Biogeochemistry **67:** 353–368, 2004.

$\delta^{15}N$ as an indicator of N_2-fixation by cyanobacterial mats in tropical marshes

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Received 6 November 2001; accepted in revised form 7 April 2003

Key words: $\delta^{15}N$, Belize, Cyanobacteria, Nitrogen fixation, Wetlands

Abstract. Cyanobacterial mats (CBM) are important components of wetland ecosystems in limestone-based regions of the Caribbean. During two sampling periods (July 1999 and January 2000) we measured N_2-fixation in samples from 23 different marshes simultaneously with measurements of relevant environmental factors. Samples were evaluated for abundance of five groups of cyanobacteria: (1) *Leptolyngbya*, (2) *Oscillatoria*, (3) Chroococcales, (4) *Nostoc*-& Stigonematales, and (5) dead sheaths. Differences in nitrogen fixation, expressed as nitrogenase activity in nmol C_2H_4 cm^{-2} h^{-1}, were best explained by the proportion of heterocyst-forming cyanobacteria. The samples were analyzed for the natural abundance of $\delta^{15}N$. $\delta^{15}N$ values ranged from −1.99 to 11.44 ‰ and were strongly negatively correlated with N_2-fixation. With all data included, $\delta^{15}N$ was also strongly correlated with nitrates in water. With the samples from Little Belize (high nitrate content marshes) excluded, the effect of nitrate became insignificant. N_2-fixation predicted from $\delta^{15}N$ measured on an independent data set from September 2000 was moderately accurate (r^2 = 0.68, 0.52 and 0.54 for predictions based on July 1999, January 2000 and combined data sets, respectively). When individual sample sets were divided into two groups with $\delta^{15}N < 2$ and $\delta^{15}N > 2$, the two groups were always highly significantly different in terms of their N_2-fixation. The presented evidence suggests that $\delta^{15}N$ can be used as a reliable indicator of N_2-fixation by CBM.

Introduction

Floating microbial mats are important components of inland wetland ecosystems in limestone-based tropical regions of the Caribbean (Yucatan peninsula, Cuba). The mats are formed by complex assemblages of cyanobacteria and we have referred to them as cyanobacterial mats (CBM) (Rejmánková and Komárková 2000). CBM are well described from the Everglades (Browder et al. 1994; McCormick and O'Dell 1996; McCormick et al. 1997, 1996, 1998; Vymazal 1994; Vymazal and Richardson 1995) where they form a thick carpet covering sediments and submersed portions of most aquatic macrophytes. Similar communities are known from many places in the Caribbean, such as Yucatan, Campeche, Cuba and Jamaica (Jiři Komárek, personal communication).

Through a process of nitrogen fixation, cyanobacterial mats in oligotrophic waters provide biologically available nitrogen (NH_4–N) to other components of the system (Doyle and Fisher 1994; Currin and Paerl 1998). We have previously reported data on N_2-fixation from five different marshes in northern Belize (Rejmánková and Komárková 2000). The mats were generally dominated by Cyanobacteria with only a negligible representation of a few species of diatoms. The cyanobacteria were represented by the *Leptolyngbya* group, followed by Chroococcales, and Nostocales with Stigonematales. In addition, *Phormidium* and *Oscillatoria* groups were present but their contribution to mat composition was minor. Nitrogen fixation ranged from low (< 5 nmol C_2H_4 cm^{-2} h^{-1}) to high (12 to 17.5 nmol C_2H_4 cm^{-2} h^{-1}). Differences could be explained by the proportion of heterocyst-forming cyanobacteria in the mat showing a linear increase of nitrogenase activity with increasing proportion of *Nostoc*- & Stigonematales. The diurnal course of nitrogen fixation at each marsh showed that the highest values of acetylene reduction occurred around midday and no nitrogen fixation was detected at night. Thus the contribution to nitrogen fixation by non-heterocystous cyanobacteria (*Leptolyngbya* spp.) was likely negligible despite their dominance in the mats.

The natural abundance of ^{15}N in plant tissues is a reflection of the plant nitrogen source and the mechanism of N metabolism (Handley and Raven 1992; Yoneyama et al. 1998). Therefore surveys of ^{15}N natural abundance can provide useful insight into the N cycling and ecosystem functioning (Nadelhoffer and Fry 1994). While most often used and reported as part of food web studies, stable isotope assays can also play an important role as monitoring tools (France et al. 1998; Michelsen et al. 1998; Fry 1999). N_2-fixation by cyanobacteria is accompanied by relatively little isotopic fractionation and as a result, nitrogen-fixing cyanobacteria should have $\delta^{15}N$ close to 0 ‰; (Goericke et al. 1994; Kline and Lewin 1999); with the range of −2 to 2 ‰ (Nadelhoffer and Fry 1994). Differences in natural abundance of ^{15}N among freshwater N_2-fixing cyanobacteria and non-fixing green algae were reported by Gu and Alexander (1993) as a reliable estimation of N_2-fixation. Similarly, Carpenter et al. (1997) found the $\delta^{15}N$ isotopic signature of a planktonic marine cyanobacterium *Trichodesmium* to be a good indicator of N_2-fixation.

