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Heterotrophic nitrogen fixation (acetylene reduction) during leaf-litter decomposition of two mangrove species from South Florida, USA

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Abstract Heterotrophic nitrogen-fixation (acetylene reduction) was measured during decomposition (under dark conditions) of *Rhizophora mangle* L. and *Avicennia germinans* (L.) Stearn leaf litter. Nitrogen-fixation rates in leaf litter increased following 24 d incubation, then decreased after ≃44 d for both species. Maximum rates of 66.2 and 64.6 nmol C_2H_4 g^{-1} dry wt h^{-1} were reached by *R. mangle* and *A. germinans* leaf litter, respectively. Higher fixation rates of leaf litter were associated with an increase in water content and sediment particles on leaf surfaces of both species. Rates of nitrogen fixation by diazotrophs attached to sediment particles were not significantly different from zero. With additions of D-glucose, ethylene production rates increased by factors of 625-, 34- and 7-fold for sediment, *R. mangle* and *A. germinans* leaf litter, respectively, compared to rates prior to enrichment. These organically enhanced rates of nitrogen fixation on leaves could be accounted for by increased activity associated with attached sediment particles and not the leaf material. Total phenolics [reported as tannic acid equivalent (TAE) units] decreased nitrogen-fixation rates when added to D-glucose-enriched sediment at >20 mg TAE l^{-1}. Phenolic compounds could explain the initial lag in rates of nitrogen fixation during leaf-litter decomposition of *R. mangle* (initial content of 110.8 mg TAE g^{-1} dry wt), but not of *A. germinans* (initial content of 23.4 mg TAE g^{-1} dry wt). The higher phenolic content and reportedly lower carbon substrate of *R. mangle* did not result in species-specific differences in either the magnitude or temporal pattern of nitrogen fixation compared to *A. germinans* leaf litter. We conclude that the availability of organic substrates leached from the leaf litter along with colonization by the heterotrophic diazotrophs (as indicated by sediment accumulation) controls nitrogen-fixation rates in a similar manner in the leaf litter of both species.

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Introduction

Immobilization is the process of nutrient enrichment of leaf litter during the initial stages of decomposition, and has been particularly well-documented for nitrogen in mangrove detritus (Rice and Tenore 1981; Newell et al. 1984; Twilley et al. 1986; Day et al. 1987; Alongi et al. 1992). The sources of nitrogen during immobilization in mangrove leaf litter include both the uptake of inorganic nitrogen during flood tides (Rivera-Monroy and Twilley 1996), and/or nitrogen fixation (Zuberer and Silver 1978; van der Valk and Attiwill 1984). Nitrogen fixation represents a "new" source of nitrogen to mangrove forests, but the contribution of this process to the nitrogen budget of mangrove wetlands remains poorly understood. Rates of nitrogen fixation in mangrove wetlands varies with species and quality of leaf litter, stages of decomposition, community types of nitrogen-fixers, concentration of organic carbon substrates, and ambient conditions such as inundation, temperature and light (Rodina 1964; Gotto and Taylor 1976; Zuberer and Silver 1978; Potts 1979; Gotto et al. 1981; van der Walk and Attiwill 1984; Mann and Steinke 1992). Together, these factors result in diverse temporal and spatial patterns of nitrogen fixation that limit our ability to estimate the areal input of new nitrogen to the forest floor. Better understanding of the regulatory mechanisms that control this temporal and spatial heterogeneity of nitrogenase activity during leaf-litter decomposition are essential for determining the role of nitrogen fixation in the nitrogen budget of mangrove wetlands.

A previous study (Pelegrí et al. 1997) indicated that nitrogen fixation was time-dependent during leaf-litter

decomposition, and was associated with the intermediate stages of leaf decay. Understanding more specific mechanisms as to the temporal change in nitrogen-fixation rates among different species of leaf litter can also provide insights into the spatial variation of this process by heterotrophic bacteria. Diazotrophic microbial populations in mangrove sediments are often carbon-limited (Zuberer and Silver 1975), and different species of mangrove leaf litter vary in the leaching of dissolved organic carbon (DOC) (Twilley 1982, 1985). The cumulative total DOC leached from senescent leaf litter (in seawater of 30 g wet wt l^{-1}) during a 48 h experiment was 27.3 mg C g^{-1} dry wt for *Avicennia germinans* compared to 9.5 mg C g^{-1} dry wt for *Rhizophora mangle* (Twilley 1982). Thus, the differential supply of organic matter by *A. germinans* leaf litter may stimulate heterotrophic diazotrophic microorganisms (Zuberer and Silver 1978; van der Valk and Attiwill 1984), and enhance the incorporation of atmospheric inorganic nitrogen into their organic pools.

Differences in the chemical composition of *Rhizophora mangle* and *Avicennia germinans* leaf litter provide an approach to test some of the expected regulatory factors of nitrogen fixation in mangrove wetlands. *R. mangle* leaf litter has lower initial nitrogen concentration (Twilley et al. 1986; Pelegrí et al. 1997), higher phenolic content (Walsh 1974; Cundell et al. 1979; Benner et al. 1986, 1990; Robertson 1988), and a thick waxy cuticle, compared to the nitrogen-rich leaf litter of *A. germinans* that has low concentrations of phenolics (Robertson 1988). Nitrogen-rich litter has been reported to decompose at higher rates than nitrogen-poor litter (Suberkropp et al. 1976; Tam et al. 1990). Various authors have found a delay in mangrove leaf-litter colonization which was attributed to their content in total phenolics (Cundell et al. 1979; González-Farias and Mee 1988). Phenolic compounds can inhibit microbial activity through the precipitation of exoenzymes. Water is needed for penetrating the leaves and solubilizing potentially-inhibitory phenolics, and desiccation appears to be one of the most important factors limiting nitrogen fixation by communities growing in exposed mangrove areas (Potts 1979). Nitrogen fixation may also be proportional to the nitrogen content of leaf litter, as indicated by C:N ratios >16. Even though *R. mangle* has higher phenolic compounds and less leaching of organic matter, there is much more demand for nitrogen during decomposition in this leaf litter than during decomposition of *A. germinans* leaf litter. It is not clear how these factors interact to control species-specific rates of nitrogen fixation in litter of mangrove wetlands.

