Cellulolytic, fermentative, and methanogenic guilds in benthic periphyton mats from the Florida Everglades

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Abstract

Phosphorus enrichment caused by runoff from agricultural areas has resulted in ecosystem-level changes in the northern Florida Everglades, including a loss of periphyton mats from nutrient-impacted areas. The potential for methanogenesis resulting from the anaerobic decomposition of cellulose and fermentation products, and the microorganisms responsible for these processes, were studied in mats from a region not impacted by nutrient enrichment. Methane was produced from periphyton incubated with cellulose, propionate, butyrate, and formate, with an accumulation of fatty acids in incubations. The accumulation of fatty acids may have been caused by the inhibition of syntrophic oxidation, a potentially significant route for methane production in soils. Sequence analysis of 16S rRNA genes characteristic of *Clostridium*, the primary genus responsible for anaerobic decomposition and fermentation in soils of the area, indicated that *Clostridium* Cluster I assemblages present in the mat differed from those in the soils of the area. Significantly, sequences characteristic of the *Clostridium* group that dominates the soils of the area, group XIV, were not detected in the mat. These results indicate that benthic periphyton is probably a significant source of methane in the Everglades, and the responsible microorganisms differ significantly from those in the soils of the area.

Introduction

The northern Florida Everglades has historically been a low-nutrient system, and much of the greater Everglades ecosystem is dominated by plants that are well adapted to low phosphorus concentrations. Plants such as sawgrass (*Cladium jamaicense*) on ridges and spike rush (*Eleocharis cellulosa*) in sloughs provide most of the carbon that drives much of the biogeochemical cycling in Everglades soils. The northern Everglades has been subject to nutrient loading associated with nearby agricultural activities for over 35 years, however, and phosphorus (P) loading has led to a number of changes in the Everglades ecosystem. A gradient in soil nutrient concentrations is well defined and documented in Water Conservation Area 2A (WCA-2A) of the northern Everglades (Koch & Reddy, 1992; Reddy *et al.*, 1993; DeBusk *et al.*, 1994).

Among the most significant changes in WCA-2A resulting from eutrophication is the replacement of the dominant sawgrass by dense stands of cattail (*Typha domingensis*). This has resulted in increased plant biomass, and hence in increased organic carbon in the soils of the eutrophic regions. The increase in the available organic carbon has led to higher microbial activity and to changes in the composition of microbial communities in eutrophic soils (Chauhan *et al.*, 2004; Uz & Ogram, 2006).

Another significant change to the nutrient-impacted regions of WCA-2A is the loss of calcareous benthic periphyton mats. Mats in the eutrophic regions are much less plentiful and have lost much of the calcareous structure characteristic of those in the oligotrophic regions (McCormick *et al.*, 1996). Benthic periphyton mats are formed from senescing periphyton and macrophytes (Noe *et al.*, 2003), and harbour complex assemblages of eukaryotic algae and cyanobacteria bacteria that may be a significant source of available carbon for microbial activity in the soil beneath the mats (Jones, 1980; Fallon *et al.*, 1985; Sharma *et al.*, 2005).

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Few studies have been conducted on carbon cycling in the benthic periphyton mats of the northern Everglades. The majority of these studies focused almost exclusively on nutrient cycling within the mats (D'Angelo & Reddy, 1994a, b; Noe *et al.*, 2003), and no studies have been conducted on the potential for anaerobic processes occurring within the mats. The relatively high amount of readily decomposable carbon from both the mat and fresh detritus strongly suggests that benthic periphyton may exhibit higher rates of anaerobic carbon cycling, including fermentation and methanogenesis, than are observed in the relatively recalcitrant peat soil below. Correspondingly, it is likely that the microbial communities responsible for anaerobic carbon cycling in these mats may differ from those in the soil as a result of the different environment that they are in contact with.

The objectives of this study were to investigate the potential fermentation and methanogenesis of various carbon sources in benthic periphyton mats from the oligotrophic region of WCA-2A, to characterize the microbial assemblages responsible for these processes, and to compare these results with similar studies conducted on WCA-2A soils. Our previous study (Uz & Ogram, 2006) in Everglades soils and other studies (Franks *et al.*, 1998; Schwarz, 2001; Weber *et al.*, 2001; Van Dyke & McCarthy, 2002) conducted in various anoxic environments have emphasized the importance of *Clostridium* species in cellulose degradation and fermentation, particularly of those species belonging to Clusters I, III, IV and XIV (Weber *et al.*, 2001; Van Dyke & McCarthy, 2002). We therefore focused on this genus to investigate the distribution of fermentative and cellulose-degrading clostridia in the benthic periphyton mats.

Studies on the microbial ecology of carbon cycling in these mats will lead to a greater understanding of the role of benthic periphyton in the overall carbon cycle of the Everglades, and of the impact of nutrient pollution on this sensitive ecosystem.

Materials and methods

Site characteristics, sampling and biogeochemical characterization

Benthic periphyton was obtained as part of soil cores collected by South Florida Water Management District personnel from a low-nutrient station (U3) of the Florida Everglades Water Conservation Area 2A (WCA-2A) under flooded conditions in Autumn 2004. Site characteristics have been previously described (Castro *et al.*, 2002; Chauhan *et al.*, 2004). Three intact cores (3-inch diameter) were shipped on ice for overnight delivery to the laboratory in Gainesville. Benthic periphyton layers were removed from the cores, and composite subsamples to be used for microcosm experiments and enumeration were stored at 4°C until initiation of the microcosm studies (within 2–7 days after sampling). Subsamples intended for DNA analysis were stored at $-70\,^{\circ}C$. Ammonium, total phosphorus, total inorganic phosphorus, extractable total organic carbon, and microbial organic carbon were determined by the Wetland Biogeochemistry Laboratory as described previously (Wright & Reddy, 2001; Castro *et al.*, 2002; Chauhan *et al.*, 2004).