Information on the amount of N contributed to the marsh ecosystems through N_2-fixation by CBM is important for estimates of nutrient budgets and understanding of ecosystem processes. The relative importance of N or P as a limiting nutrient depends on their ratio in the inputs and on several internal processes including organic material mineralization and N_2-fixation (Aerts 1997; Aerts and Chapin, III 2000; Downing et al. 1999). Microbial communities responsible for N_2-fixation are sensitive to environmental changes (e.g., light intensity, nutrient additions, salinity) and changes in their composition, e.g., elimination of N_2-fixers may modify ecosystem biogeochemistry. The problem is that contrary to macrophyte communities where species changes in a community are easily visible, identification of species composition and metabolic activities of CBM is quite demanding.

From our previous research we know that the rate of acetylene reduction (corresponding to N_2-fixation) is closely related to the proportion of *Nostoc* & Stigonematales (usually *Scytonema* spp.) in the mat. Processing the mat samples for cy-

anobacteria species composition and/or measuring N_2-fixation directly by acetylene reduction is labor intensive allowing for only a limited number of samples to be processed. On the other hand, large numbers of samples can be analyzed for $\delta^{15}N$ isotopic signature. Our goal was to investigate the potential of using the stable ^{15}N isotopic signature for detecting N_2-fixation associated with CBM. Finding a more efficient way to estimate the N_2-fixation by CBM would help to reveal local and regional patterns of contribution of N to marsh ecosystems.

Our specific objectives were:

1. Asses the regional spatial and temporal variability in CBM composition and N_2-fixation in marshes of northern Belize.
2. Measure $\delta^{15}N$ in corresponding CBM samples.
3. Evaluate the potential of $\delta^{15}N$ measurements for prediction of N_2-fixation in an independent data set.

Methods

Study site description

The study site has been described extensively elsewhere (Rejmánková et al. 1996; Rejmánková and Komárková 2000). Limestone-based herbaceous wetlands cover extensive areas on the Yucatan Peninsula (SE Mexico and northern Belize) and Honduras, and they form large ecosystems in Cuba (Borhidi 1991). These wetlands range in size from small marshes (<1 ha) to large shallow inland lagoons (> 100 km^2). Chemical analyses of ion content revealed large differences in sulfate, bicarbonate and chloride, suggesting complex and varied sources of water in these wetlands. The primary producers include emergent macrophytes, usually sparse monodominant stands of graminoids interspersed with CBM. The climate of the Yucatan peninsula is tropical wet-dry with average mean annual minimum and maximum temperatures of 22.8° to 30.2°C and mean annual rainfall of 1300–1500 mm (King et al. 1992). Most wetlands in the study area remain flooded or water saturated year-round.

The sampling was conducted in the Corozal and Orange Walk Districts of northern Belize during the wet season (July 1999, 12 marshes) and dry season (January 2000, 14 marshes). These sampling intervals are representative of the climatic range typical for this region. In September 2000, 15 additional marshes were sampled to verify the predictions that were made based on the results of July and January measurements.

Composition of cyanobacterial mats

Samples of cyanobacterial mats were preserved in formaldehyde (2% v/v) and because of extreme variability in mat structure, the following procedure was used to

select representative samples for microscopic examination. Each sample was well mixed in a Petri dish. Several subsamples (1–2 cm^3) of the mixture were placed in the Evelhjem's homogenizer and mixed with 2 ml of DI water. Several drops of 3% HNO_3 were added and the content was further homogenized. The sample was transferred to a glass vial and left for several hours till all the bubbles disappeared. The sample was then washed with 40 ml of water and left to settle overnight. Next day the surplus water was removed, the sample was shaken thoroughly and two sub-samples (slides) were used for examination under the light microscope. Each sample included several tens of species that were grouped into the following taxonomic/functional groups: (1) *Nostoc*-& Stigonematales, (2) *Leptolyngbya*, (3) *Oscillatoria*, (4) Chroococcales, and (5) dead sheaths. Ten fields were evaluated (200x) from each slide by a visual estimate of cover of these five groups. Since the group *Nostoc* & Stigonematales was represented mostly by genus *Scytonema*, we will refer to this group as "*Scytonema*" throughout the text.