The literature contains several references to the inhibitory effect of phenolics on nitrogen fixation of mangrove leaf litter; however, the specific increase in nitrogen fixation associated with a decrease in phenolic concentrations has not been documented. The inhibitory effects of phenolics compared to the stimulatory effects of increased organic substrate and micronutrient availability of mangrove-leaf leachate have been demonstrated for lignocellulose degradation rates (Benner et al. 1986; Lee et al. 1990). However, the interactions of these chemical characteristics of mangrove leaf litter and their influences on nitrogen fixation have not been directly measured.

The interaction of phenolic compounds, leaching of dissolved organic carbon, and relative C:N ratios should provide distinct species-specific and time-dependent rates of nitrogen fixation during leaf decomposition that have been observed in other studies (van der Walk and Attiwill 1984; Mann and Steinke 1992). We hypothesize that the change in the chemical concentration of phenolics and organic matter will correspond to changes in nitrogen-fixation rates, and that the lower supply of nitrogen in *Rhizophora mangle* may actually stimulate nitrogen fixation once phenolics have been leached from the leaf litter. Thus, the water content of leaf litter during decomposition and processes of leaching along with the time for diazotrophs to colonize the leaves suggest that complex mechanisms control nitrogen fixation in mangrove leaf litter. Here we present a study, carried out in the laboratory and under controlled conditions, of the evolution of heterotrophic nitrogen-fixation rates (acetylene reduction) during leaf-litter decomposition of two mangrove species, *R. mangle* L. and *Avicennia germinans* (L.) Stearn. These two species of mangroves are common in mangrove wetlands throughout the new-world tropics, and represent contrasting models as insights into the regulatory factors of heterotrophic nitrogen-fixation in mangrove wetlands.

Materials and methods

Field-study site

Mangrove forests consisting of three mangrove species, *Rhizophora mangle* L., *Avicennia germinans* (L.) Stearn and *Laguncularia racemosa* (L.) Gaertn, form a continuous band and extend from the Gulf of Mexico to the upland limit of periodic salt-water influence, a distance of about 10 to 20 km. The Everglades area is topographically flat, with an average gradient of 2.8 cm km^{-1} from the inland region to the coast (SFWMD 1992). Tides within the Everglades National Park are predominantly diurnal, with a mean amplitude of 1.1 m (Provost 1973; Wang et al. 1994). Monthly sea levels are above the mean annual height from May to November in the Gulf Coast of Florida (Provost 1973), causing seasonally high tidal inundations of mangrove wetlands in southwestern Florida from August to October (Twilley 1985). Mean annual air temperature in the Everglades National Park is 24 °C, ranging from 19 °C in January to 28 °C in August (Thomas 1974; Duever et al. 1994). Average annual precipitation in the Everglades National Park is 1320 mm, with distinct wet (June to September) and dry (November to April) seasons (Thomas 1974).

Laboratory incubations

Senescent leaves from the canopy of *Rhizophora mangle* and *Avicennia germinans* trees were collected during May 1995 at a site 9.9 km from the mouth of Shark River Estuary at the Everglades National Park (southwest Florida, USA). Leaves were collected by

gently shaking trees of both species, and were transported to the laboratory in paper bags within a container containing drierite to provide dry conditions. Intact sediment samples were collected from the same location with a shovel at 2.5 cm depth. These sediment samples were placed in aluminum containers (19 × 19 cm) and returned to the laboratory.

Fifteen aluminum containers had 10 to 15 leaves each of *Rhizophora mangle* and another 15 containers had 10 to 15 leaves each of *Avicennia germinans* placed on the surface of the sediment. The containers were stored in the dark at 25 °C in a Percival incubator, thus limiting the growth to heterotrophic populations of diazotrophs. To simulate tidal conditions, seawater (19 to 23‰ S) was added to each of the containers covering the leaves. This tidal water was allowed to soak sediment and leaves for 3 h and was then drained from the containers. The containers could be drained with a tube that was plugged with glass-fiber filters to avoid loss of sediment particles during drainage. This procedure was repeated every 4 to 6 d. The seawater contained 1.8 to 7.7 μM NH_4^+.

