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SHORT COMMUNICATION

Seasonal changes in periphyton nitrogen fixation in a protected tropical wetland

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Abstract Cyanobacteria are important for global nitrogen cycle and often form complex associations referred to as cyanobacterial mats or periphyton that are common in tropical, limestone-based wetlands. The objective of this study was to monitor the nitrogen fixation rate using the acetylene reduction assay of these cyanobacterial mats in a tropical, unfertilized, and protected wetland. To account for temporal and spatial variation of nitrogenase activity, we were interested in seasons in a hydrological cycle (dry, rains, and end of rains), sites with different vascular vegetation, and rates of nitrogenase activity in a 24-h cycle. The annual average of nitrogenase activity was 22 nmol C_2H_4 cm^{-2} h^{-1}, with a range of <6 to 35 nmol C_2H_4 cm^{-2} h^{-1}, and the annual nitrogen fixation rate of our study site (9.0 g N m^{-2} $year^{-1}$) is higher than similar estimates from other freshwater wetlands. There was a clear temporal pattern in nitrogenase activity with a maximum rate occurring during the rainy season (August) and a maximum nitrogenase activity occurring between 0600 and 1200 hours. We found spatial differences in nitrogenase activity among the four sites that could be attributed to variations in species composition within the periphyton.

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Introduction

Cyanobacteria or blue–green algae are a diverse group of prokaryotes that often form complex associations with bacteria and green algae in structures known as cyanobacterial mats (Stal 2000). However, cyanobacterial mats in wetlands are often referred to as periphyton (Vymazal and Richardson 1995). The periphyton structure consists of a dense mat of entangled cyanobacteria filaments as well as empty sheaths and mucilaginous algal colonies with adhered detritus. Cyanobacteria are able to survive in extreme environments because of unique adaptations such as their capability of fixing N_2 (Paerl et al. 1995; Bergman et al. 1997) and their resistance to desiccation (Potts 1996, 1999). Because of the ability to fix atmospheric nitrogen, cyanobacterial mats have been used as biofertilizer in modern agriculture (Mandal et al. 1999; Ladha and Reddy 2003). Moreover, cyanobacterial mats contribute to the overall ecosystem primary production and play a key role in nutrient cycle (Goldsborough and Robinson 1996; McCormick and O'Dell 1996; Scott et al. 2005).

Cyanobacteria are important for global nitrogen cycle; however, there are not many studies about the contribution of nitrogen fixation by these organisms and their associations (e.g., periphyton) in natural ecosystems worldwide, especially in tropical wetlands. Cyanobacteria are widespread in oligotrophic limestone-based tropical wetlands in the Caribbean, and similar associations of cyanobacteria flora have been identified in the Florida Everglades

(Vymazal and Richardson 1995; McCormick and Stevenson 1998; McCormick et al. 1998), Belize alkaline marshes (Rejmánková and Komárková 2000), and in Cuba, Jamaica, Venezuela, and the Yucatan peninsula in Mexico (Rejmánková et al. 2004a; Becerra-Absalón and Tavera 2003; Novelo and Tavera 2003; this study).

The spatial and temporal dynamics of periphyton (e.g., changes in species composition) in wetlands has been explained by the interaction between physical and chemical factors (Goldsborough and Robinson 1996). However, the spatial and temporal dynamics of nitrogen fixation by periphyton in tropical wetlands is still a black box in global nitrogen models (see Holland et al. 1999, for a review). The main question of our study was: Does nitrogen fixation varies in space and time in a tropical, oligotrophic, calcareous wetland that has not been affected by urban development (e.g., phosphorous enrichment)? Reference values of nitrogen fixation rate in natural protected wetlands are important because the changes in the rate could serve as an indicator of natural and anthropogenic eutrophication as has been observed by McCormick and Stevenson (1998) on P enrichment in the Everglades. The goal of this study was to measure in space (four sites with different vascular vegetation) and time (three seasons in a hydrological cycle and 24-h incubation periods during each season) the nitrogenase activity in the periphyton using the acetylene reduction assay (ARA). Because previous research has reported changes in space and time in species composition and nutrient cycling of periphyton in the Yucatan (Novelo and Tavera 2003; Vargas and Novelo 2003), we hypothesized that N fixation would be influenced by interactions between the periphyton and their environment via seasonal and vegetation changes.