N2-fixation

The acetylene reduction bell-jar technique (Stal 1988) was employed to estimate the cyanobacterial mat nitrogen fixation by the reduction of acetylene to ethylene by nitrogenase. Mat samples were carefully collected in shallow buckets and transferred to the field laboratory. Samples were collected from four randomly located plots in each marsh. Before measurement, water from each location was added to each bucket. Glass bottles (60 ml), equipped with a float, with a narrow opening and cut-off bottom were carefully pushed through the mats until approximately one-third of the bottle was above the mat. The bottle was then closed with a perforated screw cap with a septum. Acetylene, freshly generated from calcium carbide, was injected into each bottle through the septum and the bottles were incubated outdoors for 3 hours (11am-2pm). At the end of the exposure, several ml of headspace was withdrawn with an airtight syringe (Alltech) and analyzed by gas chromatograph (Shimadzu 8 GC) with a flame ionization detector and a Porapak-T column at 80°C. The results are reported as the nitrogenase activity in nmol C_2H_4 cm^{-2} h^{-1}. Controls with samples and no acetylene addition showed no endogenous ethylene production. Cyanobacterial mats enclosed in the bottles were sampled for chlorophyll and biomass determination after termination of the exposure.

Water Chemistry and Chlorophyll a

Water samples for nutrient and chlorophyll analyses were collected into HCl-rinsed plastic bottles and stored on ice until processing. Salinity was measured as a specific conductivity using Hana conductivity meter (specific conductivity [mS/cm] = 1.5∗salinity [ppt]). Water samples for NO_3-N, NH_4-N, and SRP were filtered through a 0.45 μm filter within an hour after sampling and frozen until analysis. Nitrogen and SRP were analyzed according to standard procedures (Hunter et al. 1993). Chlorophyll a concentration was measured in the mat samples used for N_2-fixation that were frozen and before analysis extracted for 24 hr with 20 ml of

methanol. Chlorophyll fluorescence was read before and after acidification on a Turner fluorometer.

Isotope analysis

Stable isotopes of N were measured by continuous flow isotope ratio mass spectrometry using a Europa ANCA elemental analyzer and a 20-20 isotope ratio mass spectrometer (PDZ Europa, Sandbach, UK). Dried samples containing approximately 5–7 μm N were packaged in tin capsules (Elemental microanalysis, Manchester, MA). The samples were combusted at 1000 C in the elemental analyzer. The ratio of $^{15}N/^{14}N$ (R15) was measured for the sample (10 mg) and for an injection of standardized N_2 gas introduced into the mass spectrometer in each sample cycle $\delta^{15}N$ was calculated from: $\delta^{15}N$ VAIR = 1000((R15sample/R15standard) $^{-1}$) and expressed on "per mil" basis.

Data analyses

Relationships among environmental variables (salinity, NH_4-N, NO_3-N, SRP) and the abundance of individual components of the CBM communities were investigated using the redundancy analysis (RDA, ter Braak and Šmilauer (2002)). RDA is a constrained ordination technique, the results of which are based on abundance of individual taxa and values of environmental data evaluated simultaneously. The ordination axes in RDA are constrained to optimize the relationship between these two multivariate data sets. The direction of individual variables is indicated in the ordination diagram by arrows with lengths proportional to their importance. The association (correlation) between taxa and environmental variables is apparent from the angles between arrows. The statistical significance of contribution from individual environmental variables was evaluated by Monte Carlo permutation tests (Lepš and Šmilauer 2000). If the composition of studied communities is significantly related to the examined environmental variables, then the eigenvalues resulting from the analysis of the original data are among the 5% highest values calculated from at least 100 (in our case 499) random permutations of environmental data.

Differences of regression slopes were assessed by the t-test for comparison of regression coefficients (Zar 1984). Simple correlation coefficients were used for quantification of association between individual variables.

Results

The cyanobacterial mats consisted predominantly of fine living and dead filaments of different species of the genus *Leptolyngbya*, that served as a float helping other species to maintain their position near the mat surface. Occasionally dead sheaths

Table 1. Morphological and taxonomical characteristics of the main groups of cyanobacteria

Group	Characteristics	Main species (genera)
Nostoc- & Stigonematales	Heterocytous filaments, mostly wide, branched, with yellowish mucilagenous sheats interspersed in the mat of *Lyptolyngbya* and other dead sheats	*Scytonema evanescens* Gardner *S. tenue* Gardner *Tolypothrix robusta* Gardner *T.* cf. *willei* Gardner *Hassalia* cf. *brevis* Gardner *Aulosira* sp. *Trichormus* sp. *Stigonema elegans* Gardner
Leptolyngbya	Mostly fine (<1 μm) filaments in sheats or without sheats producing pale greyish, cotton-like mat	*Leptolyngbya bijugata* (Kongisser) A. & Kom. *L. distincta* (Nordst.) A. & Kom. *L. lagerheimii* (Gom.) A. & Kom. *Pseudanabaena* spec. div. *Planktolyngbya* sp. *Jaaginema* sp.
Oscillatoria	Olive-green and blue-green filaments (3–15 μm wide) with or without sheats covering *Leptolyngbya* filaments	*Oscillatoria obtusa* Gardner *O. tenuis* var. *laevis* Gardner *Lyngbya martensiana* var. *minor* Gardner *L. nigra* Agardh ex Gomont *L.* cf. *ocreata* Gardner *Phormidium mucosum* (Gardner) A. & Kom. *P. tortuosum* (Gardner) A. & Kom. *Schizothrix* sp. *Polychlamydum* sp. *Spirulina* sp.
Chroococcales	Small size coccal cyanobacteria in muciloginous colonies among the filaments of *Leptolyngbya*	*Aphanocapsa intertexta* Gardner Aphanothece bacilloidea Gardner *A. opalescens* Gardner *Chlorogloea* sp. div. *Gloeocapsa acervata* Gardner *Gloeothece intersprsa* Gardner *Johannesbaptistia pellucida* (Dickie) Talor

of *Lyngbya* or *Scytonema* served the same purpose. Detailed description of the main groups of Cyanobacteria is given in Table 1.

