Distribution, Activities, and Interactions of Methanogens and Sulfate Reducing Prokaryotes in the Florida Everglades

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ABSTRACT

To gain insight into the mechanisms controlling methanogenic pathways in the Florida Everglades, the distribution and functional activities of methanogens and sulfate-reducing prokaryotes (SRP) were investigated in soils (0-2 or 0-4 cm depth) across the well-documented nutrient gradient in the Water Conservation Areas (WCA) caused by runoff from the adjacent Everglades Agriculture Area. The methyl coenzyme-M reductase (mcrA) gene sequences that were retrieved from WCA-2A, an area with relatively high concentrations of SO_4^{2-} (≥39 µM), indicated that methanogens inhabiting this area were broadly distributed within the orders Methanomicrobiales (MM), Methanosarcinales, Methanocellales, Methanobacteriales, and Methanomassiliicoccales. In more than 3 years of monitoring, qPCR using newly designed group-specific primers revealed that the hydrogenotrophic MM were more numerous than the obligatory acetotrophs Methanosetaeaceae (MST) in SO_4^{2-} rich areas of WCA-2A, while MST were dominant over MM in WCA-3A (relatively low SO_4^{2-} ≤4 µM). qPCR of dsrB sequences also indicated that SRP are present at greater numbers than methanogens in the WCAs. In an incubation study with WCA-2A soils, addition of MoO_4^{2-} (a specific inhibitor of SRP activity) resulted in increased methane production rates, lower apparent fractionation factors (α_{app}: [δ^{13}C-CO_2 + 1000]/[δ^{13}C-CH_4 + 1000]) and higher MST-mcrA transcript levels compared to controls without MoO_4^{2-}. These results indicate that SRP play crucial roles in controlling methanogenic pathways and in shaping the structures of methanogen assemblages as a function of position along the nutrient gradient.
INTRODUCTION

The Florida Everglades is a large freshwater subtropical wetland at the southern end of the Florida peninsula (Fig. S1), and it was estimated to harbor the largest single body of organic soils in the world at one time, covering over 8,000 km² (1). Wetlands, including the Everglades, are the primary source of natural global CH₄ emissions, producing more than 150 Tg of CH₄ annually (roughly 20% of global annual emissions) (2, 3). The Everglades ecosystem was historically limited in nutrients, particularly phosphorus (P); however, discharge of agricultural drainage from the adjacent Everglades Agricultural Area (EAA) led to elevated nutrient levels in the northern Everglades, particularly in Water Conservation Area 2A (WCA-2A), which is characterized by a well-documented gradient in soil and water P concentrations (4-7). The alleviation of P-limitation resulted in many changes to the WCA-2A ecosystem; primary productivity was significantly increased, and the dominant plant species changed from sawgrass to cattail. In addition, organic matter mineralization to CO₂ and CH₄ was greatly increased (8, 9).

Numerous studies have been conducted on the impacts of nutrient additions to WCA-2A soils, including analysis of methanogen community structures (10, 11, 12), and methanogenesis rates (8, 9). However, the detailed mechanisms controlling methanogenic pathways and the development of methanogenic guilds in response to shifting nutrient limitations is poorly understood.

In freshwater wetlands, CH₄ is primarily produced via two pathways: hydrogenotrophic methanogenesis (HM; CO₂ + 4 H₂ → CH₄ + 2 H₂O) and acetoclastic methanogenesis (AM; CH₃COOH → CH₄ + CO₂). From the stoichiometry of glucose fermentation, the relative proportions of the two pathways are 33% HM and 66% AM.
(13). The natural distribution of the two pathways generally follows this proportion; however, some notable exceptions have been reported in the literature. Recent work (14) indicated that the relative contributions of the methanogenic pathways in WCA-2A may be related to nutrient status in the Everglades; relatively greater HM is observed in nutrient impacted soils, and the predicted proportions are observed in the nutrient unimpacted soils.

Various mechanisms have been suggested for those cases in which the relative contributions of the HM and AM pathways deviate significantly from those predicted by stoichiometry, including soil depth (15, 16), nutrient type (17), seasonal conditions (18), pH (19, 20) and vegetation type (21). However, competition between methanogens and other functional groups of microorganisms has not been extensively studied as a possible mechanism responsible for shifts in the relative proportions of HM versus AM pathways.