Triplicate samples of *Rhizophora mangle* and *Avicennia germinans* intact leaves (1 to 3 g wet wt) and sediment samples (2 to 7 g wet wt) were incubated every 4 and 10 to 12 d, respectively, in 40 ml gas-tight glass vials. The vials were purged with argon (to provide an anoxic atmosphere), sealed with an open cap over a teflon–silicone septa, and placed back in the incubator. We induced anoxic conditions during the incubation to optimize rates, since higher nitrogen-fixation rates have been reported for mangrove sediments incubated under anoxic compared to oxic conditions (Gotto and Taylor 1976; Zuberer and Silver 1978). In addition, nitrogen fixation by aerobic bacteria can be measured in short-term incubations under anoxic conditions (Potts 1979). Nitrogenase activity was measured using the acetylene reduction technique (Hardy et al. 1968). Four ml of commercially-available acetylene (99.9%, Lincoln Big Three Inc.) were injected into each vial (0.09 atm, 9% C_2H_2 by volume) through the teflon–silicone septa. One extra set of samples was used as controls and incubated without addition of acetylene, to monitor endogenous production of ethylene. Gas samples of the vials were taken at 0, 0.5, 1, 2 and 3 h after acetylene enrichment. Three ml of gas were taken with a syringe and injected into 3.5 ml pre-evacuated vacutainers (Becton & Dickinson), which were store for < 1 wk prior to the ethylene assay. Gas samples were analyzed for ethylene using a Hewlett Packard 5890 gas chromatograph equipped with a hydrogen-flame ionization detector and a stainless-steel column (6′ × 1/8″ o.d.; 182.9 cm × 3.2 mm o.d.) packed with HayeSep T, 80- to 100-mesh. Temperature at both the injector and detector was 180 °C, while oven temperature was kept at 75 °C. Flow rates were 30, 60 and 450 ml min^{-1} for He (carrier gas), H_2 and air, respectively. Ethylene peak heights were measured and related to calibrations made with standard C_2H_4 concentrations (99.5%, Aldrich Chemical Co.). The conversion between ethylene-production rates and nitrogen fixation was calculated assuming the theoretical molar ratio of 3:1 for C_2H_4:N_2 (Stewart et al. 1967). However, previous studies using $^{15}N_2$ calibration have shown that the actual conversion factor may vary between 1.9 and 6.3 (Potts 1979; Hicks and Silvester 1985).

D-glucose-enrichment experiment

A D-glucose (dextrose)-enrichment experiment was conducted with leaf material of each species that had been incubating for 73 d. These leaves were in an advanced degree of decomposition, and rates of ethylene production had decreased to 20% of the maxima observed (see "Results–Decomposition time-series"). We selected D-glucose as an organic carbon source since glucose additions have been found to stimulate acetylene reduction in mangrove sediments (Zuberer and Silver 1978; Potts 1979), rhizosphere sediment of *Glyceria* sp. and *Typha* sp. (Bristow 1974), Arctic marine sediments (Knowles and Wishart 1977), and detritus in coastal Atlantic Ocean waters (Paerl et al. 1987; Paerl 1990). The tidal water added to the aluminum containers was enriched with D-glucose at a concentration of 2 g C l^{-1} (55.6 mM D-glucose). This enriched tidal water was allowed to soak sediment and leaves for 3 h and then drained from the containers. Twenty-four hours later, the first incubation of triplicate leaves of *Rhizophora mangle* and *Avicennia germinans* and sediment samples was performed for ethylene production as described in the preceding subsection. Successive assays of nitrogenase activity were conducted 4, 7 and 12 d following enrichment. The concentration of D-glucose added corresponded to 40- and 100-fold the amount of carbon (dissolved organic carbon) leached from *R. mangle* and *A. germinans* senescent leaves, respectively, after 3 h of soaking in our containers (50 and 20 mg C l^{-1}, respectively, calculated from Twilley 1982).

Another D-glucose-enrichment experiment was conducted with sediment by incubating samples of 7 to 10 g wet wt in 40 ml glass vials. D-glucose was added to each sediment sample to obtain final concentrations ranging from 0 to 1.5 g C l^{-1} ($n = 10$). Twenty-four hours later, the vials were incubated and assayed for ethylene production as described in the preceding subsection.

Total phenolics experiment

Senescent dry leaves of *Rhizophora mangle* and *Avicennia germinans*, collected from the field, were ground and 1 g aliquots were separately added to 10 ml filtered (GF/C) seawater. These solutions were shaken for 24 h and centrifuged for 10 min at 3000 rpm to collect the supernatant. *R. mangle* and *A. germinans* leachate contained 51.9 and 26.2 mg TAE $l,^{-1}$ respectively (see following subsection for analytical procedures). Sediment samples of 5 to 6 g wet wt were placed in 40 ml glass vials. Tannic acid (concentrations ranging from 0 to 42.4 mg TAE l^{-1}, $n = 12$), *R. mangle* leachate (diluted 4:5, concentrations ranging from 12.8 to 14.2 mg TAE l^{-1}, $n = 3$) or *A. germinans* leachate (concentrations ranging from 7.7 to 10.2 mg TAE l^{-1}, $n = 3$) were added to different sediment samples. One and a half ml of D-glucose (4 g C l^{-1} or 0.1 M D-glucose) were added to all samples. Twenty-four hours later, the vials were incubated and assayed for ethylene production as described in the earlier subsection "Laboratory incubations".

Analytical procedures

At the termination of each incubation described in an earlier subsection ("Laboratory incubations"), samples of leaf litter and sediment were freeze-dried (Labconco) overnight and analyzed for total nitrogen, carbon and phenolic content. Water content was determined from weight loss after freeze-drying the leaf samples, and oven-drying (60 °C) the sediment samples. Ash-free dry wt (AFDW) of the samples was determined from weight loss after ignition in a muffle furnace at 450 °C for 4 h. Nitrogen and carbon were analyzed on a LECO CHN-600 (System 785-500) analyzer. Phenolic compounds were extracted with 70% ethanol (Hagerman 1988; Cork and Krockenberger 1991). Total phenolics were measured spectrophotometrically after the addition of Biuret and Folin-Ciocalteau's phenol reagents (Sigma) (Lowry et al. 1951; Prive et al. 1978), and related to calibrations made with standard tannic acid (Sigma). Total phenolics are reported as tannic acid equivalent (TAE) units.