Microbial enumeration

The most probable number (MPN) technique with five replicates per dilution was used for the enumeration of cellulolytic and fermentative microorganisms. The MPN medium contained peptone ($10\,g\,L^{-1}$), NaCl ($5\,g\,L^{-1}$) and bromocresol purple ($0.0085\,g\,L^{-1}$), cysteine–sodium sulfide (2%, to provide a final redox potential of -110 to -200 mV). Glucose (20 mM) or cellulose powder (0.3% w/v; Avicell PH-101 microcrystalline cellulose; FMC Biopolymer, Philadelphia) was added to MPN tubes for the enumeration of fermentation bacteria or cellulolytic bacteria, respectively. For fermentative MPN, colour change from purple to yellow owing to acid production was deemed positive. For cellulose MPN, tubes showing a visual change in the conformation of cellulose were deemed positive. The visual change included a shift from a dispersible powder to a more dense opaque form. This observed shift in form was confirmed as degradation by incubation of the powder as sole carbon source for cellulose-degrading organisms (data not shown).

MPNs of propionate and butyrate oxidizing bacteria, and of acetotrophic and hydrogenotrophic methanogens were determined with three-tube dilution using basal carbonate yeast extract trypticase (BCYT) medium (Touzel & Albagnac, 1983). A hydrogen scavenger is required to estimate the numbers of syntrophic bacteria in soil samples (Mormile *et al.*, 1996; Horn *et al.*, 2003); therefore, each tube was amended with *Methanospirillum hungatei* JF-1. After 2 months of incubation, positive tubes were scored on the basis of methane production compared with control tubes to which exogenous organic carbon donor(s) other than BCYT were not added.

Microcosm studies

Composite periphyton samples (2 g, wet weight) were mixed with 50 mL of BCYT in triplicate 100-mL serum tubes. The medium also included resazurin (1%), cysteine-sodium sulfide (2%), and cellulose (0.16 g). All media, stock solutions, and microcosms were prepared under a nitrogen gas stream to provide anaerobic conditions. Vials were closed with rubber stoppers and aluminum seals, and incubated at $28\,^{\circ}C$. Liquid samples (1 mL) were collected weekly from microcosms. These samples were centrifuged, filtered through 0.2-μm filters, and stored at $-20\,^{\circ}C$ until analysis.

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Potential methane production was also studied in selected microcosms containing added fatty acids. Each of the 100-mL serum bottles contained 50 mL of BCYT medium with 0.1% resazurin. Periphyton (2 g, wet weight) was introduced into the medium under a constant stream of nitrogen with minimal exposure to air, and immediately crimped using butyl rubber septa and aluminum seals (Bellco Glass Inc., Vineland, NJ). Individual vials were spiked with 20 mM each of propionate, butyrate, acetate or formate from anaerobically prepared stock solutions. Cysteine–sodium sulfide (2%) was added by nitrogen-flushed syringe to reduce the medium to a final redox potential of *c.* -110 to -200 mV. All incubations were carried out at 30 ± 2 °C in the dark. Incubations with no additional carbon added served as controls, and values from these controls were subtracted from the experimental values presented.

Fatty acid and methane measurement

Liquid samples (1 mL) were collected weekly from the microcosms. These samples were centrifuged, filtered through 0.2-μm filters, and stored at -20 °C until analysis. Fatty acids were measured by HPLC (Waters Corp., Milford, MA) with a UV detector set at 210 nm. Aminex HP 87 H column (300×7.5 mm) was used with sulfuric acid (0.5 mM) as mobile phase at a flow rate of 0.6 mL min^{-1}. Methane formation in the head space was determined with a Shimadzu 8A gas chromatograph equipped with a Carboxen 1000 column (Supelco, Bellefonte, PA) and a flame ionization detector set at 110 °C. Nitrogen was used as carrier gas and the oven temperature was 160 °C. The pressure in the headspace was measured with a digital pressure device (DPI 705; Druck, New Fairfield, CT).

Extraction of DNA and PCR amplification

Before DNA extraction, a single benthic periphyton sample was homogenized by hand. DNA was extracted from the sample with a Power Soil DNA Isolation Kit (Mobio, Solana Beach, CA) according to the manufacturer's instructions. After extraction, DNA was analysed by electrophoresis through 0.7–1% agarose gels in Tris–Acetate–EDTA (TAE) buffer. DNA was stored at -20 °C until further analysis.

Owing to the great diversity within the genus *Clostridium*, no single primer set targetting all *Clostridium* species is available. Therefore, PCR primers specific to the individual *Clostridium* clusters proposed by Collins *et al*. (1994) were used in this study. Primer names, sequences, annealing temperatures, and target groups for amplification by PCR are given in Table 1 (Franks *et al*., 1998; Van Dyke & McCarthy, 2002). PCR reaction mixtures contained 10 μL of HotStarTaq master mix (Qiagen, Valencia, CA), 7 μL of distilled H_2O, 1 μL of each primer (10 pmol μL^{-1}), and 1 μL of diluted DNA solution. PCR cycling was performed at 94 °C for 1 min for denaturation and at 72 °C for 1 min for chain extension. Reaction mixtures were subjected to 40 cycles in a Perkin-Elmer Model 2400 Thermal Cycler (Perkin-Elmer, Norwalk, CT). An initial activation step of 95 °C for 15 min was required for the HotStarTaq master mix. An additional 7 min were added for chain extension at the end of reactions.