Methanogens may compete for substrates such as acetate and H$_2$ with microorganisms that use more energy-yielding alternative terminal electron acceptors such as SO$_4^{2-}$, Fe(III), NO$_3^-$, or O$_2$.

Competition between sulfate reducing prokaryotes (SRP) and methanogens for the common substrates acetate and H$_2$ has been well-documented in various environments, including marine sediments (22, 23), freshwater sediments (24, 25), and bioreactors (26); however, little attention has been given to the possibility that this competition may impact the dominant methanogenic pathway. The interactions between SRP and methanogens may be complex, depending on the availability of both electron donors and acceptors. The interaction may be competitive when sufficient sulfate is available to serve as terminal electron acceptor for SRP; however, it may be more likely
to be cooperative via a syntrophic relationship when \( \text{SO}_4^{2-} \) is limiting (27, 28). \( \text{SO}_4^{2-} \) has been recognized as an important contaminant in the northern WCAs (29). Hence, the SRP-methanogen interaction may be a crucial factor determining the methanogenic pathway in the WCAs.

The objective of this research was to determine the distribution and population dynamics of methanogens and SRP across nutrient gradients in WCAs, and to evaluate the potential interactions between SRP and methanogens as a driving force shaping methanogenic community structures and pathways. For this study, we selected sites distinct from each other with respect to the concentrations of P and \( \text{SO}_4^{2-} \), key geochemical parameters that have been shown to methanogenesis and \( \text{SO}_4^{2-} \) reduction in Everglades wetland soils (9, 10, 29). This study extends our knowledge of mechanisms related to methane production, and of the interplay between methanogens and SRP in freshwater wetlands.

**MATERIALS AND METHODS**

**Sampling and sample processing.** Replicate soil cores (≥3 cores for each site within approximately 25 m²) were obtained from sites F1, F4 and U3 within WCA-2A (in October 2009, April 2010, August 2011, and January, August, and December 2012), and site W3 within WCA-3A (in February 2012, and March and April 2013). Soil cores were sectioned to an interval of 2 cm or 4 cm from the top after removing floc to minimize inclusion of the major \( \text{O}_2 \) interface regions. A portion of soils (approximately 50 to 100 grams) was immediately frozen on dry ice and transported to the laboratory in
Gainesville where the soils were stored at -80°C until the isolation of nucleic acids or geochemical analysis. The remaining soils were used for incubation studies within one week. In addition, 5 to 10 liters of surface water were collected from each site in 10-L polypropylene bottles, which served as the source water for incubation studies. Pore waters were collected from the each sampling location and stored as described by Holmes et al. (14).

Nucleic acid isolation, PCR, clone library construction, and sequence analyses. Soil DNA was isolated from 0.2 gram (wet weight) of soils using Power Soil DNA isolation kits (MoBio Laboratories, Carlsbad, CA). Total RNA was isolated from 2.0 grams of soil using MoBio PowerSoil total RNA isolation kit. Residual DNA in RNA extracts was removed using the MoBio RTS DNase Kit. RNA was converted to cDNA using the SuperScriptIII First-Strand Synthesis SuperMix including random hexamers as reverse transcriptase PCR primers (Invitrogen, Carlsbad, CA). Nucleic acids were stored at -80°C until use.