An analysis of the variance (one-way ANOVA) was performed to test for significant differences among the treatment effects on nitrogenase activity.

Results

Decomposition time-series

Endogenous ethylene production was not detected in the control samples that were incubated without acetylene addition in the various experiments. The concentration of ethylene in the 40 ml vials at time zero was 0.2 ± 0.1 μM for all samples.

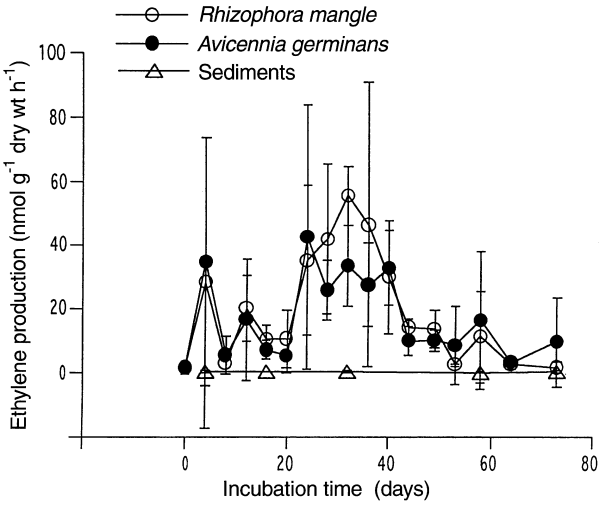

Fig. 1 *Rhizophora mangle* and *Avicennia germinans*. Rates of ethylene production for litter leaves and sediments during decomposition (*Data points* means of three replicates; *error bars* standard errors)

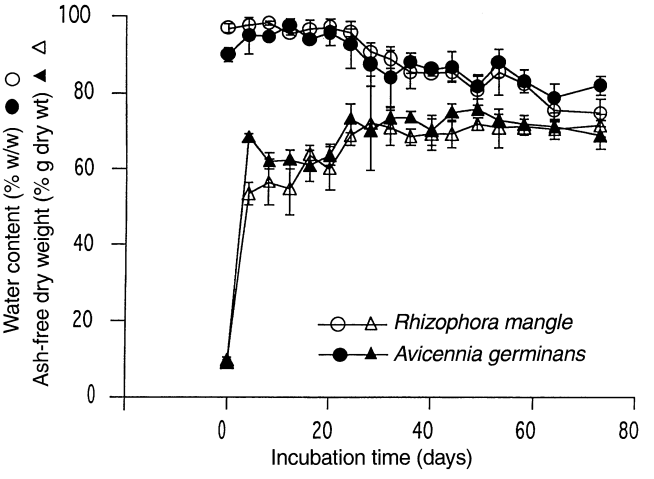

Fig. 2 *Rhizophora mangle* and *Avicennia germinans*. Water content and ash-free dry wt of litter leaves during decomposition (*Data points* means of three replicates; *error bars* standard errors)

Rates of ethylene production varied during the 73 d decomposition study of *Rhizophora mangle* and *Avicennia germinans* leaf litter, whereas nitrogenase activity was constantly low in the sediment (Fig. 1). Rates of ethylene production followed a similar pattern for both litter species, with relatively higher rates from 24 to 44 d of incubation compared to either 8 to 20 d ($p < 0.005, n = 12$) or 48 to 60 d ($p < 0.001, n = 12$) from initiation of the study. The highest rates observed were 66.2 and 64.6 nmol C_2H_4 g^{-1} dry wt h^{-1} for *R. mangle* (32 d incubation) and *A. germinans* (28 d incubation), respectively. There was no statistically-significant difference in nitrogenase activity between both species during the incubation ($p \geq 0.08$). Rates of ethylene production in the sediment ranged from 0.1 to 0.4 nmol C_2H_4 g^{-1} dry wt h^{-1}, which is not significantly different from zero ($p < 0.001$).

The water content of the leaf litter during the experiment increased from the initial value of ~5% for both species, to a maximum level of 69.2 ± 2.9% and 73.5 ± 3.9% for *Rhizophora mangle* and *Avicennia germinans*, respectively (Fig. 2). These values remained constant during the rest of the experiment for both species. This higher moisture level was reached around 24 d of incubation for both species, coinciding with the increase in ethylene-production rates. There was a significant ($p < 0.001$) difference between water-content levels 8 to 20 d after setup ($n = 12$) and 24 to 36 d after setup ($n = 12$) for both species. Water content of the sediment oscillated between 65.4 ± 5.9% and 76.2% ± 1.6% during the experiment. Ash-free dry wt ranged from 43.8 to 50.3% for sediment samples, but decreased from 91.5 to 74.9% for *R. mangle* and from 87.4 to 82.1% for *A. germinans* leaf-litter samples during the incubation (Fig. 2).

Total phenolics in the sediment ranged from 0.2 to 0.5 mg TAE g^{-1} dry wt during the experiment (significantly different from zero, $p < 0.03$) (Fig. 3). The initial total phenolic content of *Rhizophora mangle* leaf litter (110.8 mg TAE g^{-1} dry wt) was five-fold higher than *Avicennia germinans* leaf litter (23.4 mg TAE g^{-1} dry wt). The concentration of total phenolics in leaf litter decreased during the incubation to < 1 mg TAE g^{-1} dry wt at 58 d incubation for *R. mangle*. Total phenolic concentration in *A. germinans* decreased to <10 mg TAE g^{-1} dry wt after 4 d incubation, and was <1 mg TAE g^{-1} dry wt at 49 d of incubation. After 24 d incubation, when nitrogen-fixation rates increased, total phenolic content of both *R. mangle* and *A. germinans* leaf litter had decreased by 88% of the original concentration.