Cloning of 16S rRNA genes and RFLP analysis

Fresh PCR products were ligated into a pCRII-TOPO cloning vector (Invitrogen, Carlsbad, CA) and transformed into chemically competent *Escherichia coli* cells (TOP10F′) according to the vendor's instructions. Individual colonies were screened by direct PCR amplification, and restriction fragment length polymorphism (RFLP) analysis was performed using digestion enzymes HhaI+EcoRV for Cluster I, AluI for Cluster III, and MspI for Cluster IV and Cluster XIVab clones. The selection of digestion enzymes for RFLP was based on *in silico* analysis of previously identified 16S rRNA genes of *Clostridium* species in the National Center Biotechnology Information (NCBI) database using

Table 1. Primers and annealing temperatures used in this study

Primer (sequence 5′–3′)*	Target gene†	Annealing temperature (°C)	Reference
Chis-0150-a-S-23 (AAAGGRAGATTAATACCGCATAA)	*Clostridium* Cluster I 16S rRNA gene	58‡	Franks *et al*. (1998)
Cbot-0983-a-A-21 (CARGRGATGTCAAGYCYAGGT)	*Clostridium* Cluster I 16S rRNA gene		Van Dyke & McCarthy (2002)
Cther-0650-a-S-23 (TCTTGAGTGYYGGAGAGGAAAGC)	*Clostridium* Cluster III 16S rRNA gene	60	Van Dyke & McCarthy (2002)
Cther-1352-a-A-19 (GRCAGTATDCTGACCTRCC)	*Clostridium* Cluster III 16S rRNA gene		Van Dyke & McCarthy (2002)
Clos-0561-a-S-17 (TTACTGGGTGTAAAGGG)	*Clostridium* Cluster IV 16S rRNA gene	60	Van Dyke & McCarthy (2002)
Clept-1129-a-A-17 (TAGAGTGCTCTTGCGTA)	*Clostridium* Cluster IV 16S rRNA gene		Van Dyke & McCarthy (2002)
Erec-0482-a-S-19 (CGGTACYTGACTAAGAAGC)	*Clostridium* Cluster XIVab 16S rRNA gene	55	Franks *et al*. (1998)
Ccoc-1112-a-A-19 (TGGCTACTRDRVAYARGGG)	*Clostridium* Cluster XIVab 16S rRNA gene		Van Dyke & McCarthy (2002)

*Y, T/C; V, G/C/A; R, A/G.

†Based on the clustering system described by Collins *et al*. (1994).

‡Originally reported as 65 °C.

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Table 2. Selected biogeochemical parameters for benthic periphyton and soils of WCA-2A*

Sampling site	Moisture content (%)	TP (mg kg^{-1})	TP_i (mg kg^{-1})	TC (g kg^{-1})	MBC (mg kg^{-1})	Extractable TOC (mg kg^{-1})	TN (g kg^{-1})	NH_4-N (mg kg^{-1})
Periphyton	91 (2)	354 (19)	141 (14)	360 (40)	1689 (1046)	3002 (469)	24.1 (2.8)	114 (25)
U3 soil	93 (2)	449 (161)	221 (131)	230 (42)	2627 (128)	1973 (450)	32.8 (2.8)	103 (33)
F1 soil	92 (1)	1110 (352)	366 (128)	446 (24)	7705 (1534)	2404 (204)	28.8 (2.1)	90 (13)
F4 soil	93 (1)	767 (49)	310 (72)	357 (10)	8933 (1529)	2436 (284)	25.3 (2.6)	107 (16)

U3 soil is from the region from which periphyton was collected; F1 and F4 are nutrient-impacted soils (Uz & Ogram, 2006).
*SDs are presented in parentheses. Concentrations are expressed per kg (dry weight) soil. TP, total phosphorus; TP_i, total inorganic phosphorus; TC, total carbon; MBC, microbial biomass carbon; TOC, total organic carbon; TN, total nitrogen; NH_4-N, extractable ammonium. Values for F1, F4, and U3 soils were previously reported in Uz & Ogram (2006).

CLONEMAP software (version 2.11, CGC Scientific Inc, Ballwin, MO). Digestion reactions were analysed by electrophoresis through 2% agarose gels. Clone libraries were analysed by analytical rarefaction with the software ARAREFACTWIN (version 1.3, S Holland, Stratigraphy Lab, University of Georgia, Athens, GA [http://www.uga.edu/~strata/software]) to confirm that sufficient numbers of RFLP groups were selected to represent the clone libraries.

Sequencing and phylogenetic analysis

Selected clones representing different RFLP patterns were sequenced by the University of Florida's Interdisciplinary Center for Biotechnology Research core sequencing facility. Sequences were compared with previously identified sequences in the NCBI database using BLAST (Altschul *et al.*, 1990). The sequences obtained in this study were initially aligned with closely matched sequences from the NCBI database and clone sequences obtained from soils of eutrophic (F1), transition (F4) and oligotrophic (U3) regions of the Florida Everglades in a previous study (Uz & Ogram, 2006) using the PILEUP function of GCG Package (Accelrys Inc., San Diego, CA) and adjusted manually with CLUSTALX version 1.8 (Thompson *et al.*, 1997). Phylogenetic trees were generated with TREECON (Van de Peer & De Wachter, 1994, 1997) using a neighbour-joining method. Bootstrap analysis was performed with 100 resamplings of the DNA sequences to estimate the confidence of the tree topology. Clone libraries were compared with TREECLIMBER (Schloss & Handelsman, 2006). Trees for TREECLIMBER were generated with the DNAML tree-searching program of PHYLIP version 3.66 (Felsenstein & Churchill, 1996) using default settings.