Clone libraries were constructed for analysis of the methyl coenzyme-M reductase gene, *mcrA*, and the dissimilatory (bi)sulfite reductase gene, *dsrB*. Gene *mcrA* was amplified in soil DNAs from sites F1, F4 and U3 (sampled in October 2009, 0 to 2 cm depth) using primers mlas/mcrA-rev as previously described by Steinberg and Regan (30). Reverse transcription PCR (RT-PCR) was performed to amplify *dsrB* from cDNA derived from F4 soils (August 2012, 0 to 4 cm depth) using primers DSRp2060F/DSR4R as described by Foti et al. (31). The PCR products were cloned, and subsequently transformed into *Escherichia coli* TOP10 TOPO TA cloning kit for sequencing.
The transformants were randomly selected on Luria-Bertani (LB) agar plates containing kanamycin (50 µg·mL\(^{-1}\)) and sent to the University of Florida Sequencing Core Laboratory (http://www.biotech.ufl.edu/) for sequencing of inserts. All DNA sequences determined in this study were converted \textit{in silico} into corresponding amino acids in BioEdit v7.1.3 (32). For phylogenetic analysis, reference sequences of \textit{mcrA} or \textit{dsrB} were collected, with a high similarity on BLAST searching results against sequences in NCBI database (http://www.ncbi.nlm.nih.gov/). References were also obtained from a variety of taxa in website FunGene (http://fungene.cme.msu.edu/), and from environmental sequences in previously published literature. Selected sequences representing OTUs with 5% difference in amino acid sequences were pooled with the reference sequences, and aligned with ClustalX v.2.0 (33). The alignment was used as input file for phylogenetic analysis in MEGA v.5.2 (34). The phylogenetic tree was constructed using the Maximum Likelihood method with Bootstrap analysis (1,000 re-samplings).

The deduced amino acid sequences were assigned to operational taxonomic units (OTUs) based on the per cent differences between the sequences (e.g., 1%, 5%, 10%) using the furthest-neighbor method in the program Mothur v.1.31.2 (35). The Mothur program was also used to estimate diversity of OTUs, coverage of OTUs sampled within each clone library, and to create a Venn diagram showing the shared number of OTUs between clone libraries. Fast UniFrac online analysis (36) was performed for a principal coordinates analysis (PCoA), phylogenetic test (P-test) (37), UniFrac significance test (38), and hierarchical cluster analysis (38).
(RT-)qPCR for mcrA. Copy numbers of mcrA copies were estimated using qPCR with the universal primer set targeting total methanogens, mlas/mcrA-rev (39). Three forward primers MM-F (5′-CAA GTW YGG MGG ATT CGC CAA GG-3′), MST-F (5′-CAA GTW YGG MGG ATT CGC CAA GG-3′) and MB-F (5′-AAG CAC CWA ACA MCA TGG AHA CHG T-3′) were designed to enumerate groups Methanomicrobiales (MM), Methanosarcinales (MST) and Methanobacteria (MB). The conserved sequence region for each group was used for the primer design (Fig. S2). These forward primers made a pair with the universal “mcrA-rev” in PCR reactions (Table S1). The specificity of primers was verified by analysis of sequences amplified by the group specific primers. A total of 21, 28 and 26 clones were randomly selected from clone libraries constructed from PCR products using the primers MM-F, MST-F and MB-F combined with mcrA-rev, respectively. All sequences from selected clones were matched to the target groups. All qPCRs were conducted using iQ SYBR green supermix (Bio-Rad Laboratories, Hercules, CA) in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). The reaction mixture contained 10 μl of iQ SYBR Green supermix, 2 μl of primers (each, 10 pmol·μl⁻¹), and 2 μl of DNA (cDNA for RT-qPCR) in a 20 μl of final volume. qPCR cycling parameters were: 3.5 min at 95°C, followed by six cycles of touchdown PCR (30 s at 95°C, 45 s with a 1°C per cycle decrement from 60°C to the final annealing temperature, 30 s at 72°C) and 34 cycles of main PCR (denaturation at 95°C for 30s, annealing at 55°C for 45 s, extension at 72°C for 30 s, image capture at 80°C for 15s, and final extension at 72°C for 7 min). All qPCR runs included an image capture step (15 seconds at 80°C) after a final extension step of each cycle, and a melt...
curve analysis (increasing the temperature from 60 to 95°C in 0.5°C increments every 10 seconds) when the PCR amplification was completed.

Copy numbers of \( dsrB \) were estimated using primers DSRp2060F/DSR4 under the cycling conditions described by Foti et al. (31). The reaction mixture was prepared as described above for \( mcrA \)-qPCR, except for using the \( dsrB \) primers.