The nutrient chemistry of leaf litter also changed during the decomposition study (Fig. 4). Nitrogen content increased during the incubation period from 5.2 to 8.9 and from 7.3 to 14.9 mg N g^{-1} dry wt for *Rhizophora mangle* and *Avicennia germinans*, respectively (Fig. 4A). Carbon content of leaf litter decreased from 442.5 to

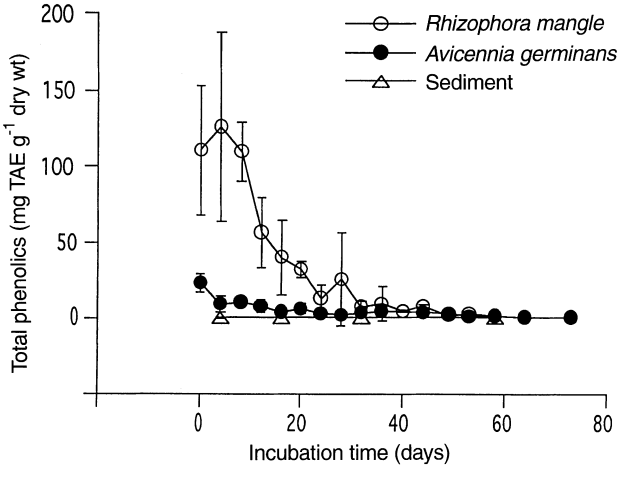

Fig. 3 *Rhizophora mangle* and *Avicennia germinans*. Total phenolic content of litter leaves and sediments during decomposition (*Data points* means of three replicates; *error bars* standard errors)

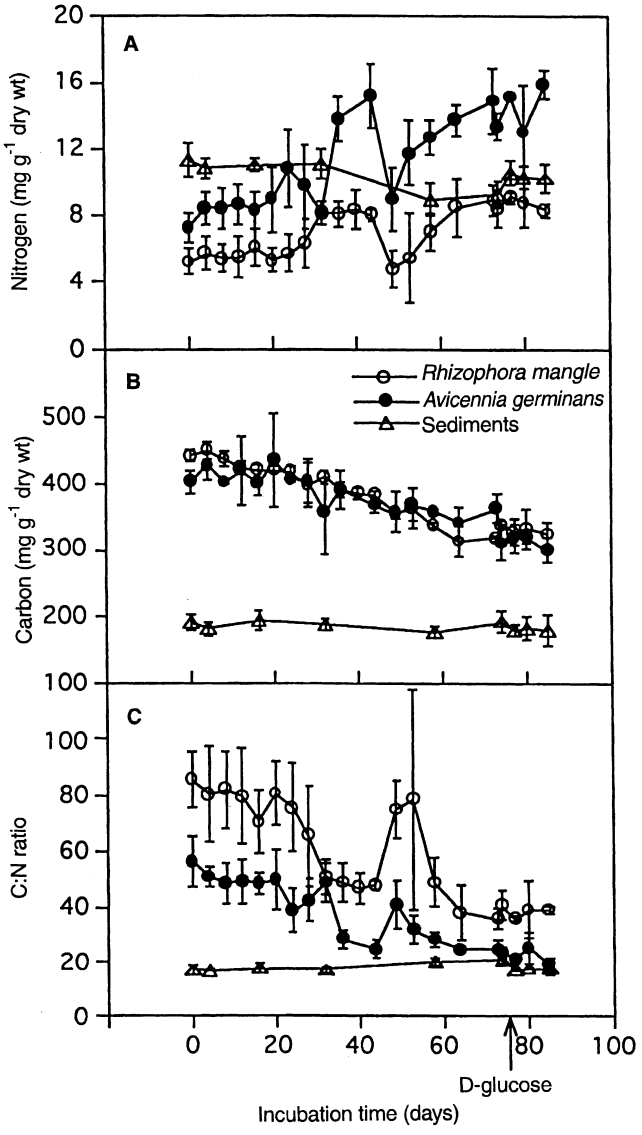

Fig. 4 *Rhizophora mangle* and *Avicennia germinans*. Nitrogen content (**A**), carbon content (**B**) and C:N ratio (mg C:mg N) (**C**) of litter leaves and sediments during decomposition (*Data points* means of three replicates; *error bars* standard errors)

319.5 for *R. mangle* and from 404.8 to 364.4 mg C g^{-1} dry wt for *A. germinans* (Fig. 4B). This led to a decrease in leaf litter C:N ratio from 85 to 39 in *R. mangle*, and from 56 to 19 in *A. germinans* during the incubation (Fig. 4C). The nitrogen content of sediment ranged between 8.9 and 11.3 mg N g^{-1} dry wt, carbon content between 178.4 and 195.1 mg C g^{-1} dry wt, and C:N ratio between 16.9 and 20.1. No significant correlation ($p > 0.5$) was found between nitrogen or C:N ratios and ethylene production rates during the decomposition study (data not shown). There was a linear relationship ($r^2 = 0.85$, $p < 0.0001$, $n = 85$) between AFDW (Y, % dry wt) and carbon content (X, mg g^{-1} dry wt) of all the samples that can be described as $Y = 21.9 + 0.2X$ (data not shown).