Nitrogen fixation and $\delta^{15}N$ values, together with other response variables, proportions of individual groups of cyanobacteria and environmental factors for the wet and dry season data sets are listed in Table 2. Nitrogen fixation was higher in the wet season (July 1999) with the average, maximum and minimum values of 7.44, 16.40 and 0.40 nmol C_2H_4 cm^{-2} h^{-1}. The corresponding values for the dry

season (January 2000) were 3.64, 8.77, and 0.18 nmol C_2H_4 cm^{-2} h^{-1}. The $\delta^{15}N$ values were somewhat lower during the wet season but the averages for the two seasons were not significantly different. In the wet season, the average, maximum and minimum $\delta^{15}N$ values were 1.54, 7.53 and −1.99 ‰ respectively. The corresponding values in the dry season were: 2.04, 11.44 and −0.41 ‰, respectively. The chlorophyll a content, dry weight of samples, and amount of nitrogen were similar for both seasons. The proportion of *Scytonema* was higher in the wet season but the difference was not statistically significant. The proportion of *Leptolyngbya* was higher in the dry season. Regarding environmental factors, NH_4-N was significantly higher in the dry season while SRP was higher in the wet season. Little Belize marshes (LBw and Lbe) showed consistently high NO_3-N concentrations and, together with Chan Chen and Doubloon marshes, were the most saline (specific conductivity 2.43 to 5.58 mS).

The redundancy analysis, RDA, was used to relate the environmental variables and the composition of cyanobacterial sampling units to find which of the environmental variables significantly contribute to structuring of these cyanobacterial communities (Figure 1). The analysis did not exclude any of the 5 variables (specific conductivity, SRP, NH_4-N, NO_3-N and pH) because of colinearity and the Monte Carlo permutation used to assess the significance of individual environmental factors showed the specific conductivity and SRP as being highly significant ($p = 0.002$ and 0.045, respectively; $F = 7.74$ and 4.78; 499 permutations of residuals). NH_4-N and NO_3-N were clearly influential (see the length of arrows) but not significant. *Scytonema* group of CBM was shown to be negatively associated with the specific conductivity.

In both seasons we found a significant positive correlation between the proportion of *Scytonema* group and N_2-fixation (Fig. 2A). Because of higher N_2-fixation in July, the regressions coefficients (slopes) for the two seasons were significantly different ($t = 2.36$; $DF = 22$; $P = 0.05$) as assessed by the t-test for comparison of regression coefficients ((Zar 1984)). We also found a significant negative correlation between the *Scytonema* group and $\delta^{15}N$ (Fig. 2B) and between the N_2 fixation and $\delta^{15}N$ (Fig. 2C). The regression slopes for these two relationships were not significantly different. The correlation between the N_2 fixation and $\delta^{15}N$ remained significant even if the two most positive delta values were excluded (for July: $r^2 = 0.587$; $P = 0.006$, for January: $r^2 = 0.697$; $P = 0.0004$).

The hypothetical causal relationship among the variables of interest was summarized in Figure 3. The importance of the relationships was indicated by values of simple correlation coefficients. The scheme suggests strong causal links between *Scytonema*, N2-fixation and $\delta^{15}N$. With all data included, $\delta^{15}N$ is also strongly correlated with nitrates in water. With the samples from Little Belize (high nitrate content marshes) excluded, the effect of nitrate becomes insignificant (data not shown).

When nitrogen fixation was predicted from $\delta^{15}N$ values in the independent data set of samples collected in September 2000 using the regression equation for July 1999, January 2000, and combined data, the predicted values were significantly positively correlated with the actual measured N_2-fixation ($r^2 = 0.676$, $P = 0.001$;

Table 2. Response variables, proportions of main groups of Cyanobacteria and environmental factors measured in the marshes in July 1999 (A) and January 2000 (B). Nitrogen fixation is expressed as nitrogenase activity (nmolC2H4/cm2/h), nitrogen (N) and chlorophyll a (CH) are expressed based on ash free dry weight, NO3-N, NH4-N and SRP are in ppb. BS = Big Snail; BV = Buena Vista; CH = Chan Chen; DB = Doubloon; EL = Eli's; HC = Honey Camp; LB = Little Belize; NE = New; SP = San Pablo; RO = Round; ON = Old Northern Highway; CA = Cane; CB = Cobweb; PA = Patchacan; TI = Typha-island

