-gray pneumatophore surface, and (*ii*) we found typically high chlorophyll *a* peaks and the negligible amounts of other chlorophyll types in the absorption spectra of the pneumatophore extracts (data not shown). Note that chlorophyll containing cells on the aerial roots have been located below the epidermis and the hypodermic and not in the bark (Dawes 1981).

Measurements of environmental conditions during the incubation of pneumatophores

Light intensity in the sampling site was measured concomitantly with the gas sampling using a Li-Cor 188B Quantum radiometer-photometer (Li-Cor, U.S.A.). Water temperature was determined using a digital Corning PS-6 (Corning Scientific Instruments, Medfield, Mass.) thermometer with an L-type probe. Dissolved oxygen was measured in small pools (at low tide) or during full tide in the pneumatophore area by immersing an oximeter YSI model 57 (Yellow Spring, Ohio) probe, and pH measurements were taken from the same place with a portable pH meter (Beckman model 21). Black mangroves reside in the upper intertidal zone and therefore the pneumatophores are covered only a few hours per day with seawater.

Light and scanning electron microscopic (SEM) analyses of pneumatophores

To observe the growth and colonization of epiphytic microbial communities, 20 glass pipettes and 10 similar-sized wood sticks were inserted into the mud close to the pneumatophores and left there for 18 months. Their height above the sediment was similar to those of natural pneumatophores. Samples were taken every 3 months from glass, wood, and pneumatophore surfaces. Excised pneumatophores and 2-cm^2 samples of these materials were first observed by light microscopy and then fixed for SEM analysis with Karnovsky fixative (2.5% glutaraldehyde, 2% paraformaldehyde in sodium cacodylate buffer 0.2 M, pH 7.2). Samples were kept at 4°C in the fixative until they were dehydrated in a graded acetone series. Finally, they were dried in a critical-point drier (CPD model CPD 020, Balzers Union, Liechtenstein) and attached to stubs with glue (Duco cement, Devcon Corp., Ill.). The dry samples were coated with both gold and a gold-palladium mixture, either in a vacuum evaporator (VE-10 Varian) or in a sputter coater (Edwards S 150B, England). We used an AmRay model 1000-A microscope (U.S.A.) at 10 kV for SEM analysis.

Experimental design and statistical analysis

All in situ experiments were performed in 10 replicates. A replicate consisted of a single test-tube assembly or a separate determination of the environmental conditions. Light microscopy and SEM analyses were done on five replicates; each consisted of a single fragment of bark on a single stub and at least 10 different photographed observations were made from each sample. Statistical analysis was done by one-way ANOVA at $P \leq 0.05$. Fitting models for the environmental conditions were created using third-order regressions and regressions of environmental conditions versus N_2 fixation were done by second-order regressions, both calculated using Sigmaplot© curve fit software (Jandel Corporation, U.S.A.).