Nucleic acid accession numbers

The GenBank accession numbers obtained in this study for *Clostridium* 16S rRNA gene sequences are DQ193913 – DQ193955.

Results and discussion

Biogeochemical characterization

Biogeochemical parameters for phosphorus, carbon and nitrogen in benthic periphyton mat and soils from WCA-2A are presented in Table 2 (Uz & Ogram, 2006). The higher levels of available nutrients in the mat relative to the soils may promote more rapid carbon cycling than is observed in the soils. Benthic periphyton samples contained lower total phosphorus and total inorganic phosphorus than did the U3 soil samples; however, total carbon and total extractable organic carbon concentrations in the benthic mat were higher than in U3 soil. Benthic periphyton mats showed the lowest microbial biomass of carbon, although with a relatively high SD. The total nitrogen in benthic periphyton samples was slightly lower than in U3 soil.

Enumeration of cellulolytic and fermentative bacteria

No significant difference in the MPNs of cellulolytic bacteria was observed between the benthic periphyton mat and U3 soil (Table 3) (Uz & Ogram, 2006). The MPN of microorganisms growing on solid substrates such as cellulose is problematic, however, and may significantly underestimate the true numbers. The MPNs of fermentative bacteria were 100-fold higher in the mat than in U3 soil. No significant difference was observed between the MPNs of propionate-, butyrate- and formate-oxidizing bacteria in the mat. Methanogenesis derived from propionate and butyrate requires the action of syntrophic consortia, in which H_2-producing, fatty acid-consuming bacteria ('syntrophs') ferment propionate to acetate and H_2, and methanogens utilize the acetate and H_2 to produce methane (Schink, 1997). Because of the requirement for the close proximity of syntrophs with methanogens, the MPNs may significantly underestimate the numbers of syntrophs. The mixing and dilution required for MPN enumeration may disrupt syntrophic consortia. Regardless, high MPN propionate-oxidizing syntrophs relative to U3 soil

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Table 3. Most probable numbers (MPNs) of cellulolytic, fermentative, syntrophic, and methanogenic bacteria in benthic periphyton and soils of WCA-2A (95% confidence interval)

	Cellulose-degrading bacteria	Fermentative bacteria	Propionate-oxidizing syntrophic bacteria	Butyrate-oxidizing syntrophic bacteria	Acetate-utilizing methanogens	Formate-utilizing methanogens
Benthic periphyton	3.2×10^4 (1.1 – 9.2)	7.9×10^8 (2.5 – 18.9)	2.2×10^6 (0.02-5.1)	2.2×10^6 (1.1 – 8)	1.0×10^6 (0.1 – 3)	1.6×10^6 (1.2 – 12.7)
U3 soil	2.4×10^4 (0.8 – 7.4)*	1.7×10^6 (0.4 – 5.0)*	0.2×10^6 (0.02 – 0.6)	0.3×10^5 (0.07 – 1)†	0.1×10^5 (0.01 – 0.3)†	0.9×10^6 (0.2 – 2.7)†
F1 soil	2.4×10^5 (0.8 – 7.6)*	5.4×10^6 (1.8 – 14.2)*	4.6×10^6 (1.1 – 15)	4.6×10^5 (1.1 – 15)†	0.09×10^5 (0.005 – 0.3)†	11×10^6 (2.2 – 30.8)†
F4 soil	3.5×10^5 (1.2 – 10.2)*	9.2×10^6 (2.7 – 22.0)*	4.6×10^6 (1.1 – 15)	4.6×10^5 (1.1 – 15)†	0.9×10^5 (0.2 – 2.7)†	4.6×10^6 (1.1 – 15)†

U3 soil is from the region from which periphyton was collected; F1 and F4 are nutrient-impacted soils (Uz & Ogram, 2006).
*Cellulose-degrading and fermentative bacteria MPNs for U3, F1, and F4 soils were previously reported by Uz & Ogram (2006).
†Propionate- and butyrate-oxidizer, and acetate- and formate-utilizing methanogen MPNs for U3, F1, and F4 soils were previously reported by Chauhan *et al*. (2004).

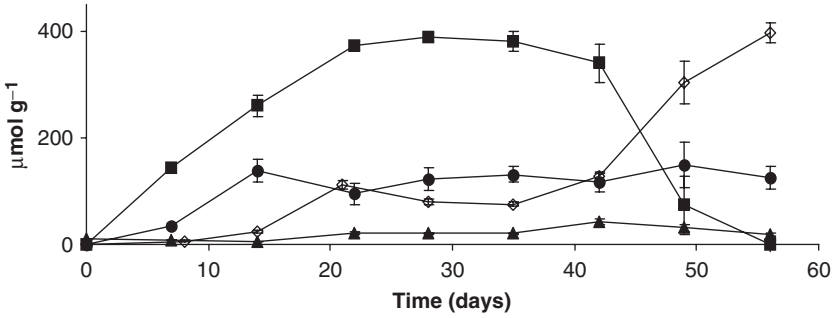


Fig. 1. Decomposition of cellulose to fermentation products and methane in benthic periphyton from the oligotrophic region of the Florida Everglades. Error bars represent SEs based on five replicates. Control values (no carbon added other than BCYT) were subtracted from the values reported here. Symbols: square, acetate; triangle, butyrate; circle, propionate; open diamond, methane.

(2.2×10^6 per gram mat vs. 1.5×10^5 per gram soil; Table 3) (Chauhan *et al.*, 2004) were observed in the mats.