Materials and methods

The study was undertaken at the El Eden Ecological Reserve, a tropical wetland surrounded by tropical seasonal forests (Gomez-Pompa et al. 2003). The El Eden Reserve was established in 1990 and is located in the Yalahau region at the northeast part of the Yucatan peninsula in Quintana Roo, Mexico (located between the latitudes 21°11′30″ N and 21°14′00″ N and the longitudes 87°10′30″ W and 87°12′30″ W). The annual precipitation is 1,650 mm and the average temperature is 24.2°C. During 4 months (July–October), at least one third of the surface of the wetland is flooded. It has an elevation between 5 and 10 m above the sea level, and it stands on limestone bedrock and shallow soils (maximum depth 20 cm).

The study site was visited three times during 2000: April (dry season, average precipitation=37 mm, temperature=25.5°C), August (rainy season, average precipitation=258 mm, temperature=25.8°C), and November (end of rains, average precipitation=89 mm, temperature=22.4°C). To capture spatial variation within the wetland, we selected four sites with different dominant vascular vegetation [site P1 *Solanum donianum* Walp.; site P2 *Cladium jamaicensis* Crantz.; site P3 *Haematoxylon campechianum* (L.); and site P4 *H. campechianum*, *Erythroxylon confusum* (L.), *Manilkara zapota* (L.) P. Rogen, and *Crescentia cujete* L.]

At each site, a representative area of 4×8 m was selected and we randomly sample four replicates (1 cm^2) of periphyton during each season to evaluate nitrogenase activity by ARA (Weaver and Danso 1994). We included one blank assay containing also 1 cm^2 of periphyton without acetylene to evaluate if periphyton naturally forms ethylene. All samples were incubated in situ for 24 h in 120-ml flasks with 12 ml of acetylene (generated from calcium carbide to create a 10% acetylene atmosphere). Gas samples were taken every 6 h corresponding to 6–12 and 12–18 h (daytime) and 18–24 and 24–6 h (nighttime) periods. After each 6-h period, a 5-ml gas sample was removed by syringe and injected into a Vacutainer tube, vacuum-sealed, and stored at ambient temperature. The gas samples were analyzed with a Varian 3300 gas chromatograph fitted with a hydrogen flame ionization detector. The stainless steel column used was 0.32 cm in outer diameter and 200 cm in length, packed with Porapak N (80–100 mesh). Data were recorded using a Varian 4290 integrator. Nitrogen fixation rates were reported as nanomoles of C_2H_4 per square centimeters per hour.

During each season, at the each one of the four sites (P1 to P4), we sampled four additional replicates of 1 cm^2 of the periphyton. Chlorophyll *a* was quantified in the field laboratory by a modified fluorometric method 445.0 with an extraction without acidification, using a Turner AU10 fluorometer (USEPA 1997).

The taxonomic groups in the periphyton were characterized at all sites throughout the study using a Nikon Eclipse E600 microscope (×40–×60). The taxonomic groups were defined as: (a) Nostocales and Stigonematales (heterocystous), (b) Oscillatoriales (non-heterocystous), and (c) Chroococcales (unicellular colonial). Proportions were recorded as categories: <5, 5–10, 20–60, and >60%.