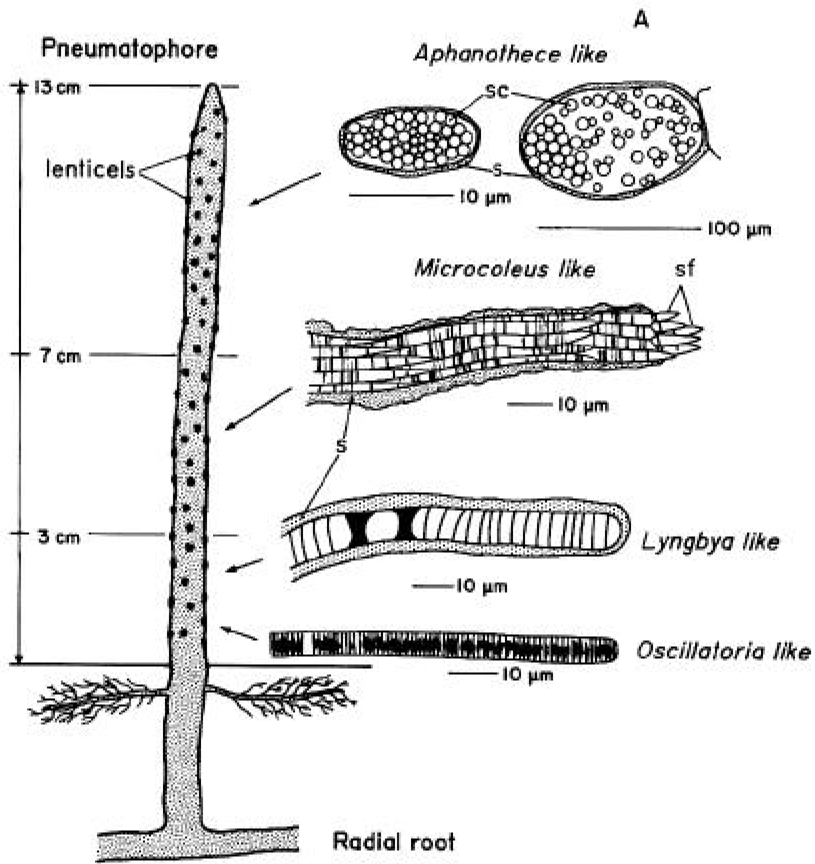
Results

Qualitative cyanobacterial colonization of black mangrove pneumatophores

The main characteristic of the pneumatophore colonization was that the entire surface was colonized by microorganisms, mainly cyanobacteria, diatoms, green algae, and to a lesser extent of microscopically observed bacteria. Not a single surface of the pneumatophore was uncolonized (Figs. 2B-2D). Under low magnification light microscopy or even with the unaided eye, small marine fauna such as nematodes and barnacles were also observed (data not shown). Different cyanobacterial populations colonized particular sites along the pneumatophore. Filaments resembling *Lyngbya* sp. and *Oscillatoria* sp. were commonly observed on the lower part of the pneumatophores from the soil surface line up to 3 cm (Figs. 2A, 3A, and 3B). Above this zone, filaments resembling *Microcoleus* sp. were the dominant microorganisms, with a wider colonization zone of about 7 cm in the middle part of the pneumatophore (Figs. 2A, 3B, and 3C). On the upper portion (approximately the top 7 cm) of the pneumatophore, coccoidal cyanobacterial colonies resembling *Aphanothece* sp. were observed through the year but were absent from other parts of the pneumatophore (Fig. 2A). However, in February 1993 they were visibly more abundant. These colonies varied in size from 10 µm (Figs. 2A and 4A-4C) to 135 km (Fig. 4D), were mixed with unidentified filamentous cyanobacteria in common biofilms (Fig. 4D), and had a cavity at the top of the colony (Figs. 4D and 4E, arrows at colony top). The microorganisms on the upper part of the pneumatophore had well-developed sheaths as a common feature (Fig. 4). The general zonation (lower, middle, and upper) was stable, with slight seasonal variations during the 18 months of sampling.

Colonization of glass pipettes submerged in the pneumatophore vicinity did not show the vertical zonation pattern observed on the pneumatophores. The colonization was random and dominated mainly by abundant diatoms, possibly heterotrophic bacteria and green algae all mixed in a mucilagenous biofilm (Figs. 5A and 5B; confirmation of chloroplast presence in the algae cells was done by light microscopy). A few cyanobacterial LPP-A and LPP-B filaments were also present (Fig. 5A). Some areas were apparently not colonized by any organism 18 months after the glass tubes were planted in the mud (Fig. 5B, arrow). This was in contrast to complete colonization of pneumatophores (compare Fig. 5B with Figs. 3 and 4). The community on the glass surface was also very diverse. The wooden sticks were randomly colonized from top to bottom by a mixture of filamentous cyanobacteria (resembling LPP-B morphotype) and unidentified microorganisms (Figs. 5C and 5D). Like the glass surface, no colony zonation was detected on the wooden sticks. Interestingly, the microorganisms on the wood were different from those observed on the glass surfaces nearby. *Microcoleus*-like filaments, the common colonizers of pneumatophores, were absent from both the glass and the wood surfaces. Biofilms on the surrounding sediment in the vicinity of the pneumatophores changed their microbial composition with the time of year. In November 1992, the sediment was dominated by heterocystous *Anabaena* sp. (Figs. 6A and 6B), while in the summer of 1993, no heterocystous cyanobacteria were found in the same place (Fig. 6C).

The absorption spectra of pigments extracted from the pneumatophore surfaces showed the distinctive peaks at 665 and 436 nm that are characteristic of chlorophyll *a*. Very small amounts of chlorophyll *b* were detected probably from green algae. Other pigments such as phycobiliproteins are water soluble and thus not detected in the methanolic extracts (data not shown).


A
Pneumatophore
13 cm
7 cm
3 cm
lenticels
Aphanothece like
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s
10 µm
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Microcoleus like
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Lyngbya like
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Oscillatoria like
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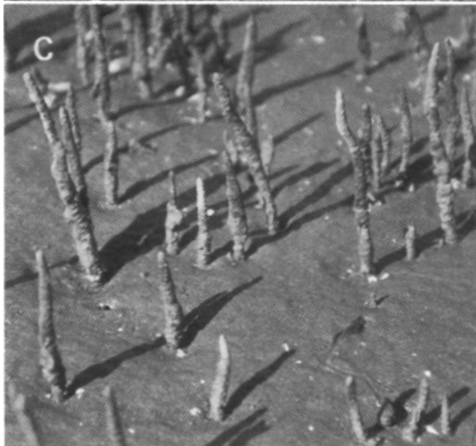
B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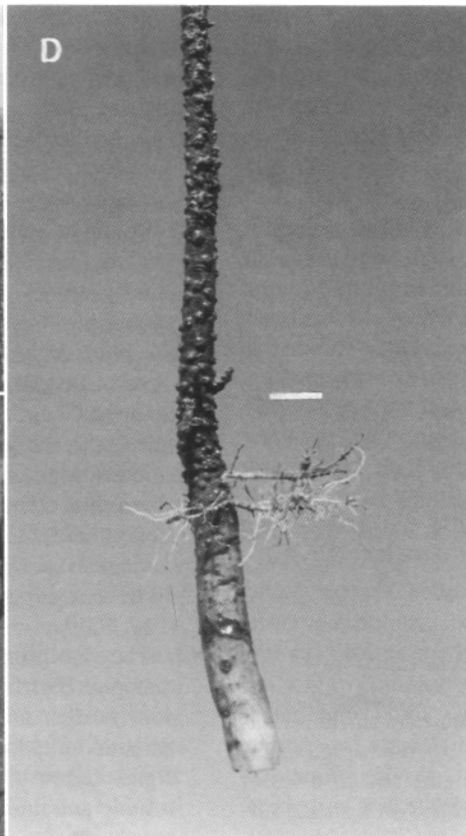
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Fig. 2. (A) Schematic representation of the pattern of colonization by cyanobacteria along a pneumatophore of black mangrove. *Aphanothece*-like colonies with single cells (*sc*). Note that *Microcoleus*-like filaments share a common sheath (*s*) (*sf*; single filaments). *Lyngbya*-like filaments embedded in a thick sheath (*s*). (B) Typical distribution of pneumatophores around a black mangrove tree. (C) Close-up of the pneumatophore area. (D) Close-up of a pneumatophore covered with cyanobacteria and numerous other marine life forms. Pneumatophore size ranged from 10-17 cm. Scale bar = 1 cm.

