

Diversity of *nifH* Genotypes in Floating Periphyton Mats Along a Nutrient Gradient in the Florida Everglades

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Abstract Periphyton mats are an important component of many wetland ecosystems, performing a range of vital ecosystem functions, including nitrogen fixation. The composition and integrity of these mats are affected by nutrient additions, which might result in changes in their function. The overall objective of this study was to investigate the distribution of *nifH* sequences in floating periphyton mats collected along a nutrient gradient in the Florida Everglades. Distribution of *nifH* clone libraries indicated nutrient enrichment selected primarily for sequences branching deeply within the heterocystous cyanobacteria and within a novel group of cyanobacteria; sequences from low-nutrient sites were broadly distributed, with no clear dominance of sequences associated with heterocystous and nonheterocystous cyanobacteria and alpha-, gamma-, and delta-proteobacteria. The dominance of heterocystous cyanobacteria in nutrient-enriched sites and the lack of clear dominance by heterocystous cyanobacteria is consistent with previously reported diurnal cycles of nitrogen fixation rates in these systems. Sequences clustering with those harbored by methanotrophs were also identified; sequences from nutrient-impacted and transition regions clustered with those characteristic of type II methanotrophs, and sequences from oligotrophic regions clustered with type I methanotrophs.

Introduction

The Florida Everglades is one of the largest freshwater marshes in North America and was historically characterized by very low phosphorus (P) concentrations. Runoff from agricultural activities in the Everglades Agricultural Area (EAA) resulted in significant input of P into regions adjacent to the EAA, such that P concentrations in the water column range between 5 and 10 $\mu\text{g/L}$ in nutrient-unimpacted areas, whereas the values are significantly higher in nutrient-impacted sites [20, 27]. Increased nutrient loading led to significant changes in the biogeochemistry of the marsh [9, 14] and in the composition and integrity of floating periphyton mats [19].

Floating periphyton communities are important ecosystem components and cover a significant proportion of the open waters of the Everglades ecosystem not impacted by nutrient enrichment. Periphyton supports high levels of productivity and serves as the base of the marsh's food web [3, 17]. These mats are composed of taxonomically and metabolically diverse microbial groups [18], and previous studies have reported periphyton structural and functional changes related to increases in P concentrations [19]. The distribution of cyanobacterial species within the mats has been shown to be a sensitive indicator of nutrient enrichment in Water Conservation Area 2A (WCA-2A) of the northern Everglades [3].

Various biogeochemical properties and processes are influenced by the activities of periphyton mats [11], particularly nitrogen fixation. Craft and Richardson [8] reported that biological nitrogen fixation from these mats might account for 75% of the nitrogen inputs to WCA-2A, and Inglett et al. [13] estimated nitrogen fixation rates in periphyton mats of $\sim 10 \text{ g N/m}^2$ in nutrient-unimpacted WCA-2A floating periphyton. Little is known of the

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distribution of nitrogen-fixing prokaryotes within these mats, however, or of the impact of nutrient enrichment on the distribution of nitrogen-fixing species within the mats.

Characterization of the composition of diazotrophic assemblages is needed to more fully understand the impact of nutrient additions on wetland ecosystem structure and function. Sequence analysis of *nifH* (encoding the highly conserved dinitrogenase reductase) has been used as an indicator of the diversity of nitrogen-fixing organisms in diverse terrestrial and marine environments [15, 25, 28, 32, 34]. In this study, the distribution of *nifH* genotypes in floating periphyton in nutrient-impacted (station F1), transition (station F4), and nutrient-unimpacted (station U3) areas of WCA-2A was studied and related to previous findings regarding the composition and activities of WCA-2A periphyton.

Materials and Methods

Site Description and Sample Collection

Floating periphyton samples were collected in WCA-2A (for a map, see [4]), an impounded wetland located in the northern Florida Everglades. The agricultural drainage enriched in P and other nutrients that drain agricultural lands are discharged slowly southward across the marsh, which creates a complex water quality gradient as described in McCormick et al. [16]. Total phosphorus (TP) concentrations in canal waters have ranged between 50 and 300 $\mu\text{g/L}$ in recent decades compared with TP concentrations of ≤ 10 $\mu\text{g/L}$ in the marsh interior [2, 16].

Floating periphyton samples were collected from three stations in WCA-2A that represented the range of vegetation and nutrient conditions along the gradient: F1 (nutrient-impacted; cattail-dominated), U3 (unimpacted by nutrient additions; sawgrass-dominated), and the transition region, F4. Monthly water column P concentrations between 1997 and 2003 averaged as follows: F1, 38 $\mu\text{g/L}$; F4, 8 $\mu\text{g/L}$; U3, 5 $\mu\text{g/L}$ [27]. Samples of three mats were collected from each of the three stations on October 9, 2002. This date was chosen as representative of a wet season sample. Between three and five subsamples of ~ 1 g each were randomly collected from each mat, combined, and manually homogenized. Samples were stored on ice and transported to the laboratories at University of Florida, Gainesville. Subsamples for DNA analyses were frozen at -70°C until analyzed.