A three-way ANOVA was used to analyze the effects of site (P1, P2, P3, and P4), seasons (dry, rainy, and end of rains), and incubation periods (0–6, 6–12, 12–18, and 18–24 h) on nitrogen fixation rates, followed by Tukey tests ($p<0.05$). Non-parametric Kruskal–Wallis tests were used to analyze taxonomic group proportions on sites and seasons because the data were not normally distributed even after

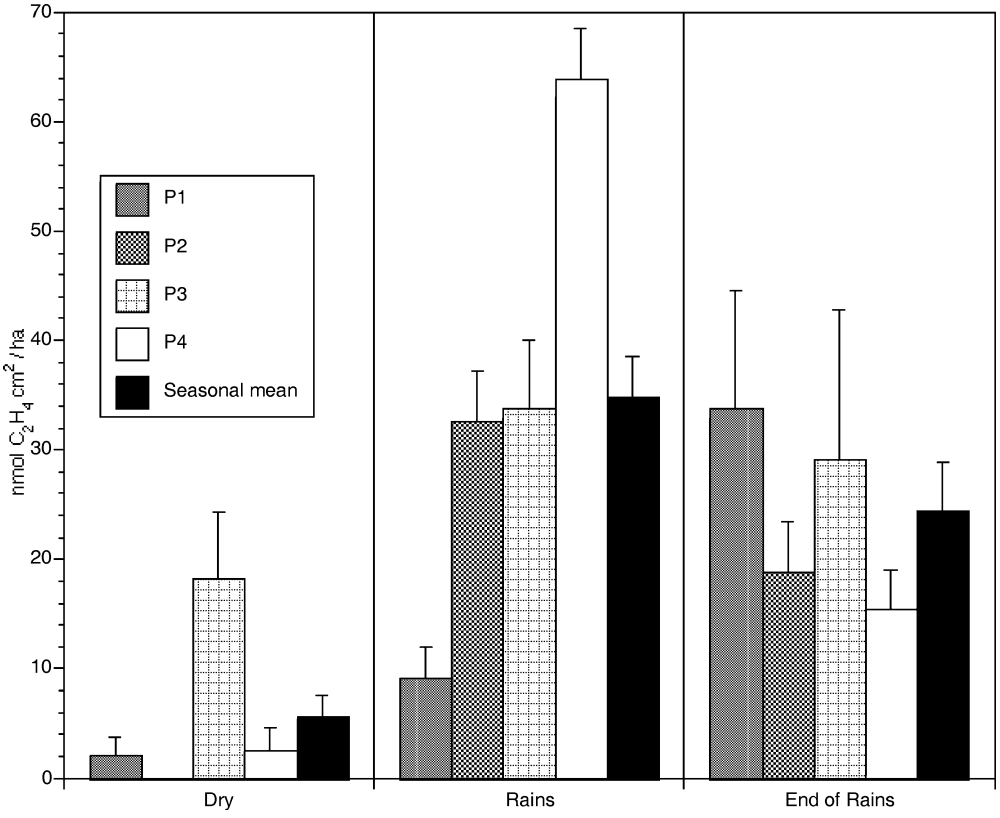

Fig. 1 Nitrogen fixation rates (nM of C_2H_4 m^{-2} h^{-1}) in four sites with different vascular vegetation (P1 to P4 and seasonal mean) across three different seasons. *Bars* represent means±1 SE

transformations. All tests were performed using SPSS v11 (SPSS, Chicago, IL, USA).

Results and discussion

Periphyton samples were mainly composed of cyanobacteria (>90%) with a small amount of chlorophytes and diatoms (<5%) during all seasons. Periphyton structure during the rainy season consisted of a dense net of entangled cyanobacteria filaments of the orders Nostocales, Stigonematales, Oscillatoriales, Chroococcales, and empty sheaths. The most common Cyanobacteria species at the surface of the periphyton were *Scytonema* spp. and *Stigonema* spp. Unicellular colonies of *Cyanokybus* sp. were common at the bottom of the mats. As the wetland dried, the periphyton acquired a crusty appearance, forming a gray crust on top of the soil characteristic of the dry period. The distribution of organisms in the periphyton suggests that cyanobacteria might be layered according to their individual metabolic needs (Stal 1995). As an example, the dominance of species *Scytonema* at the surface of the periphyton may be explained by the production of scytonemine pigments that filter solar radiation (García-Pichel and Castenholz 1991). Similar species distributions at the site have been reported by Novelo and Tavera (2003) and Becerra-Absalón and Tavera (2003).