In situ N_2-fixation activity in pneumatophores

Observations regarding diurnal and seasonal changes in N_2 fixation on the pneumatophores revealed the following. We found that rates of N_2 fixation were affected by season. During the winter (November 1993 and February 1994 samplings), the rates of N_2 fixation per 24-h period were low. Daily accumulated ethylene production (nitrogenase activity) was 65.81 and 86.56 nmol ethylene·µg chlorophyll a^{-1} · pneumatophore^{-1}· 24 h^{-1} in November and February, respectively. In May the daily accumulated ethylene was 166.71 nmol ethylene· µg chlorophyll a^{-1}· pneumatophore^{-1}· 24 h^{-1} and it was significantly different ($P \leq 0.05$) from the winter determinations. In the determinations in June the daily accumulated ethylene was the highest (603.69 nmol ethylene· µg chlorophyll a^{-1}· pneumatophore^{-1}· 24 h^{-1}) and significantly higher than in the other seasons.

Diurnal changes in N_2 fixation during the winter were insignificant owing to the low level of N_2 fixation detected and the relatively large variation within each sampling hour (Figs. 7C and 7F). However, during the summer season when significant amounts of N_2 fixation were detected, diurnal variation was evident. The two peaks of N_2 fixation occurred from early morning to midday and during the late afternoon. During the night, N_2 fixation decreased significantly, reaching minimal values around midnight (Figs. 71 and 7L).

The summer data clearly indicated that of the four environmental parameters evaluated in the pneumatophore surroundings (light intensity, water temperature, oxygen level, and water pH), high rates of N_2 fixation were associated with high light intensity and high water temperatures. Peak N_2-fixation activities were measured when the two variables coincided (compare Figs. 7G and 7J with 7I and 7L). Because of the nonlinear nature of our data, only second-order regressions between temperature versus N_2 fixation and light intensity versus N_2 fixation revealed significant correlations in both May and June samplings. Temperature versus N_2 fixation: in May, $y = 31 - 0.537x + 0.0078x^2$, $r = 0.70$ at $P \leq 0.05$; in June, $y = 24.58 - 0.01x + 0.00046x^2$, $r = 0.76$ at $P \leq 0.03$. Light intensity versus N_2 fixation: in May, $y = 2040.41 - 121.82x + 1.8x^2$, $r = 0.8521$ at $P \leq 0.02$; in June, $y = 28.65 - 9.85x + 0.1605x^2$, $r = 0.7851$ at $P \leq 0.04$.

Although it seems that light has a marked effect on the activity of the cyanobacteria, water temperature was possibly a controlling factor. When the water temperature was around 25°C or lower, N_2 fixation was reduced (Figs. 7A, 7D, and 7G), even in the presence of sufficient light. Oxygen played only a minor role in affecting N_2-fixation activity. N_2 fixation on the pneumatophore was slightly higher when the oxygen levels in the surrounding water were low. However, low oxygen levels in the water (mainly during winter samplings) did not increase N_2 fixation on the pneumatophore (Figs. 7B, 7E, 7H, and 7K).

Fig. 3. (A) SEM of the lower part of the pneumatophore showing mainly the filamentous cyanobacteria LPP-B. (B) Insert in Fig. 3A showing bundles of filamentous, biofilm material, heterotrophic bacteria, and a filament of heterocystous cyanobacteria. (C) The central part of the pneumatophore showing filaments of LPP-B cyanobacteria resembling *Microcoleus* sp. Scale bar = 10 µm. *a, Anabaena*; *b,* bacteria; *m, Microcoleus*; *o, Oscillatoria*: *s,* biofilm material.