DNA Extraction and PCR Amplification of *nifH*

Approximately 50 mg of periphyton were homogenized under liquid N_2 , and homogenates were suspended in 1 mL

of 1 N HCl to remove CaCO_3 , vortexed for 1 min, and centrifuged for 1 min and the supernatant decanted. Extraction with HCl was repeated, and the recovered pellet was resuspended in 1 mL TE (Tris-EDTA [22]) buffer. Samples were centrifuged for 1 min and supernatants decanted. Total DNA from periphyton samples was extracted with an UltraClean Plant DNA kit (MoBio, Solana Beach, CA) according to the manufacturer's instructions. DNA samples were stored at -20°C until further analysis.

A nested polymerase chain reaction (PCR) protocol was used to amplify an ~ 460 -bp segment of *nifH* [33]. For the first round of the nested reaction, primer pair *nifH3* and *nifH4* was used, and equal quantities *nifH1* and *nifH2* were used for the second round of the nested reaction [33].

Primary PCR amplification was carried out with Hot-StarTaq Master Mix (Qiagen, Valencia, CA) in an iCycler thermal cycler (Bio-Rad, Hercules, CA) with the cycling conditions described by Zani et al. [33]. The same cycling and reaction conditions were used for the second nested reaction.

Cloning of PCR Products and RFLP Analyses

Polymerase chain reaction amplification products from the two sampling dates were used to create two separate clone libraries. Amplification products were inserted into pCRII-TOPO (Invitrogen, Carlsbad, CA) cloning vectors and transformed into chemically competent *Escherichia coli* TOP10F' cells according to the manufacturer's instructions (Invitrogen). Recombinant colonies were screened for inserts of the expected size by PCR amplification. Following screening, PCR products were digested with the restriction endonuclease *HhaI* overnight at 37°C . Digested PCR products were electrophoresed through a 4% (w/v) agarose gel to visualize restriction fragment length polymorphism (RFLP) patterns. Clone libraries were analyzed by analytic rarefaction with the software aRarefactWin (version 1.3; S. Holland, Stratigraphy Lab, University of Georgia, Athens [<http://www.uga.edu/~strata/software/>]) to confirm that sufficient numbers of RFLP groups were selected to represent the richness of clone libraries from periphyton samples.

nifH Sequencing and Analysis

Clones selected after comparison of RFLP patterns were sequenced using primer *nifH1* by the DNA Sequencing Core Laboratory at the University of Florida. Sequences were compared with similar sequences in the National Center for Biotechnology Information database with BLAST [1], and sequences were aligned by ClustalX version 1.8 [26]. Phylogenetic trees were generated with

TREECON [29, 30] using a neighbor-joining method. Bootstrap analysis for 100 replicates was performed to estimate the confidence of tree topologies. *Frankia* sp. CcI3 sequence (GenBank accession No. AY059395) was used as the outgroup.

Statistical Analyses

Clone libraries were compared with TreeClimber [23]. Trees for TreeClimber were generated with the DNAmI tree-searching program of PHYLIP v. 3.66 [10] using default settings.

Nucleotide Sequence Accession Numbers

The Genbank accession numbers are as follows: DQ142674 to DQ142724 for *nifH* cyanobacterial sequences and from DQ142725 to DQ142745 for *nifH* proteobacterial sequences.

Results

Three clone libraries (libraries for F1, F4, and U3) constructed from DNA from periphyton samples were screened to determine the distribution of genotypes within the mats. Clones from F1 were grouped in 16 operational taxonomic units (OTU); for F4, 11 OTU; for U3, 15 OTU. In total, 140 clones from F1, 109 clones from F4, and 109 clones from U3 were classified into OTU by RFLP. Rarefaction analysis based on the number of clones per OTU approached saturation, such that 95% of the predicted OTU in the clone libraries were included in our analyses (data not shown).

Phylogenetic analysis of partial (~460 bp) *nifH* sequences from F1, F4, and U3 periphyton reveal distinct lineages of cyanobacterial species and other free-living diazotrophs (Fig. 1; Table 1). Sequence richness was higher in the U3 library than in either F1 or F4. The majority of clones from F1 (61%) cluster within cyanobacterial clades and 39% clustered within proteobacterial clades. From F4, the cyanobacterial cluster was represented by 86% of *nifH* sequences, and 54% of *nifH* sequences in U3 clustered within cyanobacterial clades. Comparison of tree topologies by TreeClimber [23] indicate that the cyanobacterial sequences differed significantly among the F1, F4, and U3 clone libraries ($P < 0.01$). This is supported by the traditional characterization of cyanobacteria that have shown selection for different genera at different locations along the nutrient gradient [31].