				RESPONSE VARIABLES			CYANOBACTERIA				ENVIRONMENTAL FACTORS				
MARSH	N2-fix	delta N	N (%)	CH (µg/g)	AFDW (g)	Scy (%)	Lep (%)	Osc (%)	Chr (%)	Dead (%)	pH	cond. (mS)	NO3-N	NH4-N	SRP
A															
1 - BS	7.67	1.58	2.32	186.2	0.304	40.3	36.1	0.0	2.5	16.4	7.87	0.376	0.0	8.3	3.9
2 - BVs	8.93	1.47	2.66	206.8	0.381	39.8	18.4	2.4	4.9	34.1	7.80	0.734	0.0	9.9	6.2
3 - BVn	5.18	1.46	2.39	150.6	0.311	27.5	29.1	3.7	8.6	30.1	7.80	0.734	0.0	9.9	3.7
4 - CHw	4.25	2.27	3.60	509.6	0.340	5.7	49.1	19.6	20.1	2.8	7.36	2.160	0.0	9.2	5.6
5 - DBn	16.40	0.77	3.04	297.1	0.323	40.7	29.5	2.6	16.1	11.1	7.20	3.630	0.0	20.0	7.9
6 - Eln	9.54	0.53	2.79	280.6	0.292	32.1	41.3	0.0	13.7	12.9	7.75	1.155	1.0	9.4	4.9
7 - HC	5.33	1.57	3.24	461.2	0.171	32.9	36.4	0.4	20.9	9.4	8.30	1.920	0.0	6.6	4.4
8 - LBw	0.40	7.53	3.04	340.5	0.318	3.1	66.2	2.1	27.9	0.0	8.10	2.430	61.2	23.1	4.6
9 - NE	12.04	0.54	1.91	209.8	0.213	41.0	35.1	8.7	8.9	6.2	7.78	2.440	1.2	10.7	3.5
10 - SP	1.74	2.50	3.51	411.5	0.330	10.8	35.7	31.1	7.3	15.2	7.90	1.566	0.0	13.2	4.6
11 - RO	3.06	2.20	3.25	269.9	0.257	24.4	47.5	0.0	7.5	20.5	7.77	1.750	0.5	14.9	6.1
12 - ON-1	14.73	−1.99	3.58	178.9	0.248	47.9	26.6	0.0	5.6	18.5	7.98	0.364	4.5	4.1	11.8
Mean	7.44	1.70	2.94	291.9	0.291	28.8	37.6	5.9	12.0	14.8	7.80	1.600	5.7	11.6	5.6
SD	5.06	2.19	0.54	117.3	0.059	15.0	12.4	9.7	7.7	10.1	0.29	0.980	17.5	5.5	2.3

Table 2. Continued.

MARSH	N2-fix	delta N	N (%)	RESPONSE VARIABLES CH (µg/g)	AFDW (g)	Scy (%)	CYANOBACTERIA Lep (%)	Osc (%)	Chr (%)	Dead (%)	ENVIRONMENTAL FACTORS pH	cond. (mS)	NO3-N	NH4-N	SRP
B															
13 - BSs	6.85	0.77	3.28	233.9	0.293	42.4	32.2	0.0	7.1	16.7	7.20	0.320	0.0	33.4	1.2
14 - BVs	2.55	0.39	2.76	295.8	0.240	27.9	49.6	1.9	5.3	11.5	7.70	0.620	0.0	57.4	3.2
15 - Che	1.66	2.30	2.98	276.4	0.140	7.1	55.0	29.1	7.7	1.2	7.51	1.500	0.0	81.4	1.0
16 - DBs	3.72	1.91	2.71	272.4	0.212	17.0	62.5	14.0	5.3	1.2	7.50	3.160	0.0	136.6	2.0
17 - Els	2.50	1.45	3.43	284.6	0.259	12.8	53.4	14.4	16.9	2.6	7.50	0.880	0.0	42.2	0.0
18 - HC	2.22	2.40	2.88	352.1	0.222	4.9	61.0	20.7	9.6	0.0	7.90	1.640	297.0	189.0	0.0
19 - Lbe	0.18	11.44	1.26	133.6	0.655	1.5	75.6	5.8	14.2	2.9	7.70	5.580	561.0	412.0	2.7
20 - RO	1.37	2.83	2.84	167.8	0.283	2.4	54.7	16.0	25.2	0.7	7.92	1.400	0.0	33.4	3.7
21 - CA	2.87	0.38	3.58	270.9	0.405	28.4	43.8	7.8	4.7	13.4	6.90	0.240	0.0	87.0	2.0
22 - CB	6.45	0.40	3.30	225.7	0.167	47.9	23.3	0.0	10.9	12.1	7.00	0.480	0.0	40.6	0.7
23 - LS	4.12	1.12	2.26	266.4	0.247	22.0	55.2	7.1	10.0	4.8	7.40	1.930	0.0	98.8	0.0
24 - ON-3	8.77	0.26	2.17	142.5	0.635	32.1	41.7	2.0	12.8	10.6	7.30	0.160	0.0	43.8	1.2
25 - PA	0.90	3.30	3.25	149.7	0.640	12.1	38.5	4.6	44.8	0.0	7.43	2.440	85.0	115.0	1.7
26 - TI	6.85	–0.41	2.08	103.9	0.363	42.1	40.6	0.0	8.5	7.0	7.30	0.190	0.0	32.0	1.2
Mean	3.64	2.04	2.77	226.9	0.340	21.5	49.1	8.8	13.1	6.0	7.44	1.471	67.3	100.2	1.5
SD	2.61	2.92	0.64	74.5	0.178	15.6	13.5	8.7	10.6	5.7	0.29	1.49	163.3	101.3	1.1