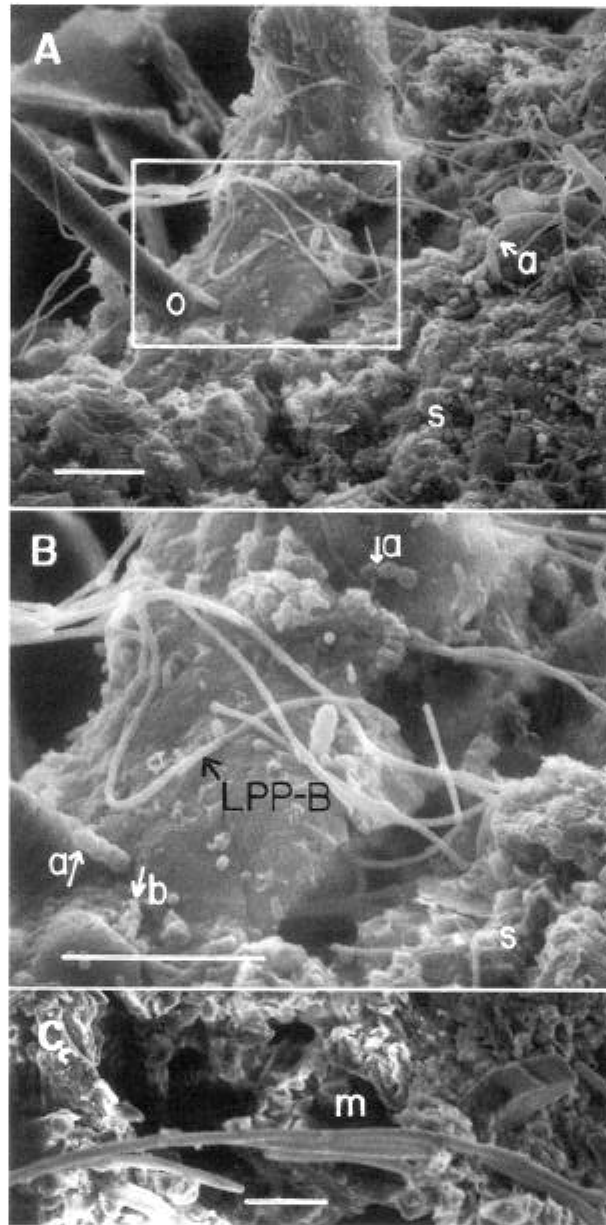
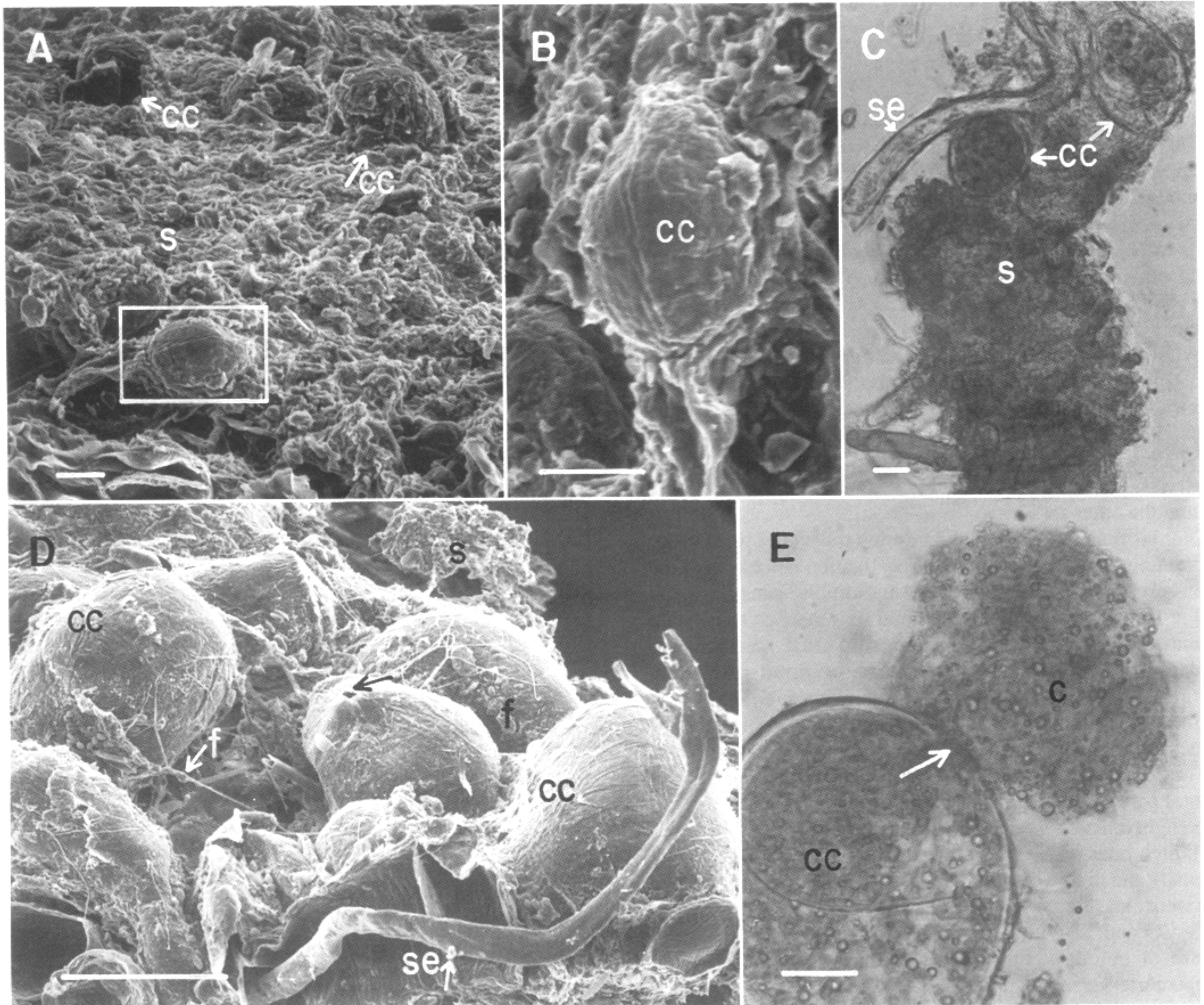