Carbon cycling potential

Cellulose microcosms were characterized by a significant accumulation of acetate, eventually leading to methanogenesis (Fig. 1). A similar accumulation of acetate was noted in previous studies of the soils of the area (Drake *et al.*, 1996; Chauhan & Ogram, 2006). It is likely that this accumulation of acetate resulted from the inhibition of syntrophic acetate oxidation (SAO), potentially a major pathway for acetate consumption and subsequent methanogenesis, in these samples (Chauhan & Ogram, 2006), and that this inhibition of the syntrophic consumption of acetate probably resulted from the accumulation of H_2 produced during fermentation in these microcosms. Low concentrations of H_2 are required for syntrophic consumption of fermentation products (Schink, 1997), and H_2 may not have been removed from these microcosms in sufficient quantities for syntrophic consumption of acetate to occur.

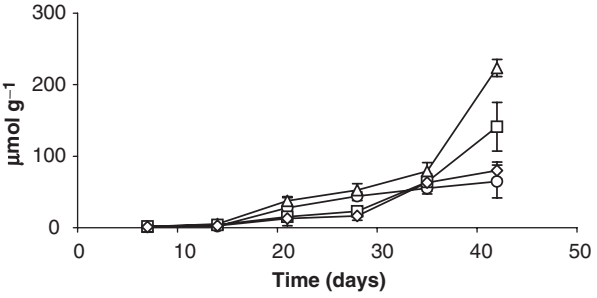


Fig. 2. Decomposition of propionate (P), butyrate (B), acetate (A) and formate (F) to methane in benthic periphyton from the oligotrophic (U3/D) regions of the Florida Everglades. Error bars represent SEs based on three replicates. Control values (no carbon added other than BCYT) were subtracted from the values reported here. Symbols: square, U3/D+A; triangle, U3/D+B; diamond, U3/D+F; circle, U3/D+P.

A significant accumulation of propionate was also noted in mat microcosms incubated with cellulose, and propionate concentrations remained stable throughout much of the

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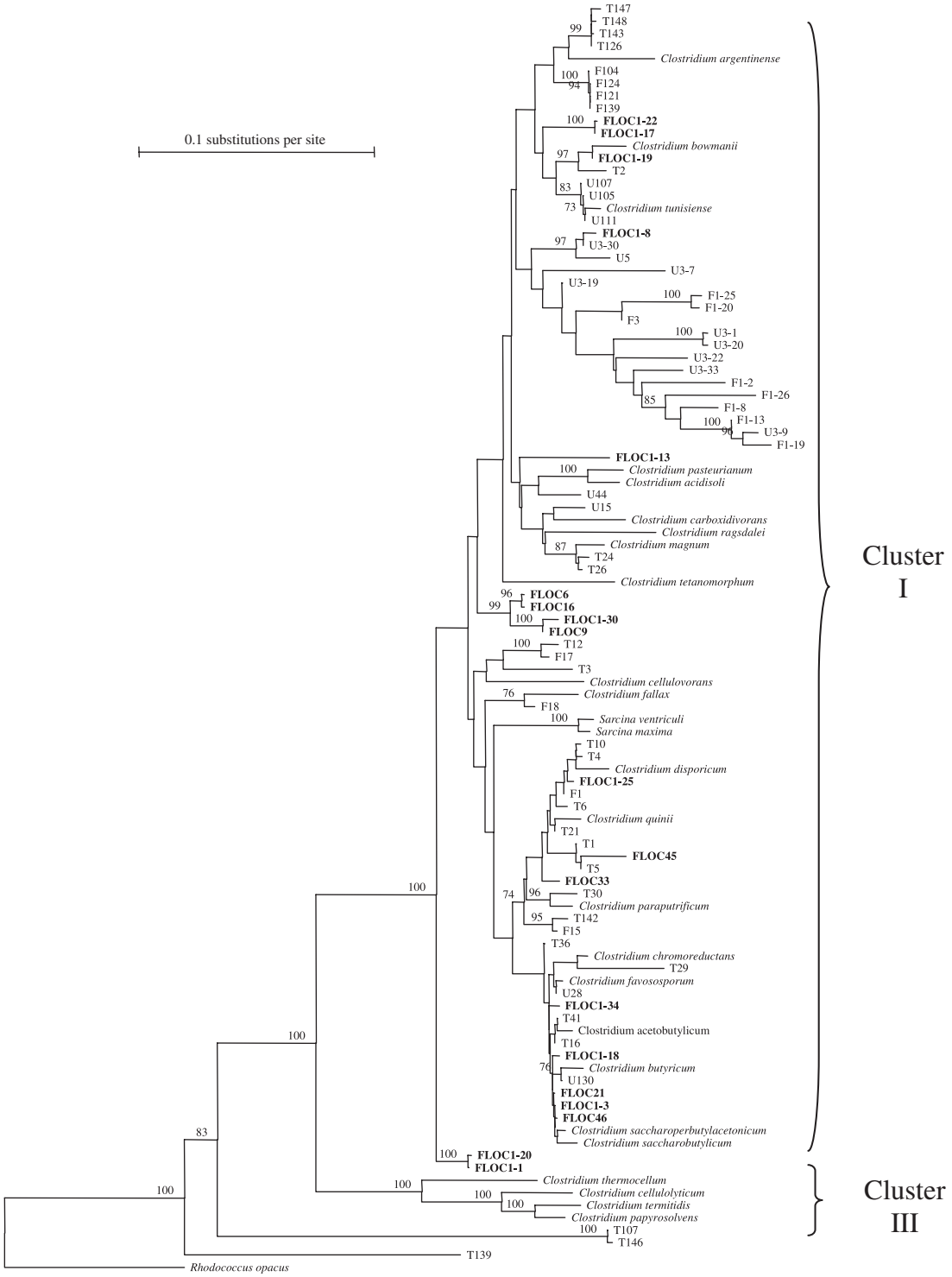


Fig. 3. Phylogenetic tree of *Clostridium* Cluster I 16S rRNA gene clone sequences obtained from benthic periphyton (FLOC) samples. Clone sequences obtained from soils of eutrophic F1 (F) and F4 (T), and oligotrophic (U) regions of the Florida Everglades are also included. Numbers at branch points refer to bootstrap analysis based on 100 resamplings.