For all (RT-)qPCRs, each soil (c)DNA was applied in triplicate to a 96-well PCR plate with a 10-fold dilution series of standard plasmid DNA carrying the gene fragment of interest. Standard DNA plasmid was prepared by cloning the target gene fragment amplified from soil samples using the same primer set used for qPCR. The insertion of the correct gene fragments of the standard plasmid DNA was confirmed by sequence analysis. The prepared standard DNAs were stored in aliquots at -80°C and a separate standard was used for each qPCR run. The PCR efficiency (E) was calculated using the formula \( E = -1 + 10^{(-1/slope)} \). PCR efficiency measured using standard plasmid DNAs under the above described condition for \( mcrA \) and \( dsrB \) was 93.4 to 96.7% (Table S2).

Soil incubation experiments. Several soil incubation experiments were conducted to measure methane production rates, sulfate reduction rates, and the isotopic compositions of \( CH_4 \) and \( CO_2 \). Each treatment was conducted in triplicate. All incubations were performed at 28°C in the dark. Bottles were shaken by inversion a few times every one or two days (for \( CH_4 \) production) or on a shaking incubator at 125 rpm (for \( SO_4^{2-} \) reduction rate).

(i) \( CH_4 \) production rate. Ten grams of surface soil (0 to 4 cm depth) were mixed with 10 ml of site water in triplicate 60-mL serum bottles closed with rubber stoppers and
aluminum seals. The headspace gas of the bottles was exchanged by flushing N\(_2\) through syringe needles for 10 min. Bottles were supplemented with 4 mM acetate or a H\(_2\)-CO\(_2\)
mixture (80\%:20\%, v:v) to 50 kPa. Bottles with no substrate addition were used as controls. CH\(_4\) concentration was analyzed on days 3, 7 and 14, as described below.

(ii) SO\(_4^{2-}\) reduction rate. Sulfate reduction rates were measured according to previously described methods (40, 41) with a slight modification as described by Castro et al. (42). Sulfate reduction rates were calculated according to Fossing and Jørgensen (43).

(iii) isotope composition of CH\(_4\) and CO\(_2\). Ten grams of soils were incubated with 10 ml surface water in 60-mL serum bottles closed with a rubber stopper and an aluminum seal. MoO\(_4^{2-}\) (20 mM) was added to half of the incubations vials. Headspace gas was taken on incubation days 5, 8, and 12 to measure the CH\(_4\) production rates. The stable isotopes of CH\(_4\) and CO\(_2\) were determined as described below with the gas samples taken on day 12 for F1 and U3, F4 vials were sampled on day 27 because there was not enough CH\(_4\) in the headspace to measure isotopically until then.

(iv) potential syntrophic acetate-oxidizing (SAO) activity. Potential SAO activity was measured as described by Hori et al. (44). Briefly, 5 grams of soil were anaerobically incubated with 15 ml surface water in 60-mL serum bottles with acetate labeled with \(^{13}\)C at either the C1 or C2 position, or both C1 and C2 (Sigma Biochemicals), to a final concentration of 0.5 mM in separate incubations. After 6 days of incubation (12 days for U3 soils), the concentrations of \(^{12}\)C- and \(^{13}\)C-CH\(_4\) in headspace gases were analyzed as described below. Briefly, this method is based on the fact that methane produced by the syntrophic acetate oxidation and acetoclastic pathways yield methane from carbons at different positions in the acetate molecule.
Analytical methods. Total phosphorus, total carbon (TC), and total Kjeldahl nitrogen (TN) concentration was determined according to methods described by Wright and Reddy (45).

CH₄ concentrations in soil incubation and porewaters were measured from the headspace using a Shimadzu 8A Gas Chromatography equipped with a Carboxen 1000 column (Supelco, Bellefonte, PA) and a flame ionization detector operating at 110°C as described previously. The CH₄ concentration in the aquatic phase was calculated using Henry’s law constant for CH₄ (1.3×10⁻³ [mol·L⁻¹·atm⁻¹ at 298 K]) (46).

H₂ concentrations in porewaters were measured from the headspace of bottles using a PEAK Performer 1 gas analyzer (Peak Laboratories, Mountain View, CA) with a reducing compound photometer. The H₂ concentration in aquatic phase was calculated using Henry’s law constant for H₂ (7.8×10⁻⁴ [mol·L⁻¹·atm⁻¹ at 298 K]) (46).