Fig. 4. SEM of bark from the higher part of the pneumatophore. (A) Small and (D) large coccoidal colonies. (B) Insert of A showing the colony embedded in biofilm material. (C) Light microscopy of a typical mixture of filaments and coccoidal colonies. (E) Release of coccoidal cells from a colony (light microscopy). Arrows in D (black) and in E (white) show the possible release exit of coccoidal cells and the scar on the far lower right side in D is possibly the attachment site of the colony to the pneumatophore. Scale bars = 10 μm (A, B, C, E) and 100 μm (D). *cc*, coccoidal colony; *c*, coccoidal cyanobacteria; *f*, filamentous cyanobacteria; *c*, biofilm material; *se*, sheath envelopes.



Furthermore, second-order regressions between O_2 concentration and N_2 fixation were statistically insignificant in both May and June samplings. Dissolved oxygen versus N_2 fixation: in May, $y = 9.14 - 0.14x + 0.00246x^2$, $r = 0.42$ at $P \leq 0.3$; in June, $y = 4.93 + 0.026x - 0.00001x^2$, $r = 0.54$ at $P \leq 0.1$.

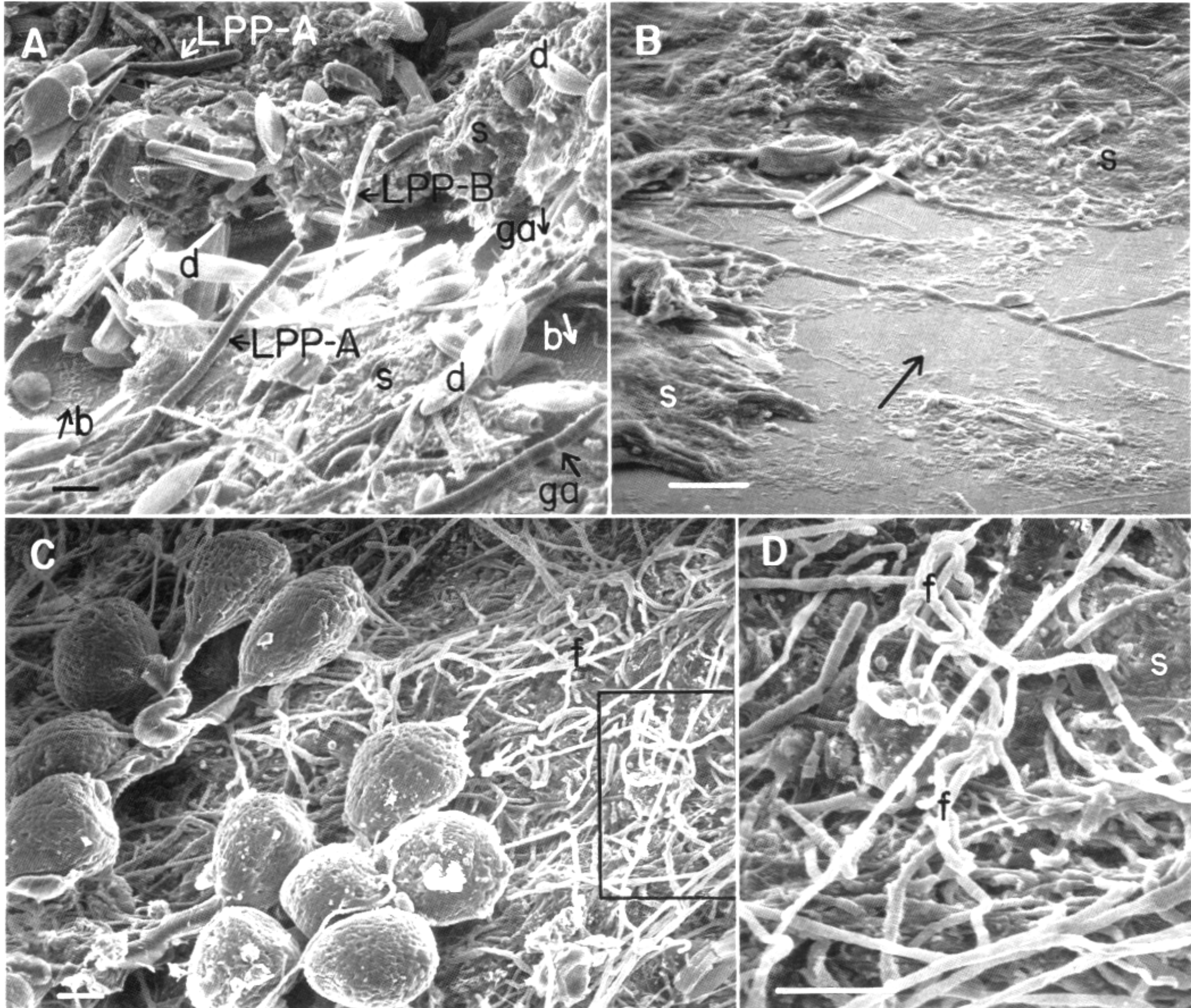
The water pH had no effect on nitrogen fixation for all samplings. Although water pH varied between the seasons, it was relatively constant within a particular sampling date (Figs. 713, 7E, 7H, and 7K).