D-glucose-enrichment experiment

Twenty-four hours after enrichment with D-glucose, ethylene-production rates were stimulated in leaf litter of both species and sediment compared to rates prior to D-glucose enrichment (Table 1). The most significant enhancement of ethylene-production rates was observed in the sediment at 250 nmol C_2H_4 g^{-1} dry wt h^{-1}, compared to 0.4 nmol C_2H_4 g^{-1} dry wt h^{-1} prior to enrichment. Rates of ethylene production increased by factors of 625-, 34- and 7-fold for sediment, *Rhizophora mangle* and *Avicennia germinans* leaf litter, respectively, after 24 h of carbon addition compared to rates prior to enrichment. These elevated rates of ethylene production decreased rapidly by 60 to 80% only 4 d after enrichment, returning to the pre-enrichment rates by 7 and 12 d after enrichment for leaf litter and sediments, respectively.

The contribution of sediment particles on the leaf litter to the patterns of ethylene production following carbon enrichment could be determined for each leaf species. Sediment-particle content on each leaf after 73 d incubation was 30.3 ± 16.2% for *Rhizophora mangle* and 35.1 ± 18% for *Avicennia germinans*. These percentages were determined by measuring differences in dry weight between seawater-cleaned and untouched halves of ten leaves of each species. Based on these percentages and the sediment ethylene-production rates in the D-glucose-amended samples (see Table 1), we calculated the contribution of the sediment particles attached to the litter to the ethylene-production rates in leaf litter amended with D-glucose (Table 1). Sediment particles could account for 50.3 ± 26.9 and 47.7 ± 24.5 nmol C_2H_4 g^{-1} dry wt h^{-1} for *R. mangle* and *A. germinans*, respectively. These rates explain nearly 100% of the elevated ethylene-production rates measured in leaf litter of *R. mangle* and *A. germinans* (70.5 ± 89.4 and 48.9 ± 15.1, respectively).

Table 1 *Rhizophora mangle* and *Avicennia germinans*. Ethylene-production rates (± SE) (nmol g^{-1} dry wt h^{-1}) for leaf litter and sediment before and after addition of seawater enriched with D-glucose (2 g C l^{-1}) to microcosms (* D-glucose addition)

Incubation time (d)	Days after D-glucose enrichment	*R. mangle*	*A. germinans*	Sediment
64	–	2.6 ± 1.3	3.3 ± 0.3	–
73*	0	1.6 ± 1.7	9.6 ± 13.9	0.4 ± 0.5
74	1	70.5 ± 89.5	48.5 ± 15.1	250 ± 103.7
77	4	13.3 ± 3.0	15.0 ± 22.4	51.2 ± 40.1
80	7	0.9 ± 1.5	7.5 ± 9.5	7.3 ± 8.9
85	12	3.2 ± 2.2	3.9 ± 1.6	0.3 ± 0.3

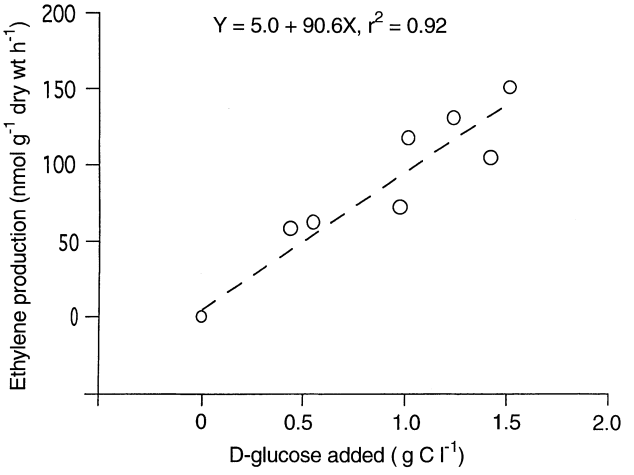

Fig. 5 Rates of ethylene production of sediment samples ($n = 10$) amended with D-glucose concentrations ranging from 0 to 1.5 g C l^{-1}. Linear relationship was found between D-glucose added and ethylene-production rates

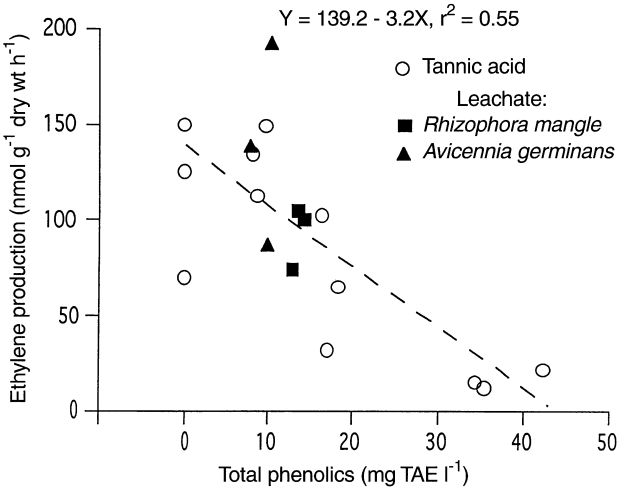

Fig. 6 Rates of ethylene production of sediment samples amended with tannic acid ($n = 12$, concentrations ranging from 0 to 42.4 mg TAE l^{-1}), *Rhizophora mangle* leachate ($n = 3$, diluted 4:5, concentrations ranging from 12.8 to 14.2 mg TAE l^{-1}), or *Avicennia germinans* leachate ($n = 3$, concentrations ranging from 7.7 to 10.2 mg TAE l^{-1}. All samples enriched with 1.5 ml D-glucose (4 g C l^{-1}). Linear relationship was found between TAE added and ethylene-production rates

Rates of ethylene production in D-glucose-enriched (up to 1.5 g C l^{-1}) sediment samples were stimulated proportionally to the amount of D-glucose added (Fig. 5). There was a significant linear relationship ($r^2 = 0.92$, $p < 0.0001$) between ethylene-production rates (Y, nmol C_2H_4 g^{-1} dry wt h^{-1}) and D-glucose additions (X, g C l^{-1}) described by $Y = 5.0 + 90.6X$.