Acetate was derivatized with 2-nitrophenylhydrazide, and the derivative was separated by the HPLC system (Waters 2695 Waters Corp., Milford, MA) equipped with a Platinum EPS C₈ column (1.6 × 250 mm) (Alltech, Deerfield, Ill) under gradient profile composed of two mobile phases as described by Albert and Martens (47). The derivative was detected at 400 nm with a UV absorbance detector (Waters 2487, Waters Corp).

The composition of δ¹³C-CH₄ and δ¹³C-CO₂ in the headspace of incubation bottles was determined using a Finnigan Mat Delta V isotope ratio mass spectrometer coupled to a gas chromatograph as described by Merritt et al. (48).

Statistical analyses. Significances in differences of gene copies, microbial activities and chemical data between study sites or treatments were computed using one-way analysis
of variance (ANOVA) or Student’s t-test with JMP 10 (SAS Institute Inc., Cary, NC, USA), followed by post hoc Tukey-Kramer honestly significant difference (HSD) test for ANOVA. P values <0.05 were considered significant. For exploring the relationships between variations in gene copy numbers and geochemical parameters, a redundancy analysis (RDA) was implemented in Canoco version 4.5 for Windows (49). The statistical significances of axis and individual parameters were evaluated using a Monte Carlo permutation full-model with 999 unrestricted permutations.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for sequences determined in this study are KR075171 to KR075423 for mcrA, and KR075424 to KR075502 for dsrB transcript sequences.

**RESULTS**

**Site descriptions and biogeochemical characteristics.** Sites F1, F4 and U3 are located within WCA-2A of the northern Everglades (Fig. S1), where agricultural drainage from EAA has resulted in a north to south gradient in soil P concentrations (50). The soil P concentrations in samples used for this study were 1.28 g·kg⁻¹ in F1 the northern-most site, 0.59 g·kg⁻¹ in F4, and 0.25 g·kg⁻¹ in U3, the southernmost site (Table 1). These values are in good agreement with previously reported results (42, 51). In contrast to the soil P concentration gradient, a sharp gradient in SO₄²⁻ concentrations was not observed in surface waters (176 to 200 µM) and soil porewaters (39 to 56 µM) (52). In WCA-2A, acetate concentrations in porewaters varied from 0 to 76 µM, and dissolved H₂
concentrations were from 0.08 to 0.60 µM (Table 1), with no significant differences observed between sites due to the high variation among samples.

Site W3 is located in the interior region of WCA-3A (Fig. S1), such that it is removed from the direct influence of surface water discharges. The site harbors relatively low soil P concentrations (0.34 g·kg⁻¹), similar to U3 in WCA-2A; however, this site is distinguished from the WCA-2A sites by much lower SO₄²⁻ concentration (≤4 µM) in surface waters and porewaters (52). W3 is therefore valuable for comparison with the WCA-2A sites. The sites selected for this study are distinct from each other with respect to the concentrations of soil P, and surface water SO₄²⁻: F1 (high P, high SO₄²⁻), F4 (intermediate P, high SO₄²⁻), U3 (low P, high SO₄²⁻), W3 (low P, low SO₄²⁻).

**Diversity and distribution of McrA sequences.** A total of 255 mcrA sequences were obtained from three clone libraries derived from F1, F4 and U3 soils in WCA-2A, and were translated *in silico* to amino acid sequences (henceforth referred to as McrA sequences) for further analysis. The McrA sequences were grouped into 96 OTUs defined by a 5% difference in amino acid sequences. The Chao1 richness estimator predicted the presence of 58 to 74 OTUs, with the highest number observed for the F1 sequences. The Shannon diversity indices (3.4 to 3.6) did not differ significantly between sites. Coverage statistics indicated that 71 to 78% of OTUs in each library were accounted for in this study (Table S3).

The 96 OTUs were distributed within the orders *Methanomicrobiales* (MM), *Methanosarcinales* (MS), *Methanocellales* (MCEL), *Methanobacteriales* (MB), and a *Methanomassiliicoccales* (MSC), which is related to recently isolated
Methanomassiliicoccus luminyensis B10 (53), and to Candidatus Methanomethylophilus alvus Mx1201 retrieved from genomes derived from a human gut (54) (Fig. 1). Detailed phylogenetic affiliation for OTUs is provided in Table S4 of supplementary materials.