Isolation and identification of diazotrophic cyanobacteria

The cyanobacterial community on the pneumatophore was dominated throughout the year by homocystous filaments belonging to the morphotypes LPP-A resembling *Lyngbya* and *Oscillatoria* and to the morphotype LPP-B resembling *Microcoleus.*

Two cyanobacteria were isolated and purified. One was a LPP-B morphotype, showing filaments with only vegetative cylindrical cells. The trichomes were straight, motile, and embedded in a sheath. On the pneumatophores, several filaments of these cyanobacteria were covered with a common sheath. The filaments showed the typical arrow-shaped apical cells measuring 7-8 x 5 μm. This morphotype resembled *Microcoleus* sp. ATCC 29128 (Rippka et al. 1979). A second isolate was a heterocystous strain, identified as *Anabaena* sp. The filaments showed the presence of both intercalary differentiated cells, spherical to ovoid vegetative cells measuring 5 x 3 μm. Nonmotile trichomes were characteristic of this isolate,

Fig. 5. (A, B) SEM of glass surface colonization showing heterogeneous colonization by diatoms, filamentous cyanobacteria, possible green algae, and bacteria. The arrow in B indicates uncolonized glass surface 18 months after placement in the pneumatophore vicinity. (C) SEM of dead-wood surface colonization by filamentous cyanobacteria and unidentified microorganisms 18 months after insertion of the wood in the pneumatophore area. (D) The insert in C shows the magnitude of the cyanobacteria colonization. Scale bars= 10 µm. *b*, bacteria; *d*, diatom; *ga*, green algae; *f*, filamentous cyanobacteria; *s*, biofilm material; LPP-A and LPP-B, filamentous cyanobacteria of these groups.



which resembled *Anabaena cylindrica* ATCC 27899 (Rippka et al. 1979). In axenic cultures both strains were nitrogen fixers and showed similar nitrogenase activity (approximately 109 nmol ethylene µg chlorophyll a^{-1}· mL cyanobacterial $culture^{-1}$ 24 h^{-1}).

Discussion

Mangrove trees are thriving in Balandra lagoon where there is almost no nutrient input from either the sea or the surrounding desert. In general, the dissolved nitrogen in the lagoon water of mangrove ecosystems and the nitrogen in the sediment are probably insufficient to fulfill the requirements of the mangrove trees (Along] and Sasekumar 1992; Zuberer and Silver 1978, 1979). Thus, N_2 fixation may play a role in the excellent health and density of these mangrove forests.

Diazotrophic cyanobacterial mats have been found in several mangrove communities throughout the world (Along] et al. 1992; Dor 1984; Hussain and Khoja 1993; Potts 1979, 1984; Potts and Whitton 1980; Sheridan 1991). Balandra lagoon also has cyanobacterial mats, in addition to the extensive cyanobacterial colonization on black mangrove aerial roots (pneumatophores). The colonization of mangroves by cyanobacteria and other diazotrophs has been observed on leaves, stems, or fallen plant parts (Gotto and Taylor 1976; van der Valk and Attiwill 1984: Zuberer and Silver 1978, 1979).

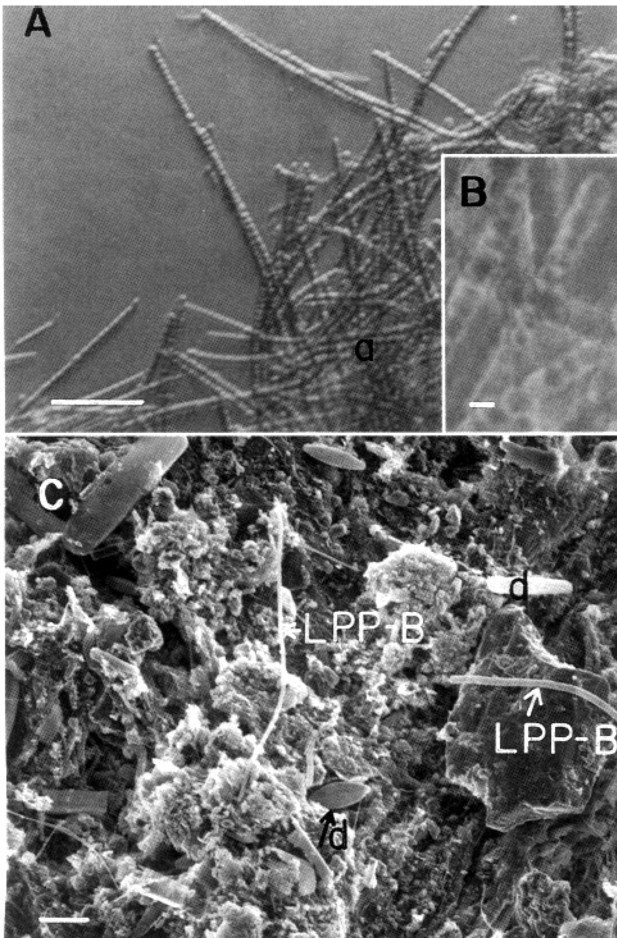


Fig. 6. Colonization of the surrounding sediment of the pneumatophore zone. (A) Light microscopy preparation (from November 1992 sampling) showing exclusive colonization by *Anabaena* sp. (B) Magnification of small portion of A showing typical *Anabaena* sp. filaments. (C) SEM of the same area in May 1993 showing random colonization by diatoms and a few LPP-B cyanobacterial filaments, all embedded in a massive biofilm. Note the absence of *Anabaena* sp. colonization. Scale bars= 100 pm (A), and 10 µm *(B, C). a, Anabaena; d,* diatom.