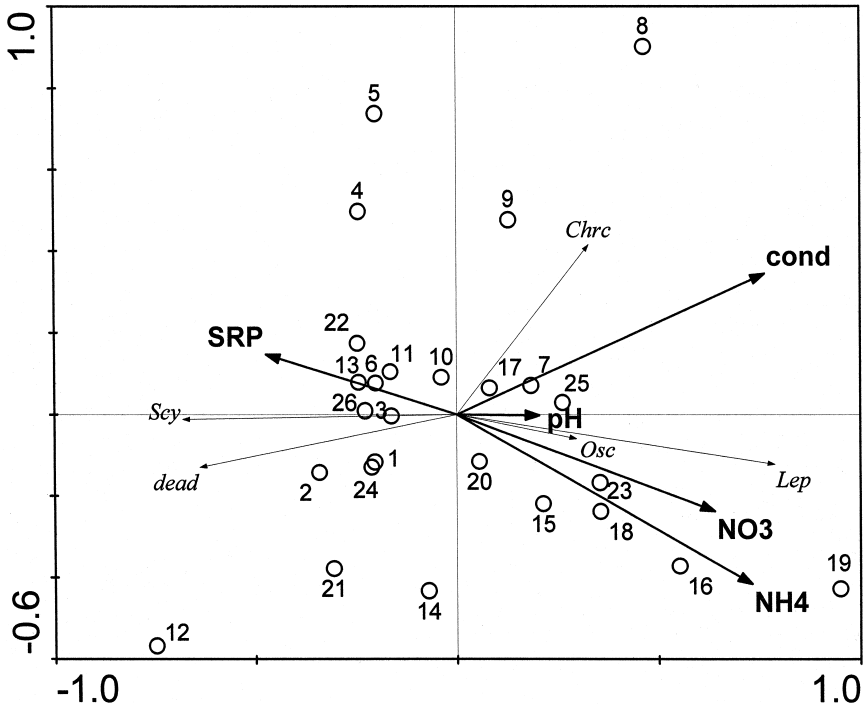

Figure 1. Redundancy analysis, RDA, ordination diagram shows the relationship between environmental variables (bold lines) and the five groups of cyanobacteria (thin lines: *Scy* = *Scytonema*, *Lep* = *Leptolyngbya*, *Osc* = *Oscillatoria*; *Chrc* = Chroococcales; *dead* = dead filaments). Circles with numbers indicate sampling locations (see also (Table 2)).

$r^2 = 0.517$, $P = 0.0025$; $r^2 = 0.544$, $P = 0.001$ for July 1999, January 2000 and the combined regression, respectively).

Rather than trying to predict the absolute values of nitrogen fixation, the estimate of high *vs.* low N_2-fixation activities may provide required information in many situations. Therefore we divided all the data sets into groups of marshes with $\delta^{15}N < 2$ ‰ (assumed to be representative of nitrogen fixing CBM; see the range of −2 to +2 ‰ given for nitrogen fixing organisms, Nadelhoffer and Fry (1994)) and marshes with $\delta^{15}N > 2$ ‰. The N_2-fixation in groups with $\delta^{15}N < 2$ ‰ was always significantly higher (Figure 4). In each case the group with $\delta^{15}N > 2$ ‰ had values of N_2-fixation well below 5 nmol C_2H_4 cm^{-2} h^{-1}, which is considered low in these types of ecosystems (Rejmánková and Komárková 2000).