Total phenolics experiment

The addition of tannic acid to D-glucose-enriched sediment samples resulted in decreased ethylene-production rates (Fig. 6). The addition of ~40 mg TAE l^{-1} reduced ethylene-production rates by ~10-fold compared to unamended samples. There was a linear relationship ($r^2 = 0.55$, $p < 0.001$) between ethylene-production rates (Y, nmol C_2H_4 g^{-1} dry wt h^{-1}) and total phenolic concentration (X, mg TAE l^{-1}), expressed as $Y = 139.2 - 3.2X$. The effect of leaf leachate on ethylene-production rates in sediment samples amended with carbon followed the pattern observed for similar phenolic concentrations using tannic acid. Based on the inverse linear model of total phenolics and ethylene production, the effect of leachates from both *Rhizophora mangle* and *Avicennia germinans* on nitrogenase activity fit the expected rates based on sediment having a phenolic content corresponding to that present in leaf-litter leachate.

Discussion

Rates of ethylene production reported in the literature range from 7.3 to 1350 nmol C_2H_4 g^{-1} dry wt h^{-1} for leaf litter of *Rhizophora mangle* (Gotto and Taylor 1976; Zuberer and Silver 1978; Pelegrí et al. 1997) and from 25 to 539.8 nmol C_2H_4 g^{-1} dry wt h^{-1} for *Avicennia germinans* (Hicks and Silvester 1985; Pelegrí et al. 1997). The heterotrophic ethylene-production rates of the present study are within the lower range of these values. Acetylene-reduction rates of just the sediment (0.1 to 0.4 nmol C_2H_4 g^{-1} dry wt h^{-1}) were in agreement with previous data obtained in mangrove sediments of south Florida (0 to 31.3 nmol C_2H_4 g^{-1} dry wt h^{-1}; Zuberer and Silver 1978; Pelegrí et al. 1997).

The highest rates of leaf-litter ethylene production measured for the mangrove wetlands of this study are 224 and 539 nmol C_2H_4 g^{-1} dry wt h^{-1} for *Rhizophora mangle* and *Avicennia germinans*, respectively (Pelegrí et al. 1997). Heterotrophic ethylene-production rates in the decomposition study reported here could account for up to 30 and 12%, respectively, of these rates. Integrated nitrogen fixation over the incubation period (73 d) was 100 and 108.4 μg N-NH_3 g^{-1} dry wt for *R. mangle* and *A. germinans*, respectively. Ethylene production accounted only for 2.7 and 1.3% of total nitrogen immobilized by 1 g of leaf litter for *R. mangle* and *A. germinans*, respectively (~9 and 16 μg N-NH_3 g^{-1} dry wt for *R. mangle* and *A. germinans*, respectively). These percentages are considerably lower than rates previously reported in the literature, such as 17 to 37% for *R. mucronata* (Woitchik et al. 1997) and 40 to 64% for *A. marina* (van der Valk and Attiwill 1984). Thus, apparently nitrogen fixation does not represent an important source of nitrogen to this mangrove ecosystem. Increases in leaf-litter nitrogen must be mostly due to uptake of ambient soluble nitrogen by attached microbes (Odum et al. 1979). However, it should be taken into consideration that our leaf-litter decomposi-

tion experiment took place under dark conditions, which would select for heterotrophic diazotrophs. Studies of cyanobacteria contributions to nitrogen fixation have reported two-fold higher nitrogen-fixation rates for mangrove sediment and *R. mangle* leaf litter incubated under light relative to dark conditions (Kimball and Teas 1975; Gotto and Taylor 1976; Hicks and Silvester 1985). Yet other studies in south Florida found that light incubations do not stimulate the nitrogenase activity of the leaf litter (Zuberer and Silver 1978). Those samples were under darkness only during the assay period and not during the whole experimental period. Thus, although some diazotrophs may have been less active in the dark, they were nevertheless present within the sample. We believe that the absence, or under-representation, of some diazotrophs, unable to grow under our incubation conditions, was responsible for the low ethylene-production rates measured.

Our glucose-enrichment experiment showed that nitrogenase activity of diazotrophs present in the sediment could be enhanced by the addition of an organic carbon source. However, we ignore if this enhancement was directly (carbon limitation) or indirectly (enhanced respiration through oxygen consumption, leading to anoxic or microaerophilic microenvironments favourable to nitrogen-fixers) linked to D-glucose addition. These enhanced nitrogen-fixation rates may be due to increases in the number or activity of the diazotrophic populations. Zuberer and Silver (1978) indicated that the lag phase (12 to 24 h) required for sediment nitrogen-fixation to increase after glucose addition was due to population increases and/or nitrogenase synthesis, as opposed to an immediate increase in the supply of an energy source. However, Döbereiner et al. (1972) noted a marked stimulation of acetylene reduction without a concomitant increase in bacterial numbers in *Paspalum notatum–Azotobacter paspali* association samples incubated in the presence of sucrose for 72 h. In our experiment, nitrogen-fixation potential (maximal nitrogen-fixation capacity) of the diazotrophs was not achieved even at the addition of 1.5 g C l^{-1} (of D-glucose), since the increase in ethylene-production rates remained proportional to D-glucose additions. Assuming a mean weight of 0.5 g dry wt per leaf, we estimate that in a 48 h leaching period one leaf of *Rhizophora mangle* or *Avicennia germinans* could contribute 5 or 14 mg C, respectively, to the underlying sediment (calculated from Twilley 1982). This could stimulate sediment ethylene-production rates by 1.1- to 1.3-fold (using the equation in Fig. 5). Leaching is more important during the first hours of soaking of the litter; thus, higher nitrogen-fixation rates could be expected during this period. These intermittent sediment-enrichment periods could select for a population of nitrogen-fixers adapted to quickly respond to the presence of a carbon source.