We studied the cyanobacterial colonization of black mangrove pneumatophores for almost 2 years. The colonization was stable with small variations among the seasons and covered the entire surface of the pneumatophore. The data we obtained from the random colonization by numerous microbial species on glass tubes and on dead-wood sticks submerged in the pneumatophore vicinity add indirect evidence for the affinity of certain cyanobacteria to colonize pneumatophores.

Zonation in colonization by marine microorganisms is a response to different microenvironmental conditions. This zonation can be endemic (composed of particular species for one site and time), but the general community structure might be comparable with other localities where different species colonize in more or less the same manner (Dor 1984; Potts 1979). In the Sinai mangroves there was a vertical zonation along the pneumatophores by mainly heterocystous cyanobacteria (Pons 1979, 1980). However, in Balandra lagoon we observed vertical zonation mainly by nonheterocystous forms. The heterocystous *Anabaena sp.* formed an abundant biofilm only on the sediment around the pneumatophores but not directly on them. Furthermore, this biofilm was short lived and disappeared within 6 months, while the nonheterocystous colonization of the pneumatophore was stable. Despite this, the possible presence of heterocystous cyanobacteria cannot be ignored, since few filaments on the pneumatophore surface were observed by SEM.

Analysis of cyanobacterial populations showed that some species in Balandra lagoon are commonly found in different mangrove ecosystems of the world. *Microcoleus* sp. seems to be a universal colonizer in all cyanobacterial communities, whether in mangrove ecosystems or microbial mats (Hussain and Khoja 1993; Potts 1980; Stal et al. 1984), and was also found in our study. Other common cyanobacterial species like *Lyngbya* and *Oscillatoria* are also well distributed; however, their dominance is more site specific (Jones 1992; Stal et al. 1984).

The environmental conditions in Balandra lagoon affect the N_2-fixation rates. N_2 fixation seemed to be light and temperature dependent, although evidence for lack of a diurnal response to light stimulation has been shown for Australian mangroves (Boto and Robertson 1990). In Balandra lagoon, the summer rates of nitrogenase activity were similar in pattern to those of microbial mats composed mainly of nonheterocystous cyanobacteria in the North Sea (Villbrandt et al. 1991; Stal et al. 1984), and differed with N_2 fixation of microbial mats near North Carolina (U.S.A.) where the main N_2-fixation activity was detected late at night (Bebout et al. 1993). We detected the first significant N_2-fixation activity from early morning until midday and a second peak in the late afternoon. The diurnal peaks we detected cannot be explained solely by light, because a gradual increase in N_2 fixation starts hours before sunrise. The effect of other environmental parameters such as root exudates, lower oxygen tension, or wetting of the pneumatophore surface by morning dew may start N_2 fixation by either (*i*) other heterotrophic N_2-fixing bacteria before dawn or (*ii*) existing populations of diazotrophic cyanobacteria utilizing storage products of photosynthesis from the previous day (Bebout et al. 1993; Jones 1992).

High oxygen tension by itself has a deleterious effect on nitrogenase activity (Fay 1992). Still, N_2 fixation was found under aerobic conditions on the pneumatophore surface, suggesting that colonizing diazotrophs have their own protecting mechanisms against oxygen inhibition. The seasonal and diurnal changes in N_2 fixation and in the environmental conditions measured in this study imply that the N_2 fixation occurring on the aerial pneumatophores should be regarded as being controlled by multiple interacting factors and may not be dominated by any one single factor. At least two factors, light intensity and water temperature, are major parameters, while oxygen tension was only a minor contributing factor. The conclusion that O_2 did not have deleterious effects on nitrogenase can be supported by the fact that O_2-resistant heterocystous cyanobacteria were also present on the pneumatophore, although in small amounts. These organisms may contribute to the overall N_2 fixation detected on the pneumatophore at periods when O_2 tension was high. However, because of the nature of natural pneumatophore colonization (being a mixture of numerous types of cyanobacteria