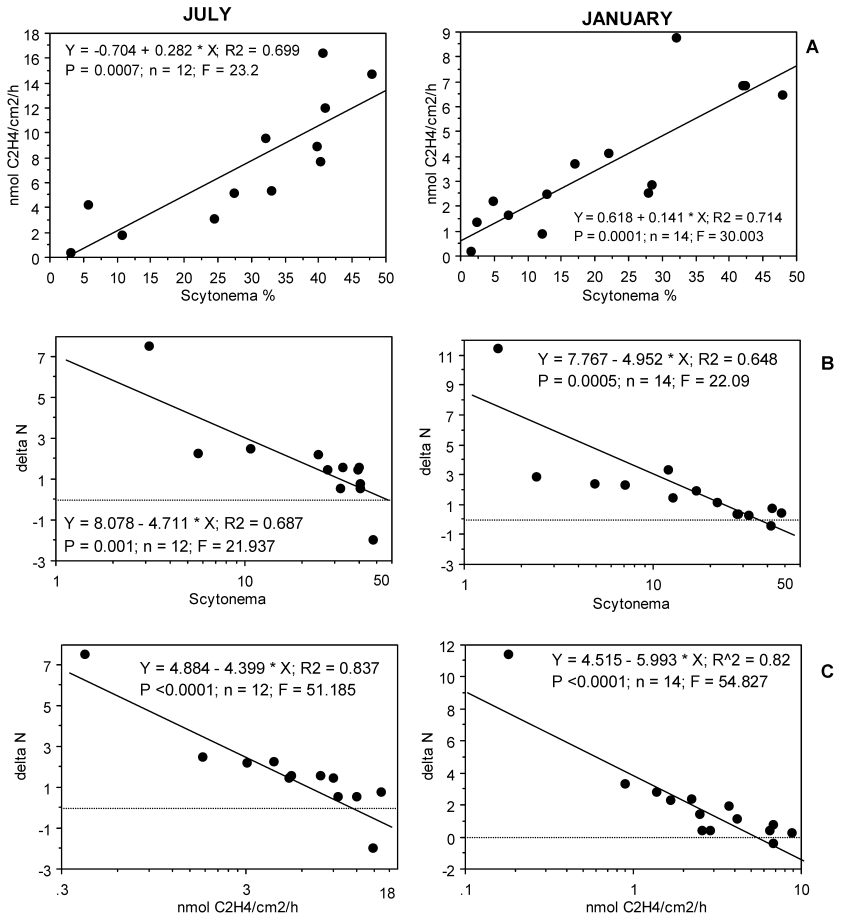

Figure 2. Relationships between: A) the proportion of *Scytonema* and N_2-fixation (expressed as nitrogenase activity in nmol C_2H_4 cm^{-2} h^{-1}) B) the proportion of *Scytonema* (log scale) and $\delta^{15}N$ C) N_2-fixation (log scale) and $\delta^{15}N$.

Discussion

Seasonal and spatial differences in N_2-fixation

We found spatial and temporal differences in nitrogen fixation among CBM from various marshes. Generally, N_2-fixation was higher during the wet season in both July 1999 and September 2000 data sets. Without experimentally testing the effects of individual environmental factors on N_2-fixation we can only speculate about which factors are responsible for this difference. Temperature may be important because summer months in Belize are warmer than winter months. Also, solar radiation was 15–20% higher in the wet season and this could have contributed to higher

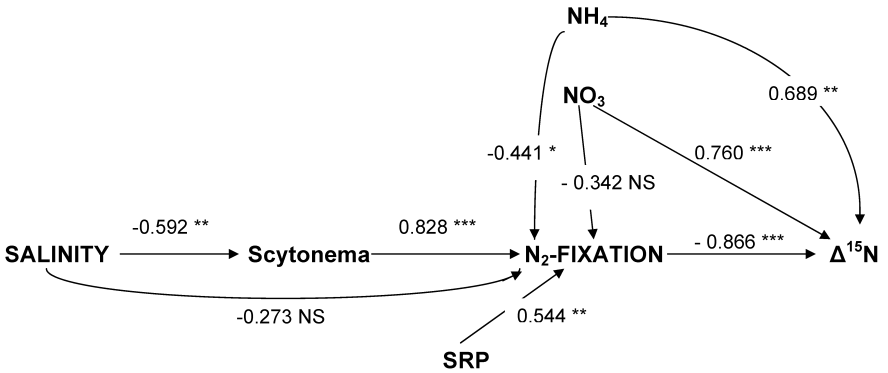

Figure 3. The positive and negative (minus signs) causal relationships among the important variables of the studied system. The numbers by arrows are simple correlation coefficients indicating the strength of the relationships. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

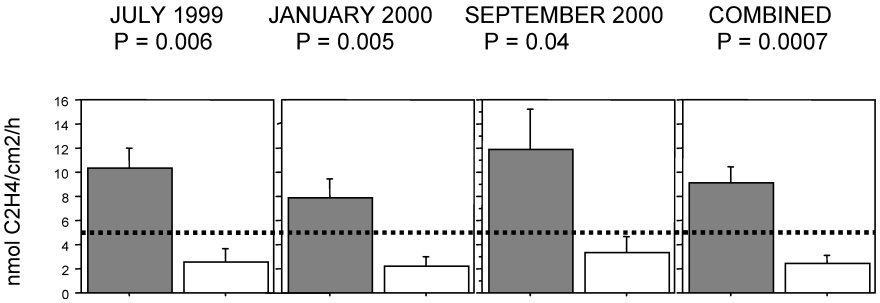

Figure 4. Mean N_2-fixation (expressed as nmol C_2H_4 $cm^{-2}h^{-1}$) for a group of marshes with $\delta^{15}N < 2$ (dark bars) and $\delta^{15}N > 2$ (empty bars) for July 1999, January 2000, September 2000 and all three data sets combined. Group means compared by t-test. The dotted line indicates the level of N_2-fixation (5 nmol C_2H_4 $cm^{-2}h^{-1}$) below which the contribution of nitrogen through N_2-fixation is considered negligible in these types of marshes.