The initial total phenolic content of senescent mangrove leaves was 11.1 and 2.3% of the dry leaf material for *Rhizophora mangle* and *Avicennia germinans*, respectively. These values are in agreement with other values reported in the literature (tannins accounted for 4.3 to 42% and 7% of dry weight for *R. mangle* and *A. germinans*, respectively: Walsh 1974; Cundell et al. 1979; Benner et al. 1986, 1990; Robertson 1988). In our experiment, concentrations of ~40 mg TAE l^{-1} appeared to decrease acetylene-reduction rates of organically-enriched (4 g D-glucose C l^{-1}) sediment samples by ten-fold relative to non phenolic-amended sediment. Based on phenolic leaf concentrations and mass percentage of leaf litter in the containers, we calculated the concentration of phenolics in our aluminum containers during the 3 h water-saturated conditions. These concentrations could have reached 40 mg TAE l^{-1} at each water saturation during the first 4 d of incubation for *A. germinans*, compared to the initial 32 d of incubation for *R. mangle*. Thus, in our decomposition experiment, total phenolics could explain the delay in the increase in nitrogen-fixation rates for *R. mangle* leaf litter in the containers, but not for *A. germinans* leaf litter.

The increase in nitrogen fixation after 24 d incubation coincided with the higher water-content levels and decreased phenolic content in *Rhizophora mangle* leaf litter. But higher rates for *Avicennia germinans* leaf litter can be associated only with higher water content, since total phenolic concentration was already low at the initiation of the incubation (23.4 mg TAE g^{-1} dry wt). The similarity in the 24 d lag of nitrogen-fixation rates for both species indicates that phenolic compounds may not be the main factor controlling litter microbial metabolism. The corresponding change in water content of leaf litter preceding peak nitrogen-fixation rates during the incubation studies for both species suggest that this process may be indicative of factors that regulate nitrogen fixation by diazotrophs in mangrove leaf litter. However, the large variation in nitrogenase activity at 24 d following incubation for both leaf species limits the ability to interpret the ecological significance of this response.

Water saturation of leaf litter by tides and precipitation can enhance the accumulation of sediment particles on the surface area of leaf litter. This was corroborated by the decrease in concentration of AFDW in leaf litter during the incubation, since sediment particles have a lower AFDW concentration. Heterotrophic diazotrophs are associated with these sediment particles, indicating the rate that they colonized leaf surfaces during the decomposition study. The significance of sediment particles on leaf surfaces to nitrogenase activity was demonstrated by comparing sediment and leaf activities in response to D-glucose enrichment. Nearly all of the elevated rates in nitrogenase activity could be accounted for by the elevated rates in nitrogen fixation of sediment particles attached to the leaf. Indeed, we found no significant ($p = 0.9$) rates of ethylene production on senescent leaves prior to incubation on sediment in the decomposition study. In a previous study, Pelegrí et al. (1997) found no significant rates of ethylene production of senescent leaves of *Rhizophora mangle* and *Avicennia germinans* in

the same area. Also, Raghukumar et al. (1995) found no bacteria on senescent leaves of *R. apiculata* still on the trees. Zuberer and Silver (1978) added various sugars to mangrove sediments and found that some caused dramatic increases in rates of acetylene reduction, while the addition of sugars to leaf litter had almost no effect on acetylene reduction under oxic and anoxic conditions.

We conclude that rates of nitrogen fixation on leaf litter are a combination of the temporal sequence of leaf colonization by diazotrophs (as indicated by sediment accumulation) along with the availability of organic substrates leached from the leaf litter which enhances nitrogenase activity. Nitrogen-fixing organisms were associated with sediment particles, but the nitrogen-fixing potential of these diazotrophic microorganisms required an organic substrate. We suggest that heterotrophic nitrogen fixation increases in leaf litter as the carbon source of the leaf becomes available associated with an increase in water content. However, once this organic substrate is leached from the leaf and decreases in concentration, rates of nitrogen fixation quickly returned to background rates. This was demonstrated both experimentally by additions of substrate, followed by decreased nitrogenase activity in 7 to 12 d; as well as by the temporal change in nitrogenase activity during the decomposition study.

Higher nitrogen content and carbon leaching from *Avicennia germinans* relative to *Rhizophora mangle* leaf litter had apparently no species-specific effect on nitrogen-fixation rates. In addition, the effect of total phenolics, although shown experimentally to decrease nitrogenase activity, did not seem to cause a species-specific difference in nitrogen fixation. Specific phenols could be responsible for the inhibitory effects observed. However, this could not be determined with the bulk tannic acid assay used in the present experiment. We calculated that there was enough total phenolics during the first 20 d reduce nitrogen fixation of *R. mangle* but not *A. germinans*. However, since both plants responded similarly in the first 20 d of the decomposition study, there may be other processes instead that control nitrogen fixation in both species. Thus, the significance of time for sediment accumulation and formation of diazotrophic populations on the leaf surface along with carbon availability from the leaf occurred after the phenolics had been reduced to levels that are not limiting nitrogenase activity. The results of this study reject our hypothesis that species-specific characteristics of leaf chemistry between *R. mangle* and *A. germinans* would result in different patterns of nitrogen fixation.

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