The rates of nitrogenase activity varied sixfold during the year with the highest rates found during the rainy season (Fig. 1). The annual average of nitrogenase activity was 22 nmol C_2H_4 cm^{-2} h^{-1}, with a range of <6–35 nmol C_2H_4 cm^{-2} h^{-1}. Our data on nitrogenase activity can be compared with values from Belize wetlands <5 to 17.5 nmol C_2H_4 cm^{-2} h^{-1} (Rejmánková and Komárková 2000) and 0.4–16.4 nmol C_2H_4 cm^{-2} h^{-1} (Rejmánková et al. 2004b), and from the Everglades 2.3–21.3 nmol C_2H_4 cm^{-2} h^{-1} (Inglett et al. 2004). Thus, it appears that the nitrogenase activity rates of periphyton mats of the Yucatan peninsula are in the high end of cyanobacterial mats of other tropical, calcareous environments.

Annual nitrogen fixation rate of periphyton mats for El Eden was 9.0 g N m^{-2} $year^{-1}$. According to Inglett et al. (2004) nitrogen fixation rates of periphyton mats of the Everglades (9.7 g N m^{-2} $year^{-1}$) are higher than similar estimates from other freshwater marshes (0.01–6.0 g N m^{-2} $year^{-1}$), peat bogs (0.05–2.1 g N m^{-2} $year^{-1}$), and cypress swamps (0.4–2.8 g N m^{-2} $year^{-1}$), but within the range reported for coastal salt marshes (0.2–15 g N m–2 $year^{-1}$) and cyanobacterial mats (1.3–76 g N m^{-2} $year^{-1}$).

For this study, we used the ARA technique with batch incubations because of its low cost and because it was easy to perform in situ of a natural protected wetland where no

electrical power is available. We understand that ARA with batch incubations has several disadvantages especially when long incubation periods are performed in closed vessels. In our 24-h incubations, oxygen may have accumulated in the light as a result of photosynthesis during daytime (6–12 and 12–18 h) or oxygen may have been depleted in the dark as a result of respiration during nighttime (18–24 and 24–6 h). A more interesting disadvantage of long batch incubations is that they may obscure any patterns of nitrogenase activity occurring in a time frame shorter than the incubation period. Despite these limitations, our results are comparable with those by previous studies using the same technique for tropical wetlands (Rejmánková and Komárková 2000; Inglett et al. 2004; Rejmánková et al. 2004b). To overcome some of the problems mentioned above, Staal et al. (2001, 2003) have shown that "on-line" monitoring of acetylene reduction is superior to "batch incubations" for natural samples (water or sediment) because it prevents the accumulation or depletion of oxygen and carbon dioxide and short response times (2 min) are required to obtain a steady-state flux of ethylene.

Our results from a three-way ANOVA test showed significant effects of all three factors (I, season; II, site; and III, incubation period) on nitrogenase activity. According to this analysis, the season appears to be the most important variable for nitrogenase activity followed by the incubation period and the site. Factor I: significant differences ($F_{2,\ 48}$=34.34, p<0.001) in nitrogenase activity were observed among the seasons, placing them in three distinct groups where maximum activity was observed during the rainy season. Factor II: we found significant differences ($F_{3,\ 48}$=4.9, p=0.003) between sites and nitrogenase activity. Site differences in nitrogen fixation can be attributed to species composition and the number of heterocysts (discussed below). Factor III: we found significant differences ($F_{3,\ 48}$=11.95, p<0.0001) in the incubation period, where the period 6–12 h showed the maximum nitrogenase activity.

Rejmánková and Komárková (2000) reported that the maximum of the nitrogen fixation rate in the wetlands of Belize occurred at noontime and it was associated with heterocystous cyanobacteria. By analyzing data with a three-way ANOVA, we found maximum activity in the period 6–12 h (p<0.05); however, because of the limitation of batch incubations, we do not know if this activity is higher in the early or late part of the incubation period. It is interesting that we found high nitrogenase activity in nighttime and this contradicts to what was reported by Rejmánková and Komárková (2000) who found no nighttime activity. High nitrogenase activity in nighttime may be explained by the large proportion of Oscillatoriales and Chroococcales as non-heterocystous cyanobacteria (Bergman et al. 1997), but further research needs to be done to test this hypothesis.