No differences were detected between the proteobacterial clades from F1 and F4 or F4 and U3 (data not shown), although the proteobacterial clades from F1 and U3 were significantly different ($P = 0.03$). The primary difference

between F1 and U3 clusters is the dominance of gamma-proteobacteria in F1 and of alpha-proteobacteria in U3. The ecological significance of these differences is not known at this time.

Sequences from the U3 library were more evenly distributed and were not characterized by the clear level of dominance by specific cyanobacterial clades observed for F1 and F4 (Fig. 1; Table 1). Of note is the significant representation of gamma-proteobacteria in U3, a subdivision not detected in libraries from F1 and F4 (Table 1). The sequences in this division clustered primarily with the type I methanotroph *Methylomonas* and might be indicative of lower methane fluxes in this region than in F1 and F4 soils [7, 12].

Discussion

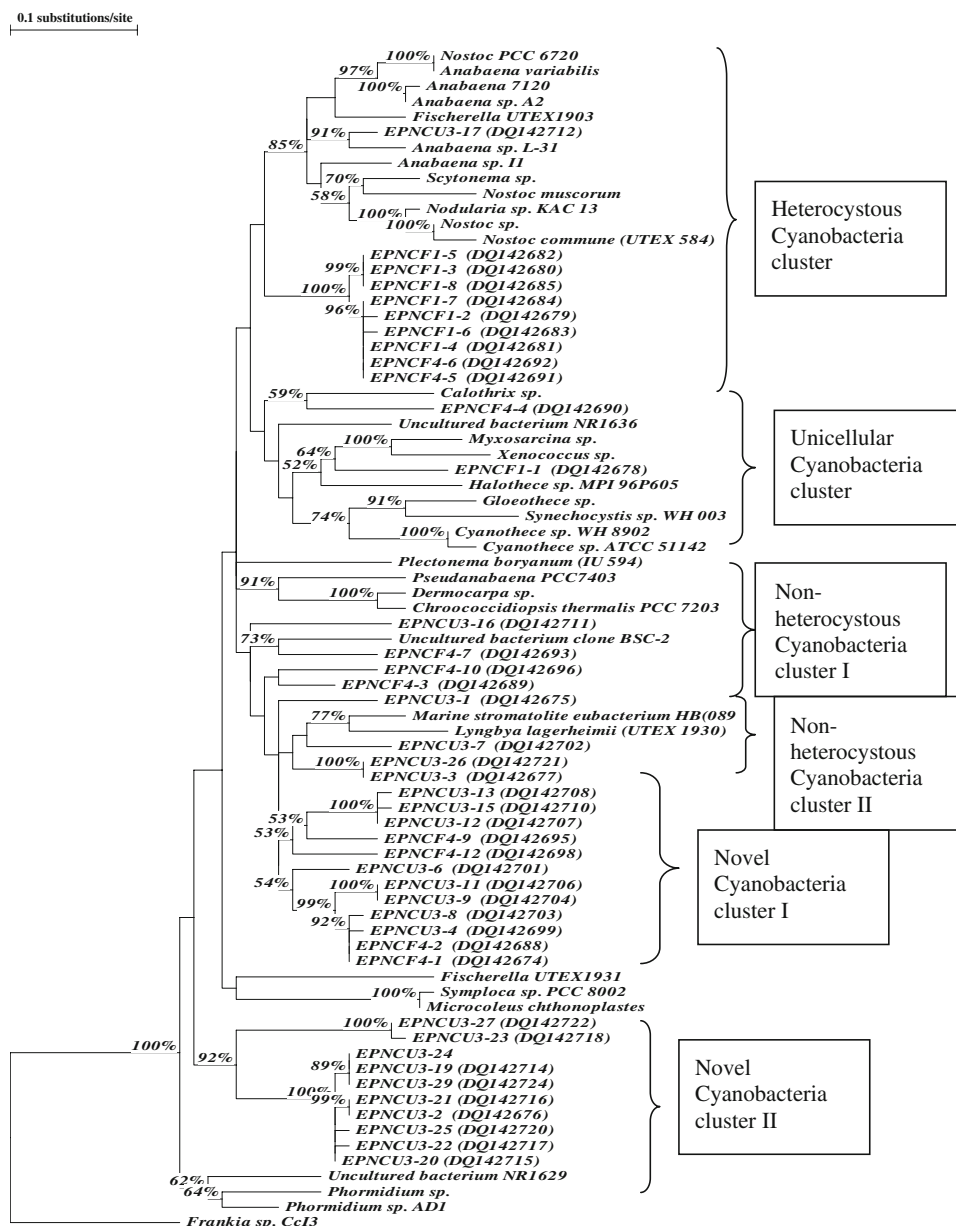
The data presented here indicate significant differences in the distribution of cyanobacterial *nifH* genotypes in floating periphyton mats as a function of position along the P gradient in WCA-2A. Heterocystous cyanobacteria genotypes dominated F1, although novel cyanobacterial clade comprised the dominant group in F4.