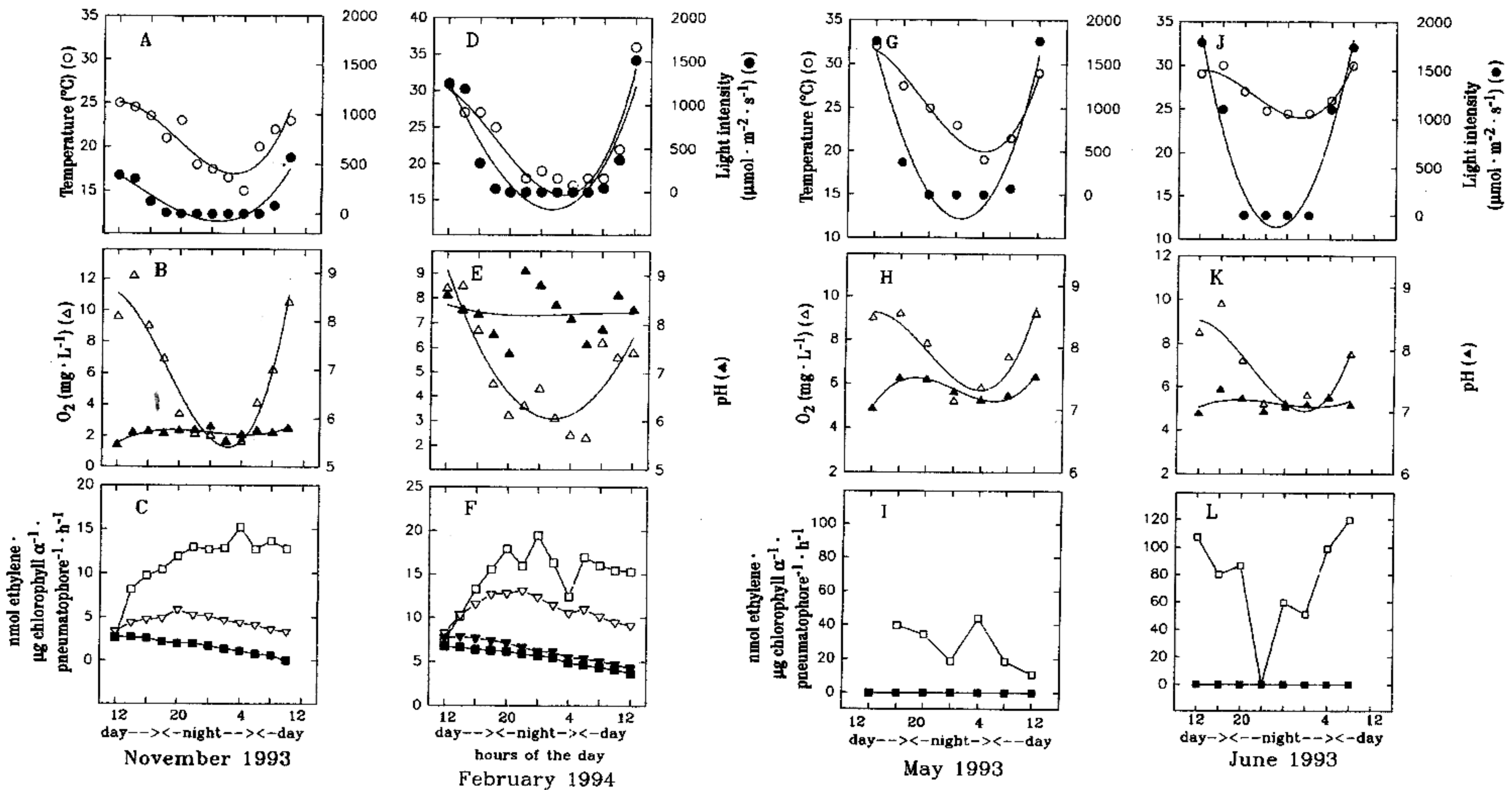


Fig. 7. Diurnal nitrogen fixation on black mangrove pneumatophores during the year and the environmental conditions that prevailed during its measurement. (C, F, I, L) □, nitrogen fixation measured; ■, negative controls; ▽, incubation in the presence of glucose; ▼, addition of glucose to negative controls (symbol can hardly be seen, since it coinsides with the ■ symbol). Continuous lines in A, B, D, E, G, H, J, and K represent third-order regressions of the environmental conditions. Regression coefficients are as follows: A, 0.9163 (○) and 0.9192 (●); B, 0.7497 (△) and 0.9654 (▲); D, 0.9212 (○) and 0.9188 (●); E, 0.8912 (△) and 0.1250 (▲); G, 0.9782 (○) and 0.9518 (●); H, 0.9817 (△) and 0.9364 (▲); J, 0.9462 (○) and 0.9800 (●); K, 0.9097 (△) and 0.3853 (▲). Figure L is from Toledo and Bashan (1994) by permission of the publisher. To simplify the complex graphs, the standard error values were not drawn and are as follows: for nitrogen fixation in May and June, ±2.52 (I); ±36.84 (L).

as well as heterotrophs), this study cannot attribute which groups of cyanobacteria (the heterocystous or the nonheterocystous) are the main contributors.

Black mangrove pneumatophores are submerged for only a few hours daily depending on the tide, yet N_2 fixation occurs continuously. During the dry hours, the fixed nitrogen is not washed away from the pneumatophore surface. If nitrogenous compounds are excreted by the cyanobacteria, it may be absorbed into the plant by the normal absorbing function of the pneumatophore. Naturally, this hypothesis should be confirmed by $^{15}N_2$ tests, which are difficult to perform under the constantly changing environmental conditions of this marine ecosystem.

In conclusion, we have described a stable vertical zonation of cyanobacterial colonization on black mangrove aerial roots in one of the northern-most distributions of mangroves in the American hemisphere. The N_2 fixation occurring on these pneumatophores was maximal in the summer and showed diurnal peaks during the daylight hours.

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