We found significant differences for the proportion of Oscillatoriales (χ^2=18.472, p>0.0001) among the three seasons, but no significant differences were found for the other two taxonomic groups. The variation in the proportion of Oscillatoriales among seasons suggests that this group is more susceptible to seasonal changes (e.g., precipitation and temperature). We did not find significant correlations between taxonomic groups and nitrogen fixation rates. These results suggest two possibilities: (a) nitrogen fixation is not dependent on a particular taxonomic group but on the metabolic activity of these groups as determined by moisture and temperature (Stewart et al. 1978) or (b) a proportion of the nitrogen fixation in this wetland may be carried out by heterotrophic bacteria (Steppe and Paerl 2002). Bacterial contribution to nitrogen fixation rates may occur at night, and it can also explain our slightly higher nitrogenase activity rates than those of wetlands in Belize and Everglades.

We found significant differences in the proportions of taxonomic groups among the sites: Nostocales and Stigonematales (χ^2=9.35, p=0.025), Oscillatoriales (χ^2=11.039, p=0.012), and Chroococcales (χ^2=11.324, p=0.01). These differences in taxonomic groups might influence differences in nitrogenase activity among sites. We could not find a significant correlation between the number of heterocysts and nitrogen fixation except when analyzed for P1 alone (r=0.99, p=0.02). This suggests that nitrogen fixation rate at site P1 may be influenced by heterocystous cyanobacteria, but this pattern was not observed in the other sites because of the different proportions of cyanobacterial taxonomic groups or heterotrophic bacteria. Further research needs to be done to determine the relative contribution of each of the taxonomic groups towards nitrogen fixation at this wetland.

Average chlorophyll *a* values were 363.9 μg l^{-1} (dry season), 497.3 μg l^{-1} (rains season), and 430.7 μg l^{-1} (end of rains season). We found a positive significant correlation (r=0.39, p<0.05) between nitrogenase activity and chlorophyll *a* values. Novelo and Tavera (2003) reported that the PO_4^{3-} concentration of the soil varied from 268.1 (dry season) to 224 mg l^{-1} (flooded period). Using these data we found a negative correlation (r=0.36, p<0.05) between the PO_4^{3-} content of soil and nitrogenase activity. It is well known that phosphate coprecipitates with calcium carbonate and the slow P release from the calcareous precipitate appears to stimulate cyanobacterial communities (Penn et al. 2000). As no agricultural runoff with high P concentrations was found in our study site, we suggest that slow P release occurred in these soils and this might have stimulated nitrogenase activity and therefore periphyton primary production.

We conclude that periphyton structure at El Eden is similar to other cyanobacterial mat communities in tropical, calcareous wetlands. At the macroscopic level, the periph-

yton appears homogeneous among sites; however, we found differences at the microscopic level (e.g., species composition) that might influence ecological processes such as nitrogen fixation. The rates of nitrogenase activity presented in this study might be at the high end of rates reported for other cyanobacterial mats in tropical wetlands and other aquatic ecosystems. Nitrogen fixation varies with time as a clear seasonal pattern exists, but a more accurate assessment of the nitrogen fixation associated with species composition and taxonomic groups (cyanobacteria vs heterotrophic bacteria) is essential to better interpret the observed diurnal nitrogen fixation patterns. This could be accomplished by characterizing the seasonal effects of a complete diurnal cycle by isolating species or taxonomic groups, by monitoring on-line ethylene, by using N stable isotopic ratios (δ^{15}N), and by using antibodies to identify and quantify the various types of nitrogenase enzymes present. Although the overall nitrogen fixation rates are similar to other tropical wetlands, we found differences among sites with different vascular vegetation and taxonomic composition of the periphyton. We suggest that large spatial scales should be studied to quantify nitrogen fixation rates in tropical wetlands to capture the heterogeneity of the site. The differences in vascular vegetation at the site could be used as an indicator of differences in periphyton microscopic structure when periphyton appears homogeneous at the macroscopic level.

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