The limited number of samples analyzed precludes a confident assessment of the relative stability of these assemblages; however, studies of other components of mats from these locations maintain relatively stable, with significant aspects of mat composition corresponding with nutrient status [20]. Analysis of the distribution of *nifH* genotypes might be a candidate for the development of a rapid indicator of nutrient status for the Everglades [5].

McCormick et al. (2001) showed that U3 water columns exhibit significantly higher TN:TP ratios than those ratios in F1 (275 vs. 26), indicating that U3 is not limited by N, unlike either F1 and F4. Twofold lower nitrogen fixation rates have consequently been observed in U3 than F1 and F4 periphyton [13], and changes in the taxa of cyanobacteria in these mats have been attributed to increased P concentrations [19]. Differences in nitrogen demand likely selected for genotypes harbored by hosts with differing efficiencies of N-fixation.

Sequences clustering within a clade with heterocystous cyanobacteria were dominant in F1 but represented only 12% of the U3 library. Heterocystous cyanobacteria typically exhibit strong diurnal patterns [24], and Inglett et al. [13] reported ratios of light acetylene reduction rates to dark acetylene reduction rates (light:dark AR) for F1 and F4 periphyton of 10.1 and 13.1, respectively. It is not known at this time what mechanism for protection from oxygen is employed by Novel Cyanobacterial Clade I, which dominated the 2002 F4 library and comprised a significant portion of the U3 library, although the observed

Fig. 1 Phylogenetic tree of cyanobacterial *nifH* clones from F1, F4, and U3. Numbers at branch points refer to bootstrap analysis based on 100 resamplings



level of dominance (89%) and the high light:dark AR suggests that they might represent heterocystous cyanobacteria. The lower light:dark AR reported for U3 (5.9) suggests heterocystous cyanobacteria are not as important, which is consistent with our results. This finding is supported by a previous study that sought to characterize shifts in WCA-2A periphyton composition as a function of P concentrations in mesocosms [21], which found an increase in the representation of heterocystous cyanobacteria from low to high P concentrations. McCormick and Stevens (1998) [17] reported an increase in the composition of filamentous cyanobacteria in periphyton from P-enriched areas of WCA-2A, which they attribute to a colimitation of P and N in those regions, although they did not report an

increase in the relative proportion of heterocystous cyanobacteria. Our study differed from those of McCormick and Stevens (1998) in that we focused on nitrogen-fixing cyanobacteria, whereas their work included green algae and diatoms.

This report suggests a dominance of heterocystous cyanobacteria in F1 periphyton, a result consistent with both nitrogen-fixation rates and light:dark AR. Heterocystous cyanobacteria were likely selected for in this area due to N-limitation relative to U3, the low-nutrient area. The physiological characteristics of Novel Cyanobacterial Group I, dominant in the nutrient-impacted F4 periphyton, are not known at this time; however, total N:P ratios, light:dark AR, and nitrogen-fixation rates suggest they

Table 1 Spatial phylogenetic distribution of *nifH* genes in floating periphyton samples from F1, F4, and U3 libraries

Diazotrophic clusters	F1 (%)	F4 (%)	U3 (%)
Heterocystous cyanobacteria	72	7	12
Nonheterocystous cyanobacteria (I)	—	1	1
Nonheterocystous cyanobacteria (II)	—	—	9
Unicellular cyanobacteria cluster	11	—	—
Novel cyanobacterial (I)	—	89	34
Novel cyanobacterial (II)	—	—	18
α -Proteobacteria	5	—	—
γ -Proteobacteria	—	—	9
δ -Proteobacteria	12	3	17

Note: A total of 92 clones from F1, 88 clones from F4, and 92 clones from U3 were compared between libraries

might represent a novel group of heterocystous cyanobacteria. F4 was converted more recently than F1 to a nutrient-impacted system due to the moving P front in WCA-2A, and a number of prokaryotic assemblages differ among F1, F4, and U3 soils [5, 6, 7]. It might be that Novel Cyanobacterial Group I, present also in U3 although not dominant, was displaced with time in F1, although the relationship between nutrient dynamics and these groups, as well as the nature of Novel Cyanobacterial Group I, requires further study.

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