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incubation period. Significant methanogenesis was observed when mats were incubated with exogenous propionate and butyrate (Fig. 2), however, indicating that syntrophic consortia capable of mineralizing propionate were present and active. The discrepancy between the consumption of exogenously added propionate and the accumulation of propionate formed from cellulose (without apparent methanogenesis) may be a result of the accumulation of H_2 produced from the fermentation of cellulose, which may have inhibited the syntrophic consumption of those fermentation products (Schink, 1997). Methane resulting from exogenously added butyrate, propionate, formate, and acetate was similar to that observed for U3 soil after 6 weeks (Chauhan *et al.*, 2004), although this is probably an underestimate owing to the observed inhibition of methane arising from acetate in the present study.

Phylogenetic analysis of *Clostridium* 16S rRNA gene sequences from the periphyton layer

Cluster I

The majority of benthic mat sequences shared $< 98\%$ similarity with 16S rRNA genes from characterized Cluster I species (Fig. 3). Clone FLOC1-19 was 99% similar to *C. bowmanii*, a psychrophilic and saccharolytic species (Spring *et al.*, 2003). Clones FLOC21, FLOC1-3, and FLOC46 grouped with *C. saccharoperbutylacetonicum*, and shared 99% similarity with 16S rRNA genes from this species. *Clostridium saccharoperbutylacetonicum* is a well–known solvent-producing species (Tashiro *et al.*, 2005), and clones that group with this species may perform similarly. In the same branch, clone FLOC1-18 grouped with *C. butylicum*, the type species for the genus *Clostridium*, with 99% sequence similarity. Clones FLOC45 and FLOC33 are 98% similar to *C. quinii,* and FLOC34 shares 99% sequence similarity with the 16S rRNA gene of *C. favososporum*.

Cluster 1 sequences in benthic periphyton differed significantly from those in the three soils analysed in this study (Table 4), and the three Cluster I soil clone libraries differed significantly from each other (data not presented). Little is known of the ecology of Cluster I *Clostridium* species in environments such as the Everglades, making conclusions for this high degree of selection based on site difficult to draw. The quality of organic carbon in the periphyton probably differs from that in soil (Table 1), such that readily available fermentable material is probably higher in periphyton than in peat soil. This is supported by the 100-fold higher MPN fermentative bacteria (Table 3) and by the higher amounts of labile carbon (Table 2) in periphyton than in any of the soils. More work is required to understand the factors strongly selecting for Cluster I phylotypes in the different environments studied here.

Table 4. Comparison of periphyton clone libraries with soil clone libraries

	U	F1	F4
Cluster I	0.009	0.004	0.014
Cluster III	0.46	0.827	0.6
Cluster IV	0.178	0.073	0.006

P-values < 0.01 are assumed to be highly significantly different, and those < 0.05 are assumed to be significantly different.
P-values generated with TREECLIMBER (Schloss & Handelsman, 2006) are presented.

Cluster III

A phylogenetic analysis of *Clostridium* Cluster III benthic periphyton sequences is presented in Fig. 4. Clone FLOC3-5 shares 98% sequence similarity with *C. cellobioparum* and *C. termitidis*, and clone FLOC3-13 is 98% similar to *Bacteroides cellulosolvens*. All other clone sequences from benthic periphyton samples were $< 98\%$ similar to known Cluster III sequences.

The strong selection observed in Cluster I sequences was not observed in Cluster III sequences; no differences were noted between the periphyton and soil libraries (Table 4). As with Cluster I, the lack of information available on the ecology of Cluster III *Clostridium* species makes any conclusions regarding the apparent lack of selection by sample type highly speculative. It may be that this cluster is dominated by cellulolytic species, as are the few cultivated strains related to our sequences. This may suggest that Cluster III phylotypes are selected by cellulose and broad environmental factors such as temperature that do not vary across the narrow range of environments studied here. The observation of similar cellulolytic MPNs between the periphyton and U3 soil also suggests little difference between the two compartments with regard to cellulose degradation of this region, although *c.* 10-fold higher MPNs were observed in F1 and F4, the nutrient-impacted soils (Uz & Ogram, 2006).

Cluster IV

Phylogenetic analysis of *Clostridium* Cluster IV produced a branching separating known cellulolytic and noncellulolytic species (Fig. 5). The branch containing *Ruminococcus flavefaciens*, *R. albus*, and *C. cellulosi* represents the cellulolytic branch of Cluster IV, and clones FLOC39, FLOC4-30 and FLOC4-5 were placed in the branch with sequences from similar libraries constructed from DNA from U3 soil (Uz & Ogram, 2006). Benthic periphyton sequences were also