nitrogen fixation. We have reported previously (Rejmánková and Komárková 2000) that N_2-fixation is positively correlated with solar radiation. Higher N_2-fixation could also be attributed to somewhat increased concentrations of P in water (compare 1.5 ppb in January with 5.6 and 5.1 ppb in July and September, respectively). Belizean marshes are P-limited and we have already demonstrated that addition of P increases N_2-fixation (Rejmánková and Komárková 2000; Rejmánková 2001). At the same time, NH_4-N concentrations were generally higher in the dry season and increased N availability might also limit N_2-fixation.

A factor that deserves more attention is salinity. Increased marsh water salinity resulting from increasing irrigation of agricultural crops is quite realistic scenario for northern Belize. Most of the coastal plain contains a fresh water lens floating on sea water and the porous limestone aquifer allows easy intrusion of sea water far inland. In both July 1999 and January 2000 data sets, the proportion of hetero-

cystous cyanobacteria was negatively correlated with salinity. This may seem contradictory to a wide range of information about nitrogen fixation in intertidal, often hypersaline cyanobacterial mats (Bebout et al. 1987; Paerl et al. 1989, 1994; Villbrandt et al. 1991). However, nitrogen fixation in those environments is predominantly performed by non-heterocystous cyanobacteria and heterocystous cyanobacteria are often absent. A strong impairment of nitrogen fixing capacity in heterocystous cyanobacteria under increased salinity was reported by Padhi et al. (1997). The low salt tolerance of *Nostoc* & Stigonematales in our samples could be explained by their mechanism of dealing with the osmotic stress. *Scytonema* and *Stigonema* were listed by Reed et al. (1986) among the least tolerant groups that accumulate disaccharide as osmolytica.

Differences in $\delta^{15}N$ values

Similar to nitrogen fixation values, the nitrogen stable isotope measurements showed mostly consistent differences among CBM from various marshes. Little Belize marshes had repeatedly high $\delta^{15}N$. They also had high NO_3-N concentration in water (4.4 and 40 μM in July and January, respectively as compared to almost non-detectable in other marshes). These marshes are located in the Mennonite settlement and are receiving large quantities of animal (and human) waste. Although the actual isotopic composition of nitrate has not been measured, we can assume that the reason for high $\delta^{15}N$ was due input from human and animal waste whose $\delta^{15}N$ has been reported to range from +10 to +20 ‰ (Fry 1999; McClelland and Valiela 1997; Wayland and Hobson 2001). The low nitrogen fixation in these particular marshes is probably a combination of high nitrogen content in water and high salinity. N_2-fixation in the high salinity but low NO_3-N marshes from the independent data set (September 2000) was in a low range but not as low as LBw and LBe. It needs to be experimentally tested which of the two factors is more important.

Indicator value of $\delta^{15}N$ measurements

There are limits to making inferences based on observations of ^{15}N abundance in one or a few pools of N in a system (Höberg 1997), nevertheless, measurements of ^{15}N might offer the advantage of giving insights into the N cycle without disturbing the system by adding an ^{15}N tracer. Our results support Robinson (2001) conclusion stating that $\delta^{15}N$ can provide a good clue about a process but not necessarily deliver conclusive evidence for its cause. Unless we test axenic cultures of cyanobacteria, we cannot be absolutely sure what the isotopic signature is. The instantaneous samples represent an integral signal over the life span of a particular organism (Handley et al. 1998). The individual cyanobacteria in CBM are short-lived and they would thus seem to be suitable for giving a quick response to changes in environmental conditions. However the CBM is a complex mix of many different types of cyano- and other bacteria that are involved in numerous nitrogen cy-

cling processes. Without a detailed study of these processes, the $\delta^{15}N$ values will remain only an indication of nitrogen fixation.

In conclusion, using $\delta^{15}N$ for assessing nitrogen fixation by CBM in NO_3-N varying environment can only yield a semi-quantitative estimate, but it provides a new approach to addressing nitrogen fixation in the aquatic environment and shows some advantages over other available techniques.

Acknowledgements

This research was supported by the UC Davis Faculty Research Grants. The field and laboratory assistance of Daniel Rejmánek and Jian Huang is gratefully acknowledged. David Harris conducted the $\delta^{15}N$ analyses and provided valuable comments to the manuscript. Thanks are also due to Sonia and Victor Espat for their logistic support in Belize. Comments of the two anonymous reviewers greatly improved the manuscript.

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