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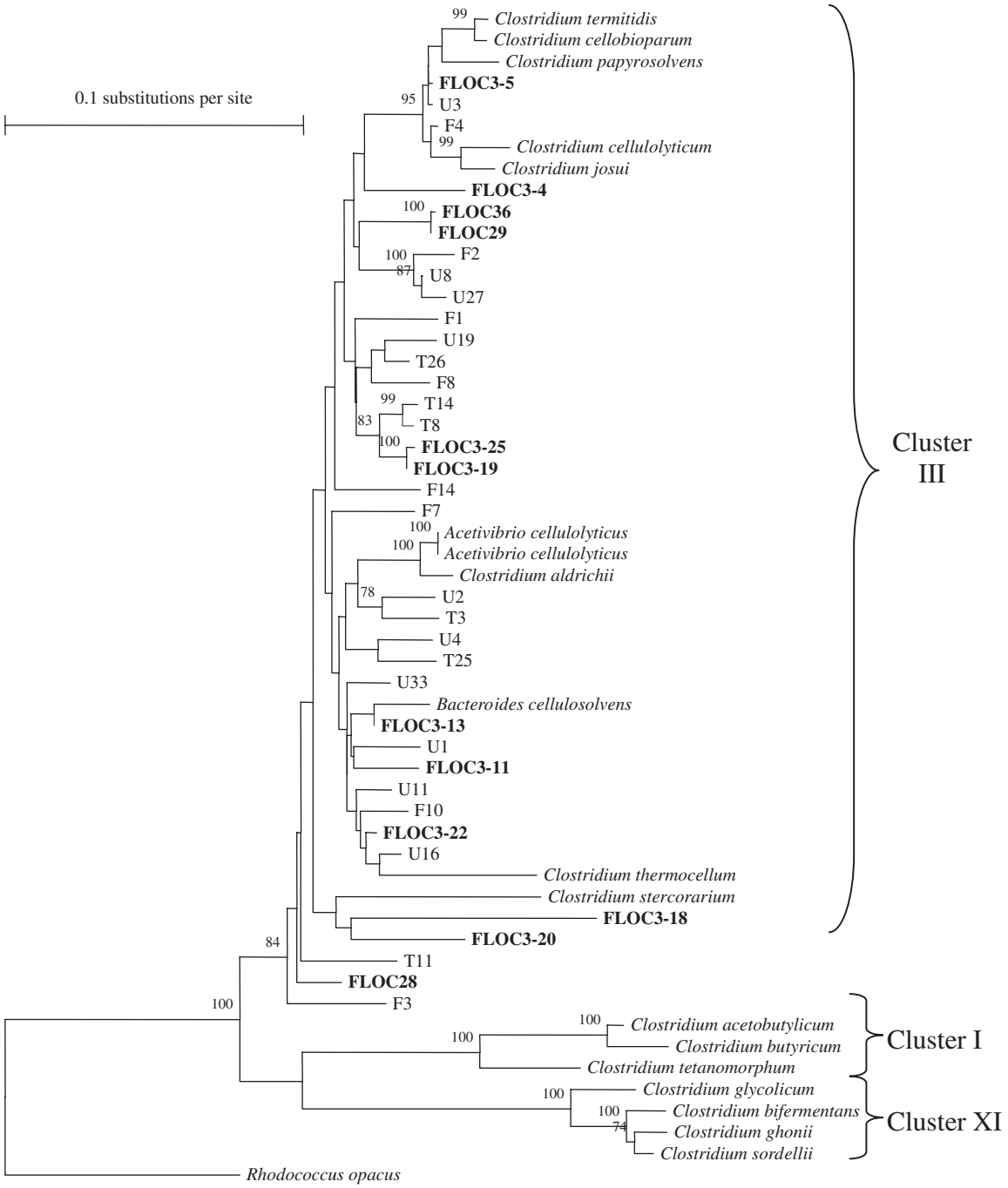


Fig. 4. Phylogenetic tree of *Clostridium* Cluster III 16S rRNA gene clone sequences obtained from benthic periphyton (FLOC) samples. Clone sequences obtained from soils of eutrophic F1 (F) and F4 (T), and oligotrophic (U) regions of the Florida Everglades are also included. Numbers at branch points refer to bootstrap analysis based on 100 resamplings.

observed in the noncellulolytic branch with sequences from soils collected from eutrophic regions of WCA-2A (Uz & Ogram, 2006).

Somewhat surprisingly, the periphyton Cluster IV library differed significantly from the F4 library (Table 4). The lack of difference noted between the periphyton and U3 and F1 libraries suggest that the difference between periphyton and F4 may be more relevant to F4 than to the periphyton. F4 is a site that experienced elevated nutrient concentrations more recently than F1, and it differs significantly from F1 with respect to methanogens and sulfate-reducing prokaryotes (Castro *et al.*, 2005).

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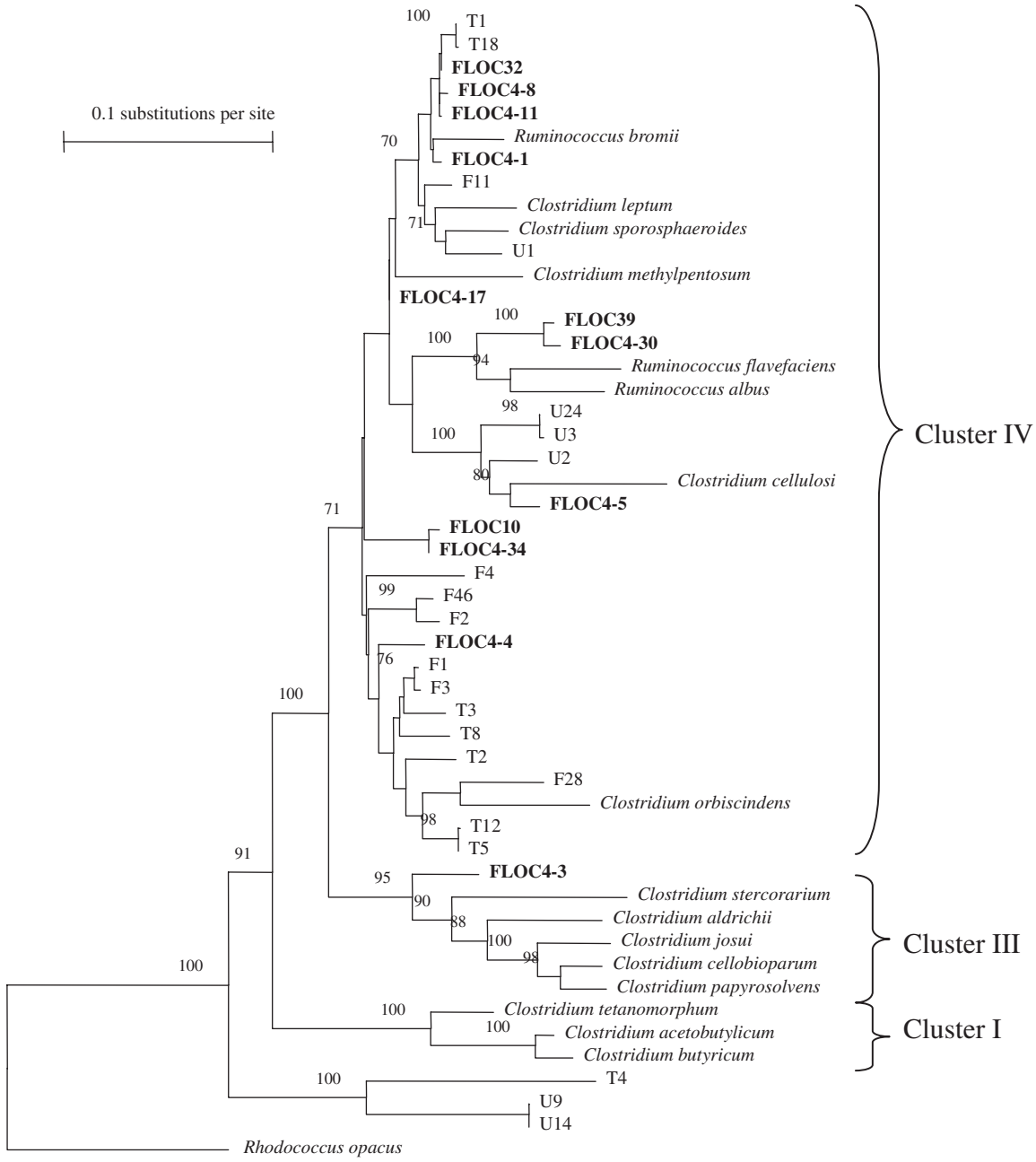


Fig. 5. Phylogenetic tree of *Clostridium* Cluster IV 16S rRNA gene clone sequences obtained from benthic periphyton (FLOC) samples. Clone sequences obtained from soils of eutrophic F1 (F) and F4 (T), and oligotrophic (U) regions of the Florida Everglades are also included. Numbers at branch points refer to bootstrap analysis based on 100 resamplings.

Cluster XIV

Perhaps the most striking observation is that DNA isolated from benthic periphyton did not yield an amplification product with primers specific for *Clostridium* Cluster XIV species. Cluster XIV, especially subcluster XVIa, contains various cellulose- and hemicellulose-degrading species. The absence of this cluster was surprising, both because of its importance in U3 soils (Uz & Ogram, 2006) and because of previously published reports indicating the importance of Cluster XIV species in environments such as rice paddy soils, landfills, and human intestines where plant material or cellulose is present (Hengstmann *et al.*, 1999; Weber *et al.*, 2001; Hayashi *et al.*, 2002; Van Dyke & McCarthy, 2002). Plant material was not visible in the mat, possibly suggesting that Cluster XIV strains were not selected for. No significance was observed between MPN cellulolytic

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microorganisms in soil and in the mat, however. In a study conducted in a methanogenic landfill leachate bioreactor, Cluster XIVa species were associated with glucose fermentation but not with cellulose degradation (Burrell *et al.*, 2004). It is not clear at this point in time why no representatives of this cluster were found in the mat.

Summary

This report documents the first study of which we are aware on potential methanogenesis from cellulose decomposition in benthic periphyton mats of the Everglades, and on the major phylogenetic groups responsible for cellulolytic and fermentative processes in the mats. These mats are sites of high microbial activity, and may be a significant source of available carbon and nitrogen for the soil below, as well as being productive sites of methanogenesis. The MPNs of fermentative bacteria per gram of mat were *c.* 100-fold higher than those in soils of the area, indicating the potential for much more rapid production of fermentation products than in the soil. A significant accumulation of acetate in microcosms incubated with cellulose was observed, possibly owing to the inhibition of syntrophic acetate oxidation resulting from inhibitory levels of H_2 formed during fermentation. Phylogenetic analysis of *Clostridium* sequences revealed significant selection of Cluster I *Clostridium* phylotypes on the basis of periphyton and nutrient impact in soils, while the largely cellulolytic Cluster III phylotypes did not differ significantly between the periphyton and soils. No sequences representing *Clostridium* Cluster XIV, the dominant cellulolytic cluster observed in soils of the area, were found in periphyton. It should be noted that only one mat was included in the phylogenetic analysis, so spatial variability may result in distributions different from those reported here. Given the broad distribution of these mats and the general connection between soil and periphyton through water movement, however, it seems unlikely that spatial variability alone accounts for the lack of detection of Cluster XIV sequences.

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