Order MM is composed of 50 OTUs, including 144 sequences (accounting for 56% of total sequences). Of the MM OTUs, 24 OTUs clustered within the family Methanomicrobiaceae (subclades MM-I [9 OTUs] and II [15 OTUs]), and 13 OTUs within the Methanoregulaceae (MM-V [6 OTUs] and MM-VII [7 OTUs]). The remaining 13 OTUs were divided into novel clades MM-III (2 OTUs), MM-IV (7 OTUs) and MM-VI (4 OTUs), which did not include previously taxonomically defined methanogens. Order MS comprised 24 OTUs including 60 sequences (accounting for 25% of total sequences), of which 22 OTUs were assigned to Methanosetaetaceae (MST) and 2 OTUs to Methanosarcinaceae (MSR). Order MB comprised 8 OTUs, including 20 sequences (7.8% of total sequences), and was present in all sites as a minor group (≤10.4%). One mrtA sequence (MB-I) was found in the F1 library, which is usually detected in MB as an isozyme of McrA (55). Methanocellales (MCEL) included 4 OTUs as a minority (≤5.7%) in libraries F1 and U3, with no sequence from F4. Group MMC includes 9 OTUs from libraries F1 (accounting for 6.9% of total F1 sequences) and U3 (14.9% of total sequences), which was further divided into subgroups MMC-I and MMC-II including strains B10 and Mx1201.

The differences in McrA assemblages between F1, F4, and U3 were clearly illustrated in the compositions and relative abundances (%) of subclades for each site as depicted in the stacked column graph of Fig. 1. Of 96 OTUs, only 4 OTUs were shared between the three sites, and fewer than 15 OTUs were shared between two sites (see
Venn diagram in Fig. S3). Significant differences between assemblages were statistically verified \((P \leq 0.001)\) by P-test (37) and Unifrac Significant test (38) in Unifrac implementation. In a scatter plot of Unweighted PCoA, all assemblages were clearly separated by either P1 (explaining 62.57\% variation) or P2 (37.43\% variation), which is in good agreement with the largely separated assemblages represented in a hierarchical tree (Fig. S3).

**qPCR enumeration of mcrA and dsrB copies.**

(i) mcrA copy numbers. The numbers of *mcrA* copies were estimated for total methanogens, and for groups MM, MST and MB. Since WCA-2A *mcrA* sequences are largely unique and diverse, new primers MM-F, MST-F and MB-F were designed to enumerate these groups. Degenerative primer MM-F targets major groups of MM included in subclades MM-II, III, IV and V, which include 73\% of WCA-2A-MM sequences (Fig. 1). Primer MST-F targets MST sequences on branches that include 70\% of the WCA-2A-MST sequences, with the exception of one branch closely related to *Methanoseta harundinacea*. Primer MB-F targets MB, and its sequence was highly matched to all the WCA-2A-MB sequences. Even though there was some variation among the sites, the relative proportions of sequences targeted by MM-F, MST-F, and MB-F were consistent with the proportions of MM, MST and MB in the order MM > MST > MB for each clone library (Fig. 1). Therefore, qPCR using these primers is believed to be appropriate for estimating the relative number of target groups within and between sites.

qPCR for *mcrA* was conducted for long term soil sampling in different seasons.
during 2009 to 2013 (Table S5). At each time point three sub samples were taken and then averaged for analysis. A one-way ANOVA blocked by time was run for each of the sites and for the different response variables. When significant differences in genes were found, a Tukey's HSD was used determine which genes were different (Fig. S4).

qPCR for groups MM, MST and MB revealed that MM and/or MST are dominant in all WCA sites; however, MM is dominant in WCA-2A and MST is dominant in WCA-3A. Within all WCA-2A sites, MM mcrA copies (2.2 to 9.6×10⁸⋅g soil⁻¹ on average) were higher than MST mcrA copies (1.7 to 5.7×10⁸⋅g soil⁻¹ on average) (Fig.2 and Table S5). A one-way ANOVA blocked by time revealed that MM copy numbers were higher than MST copies in site F1 (P<0.05), but were not significantly different in other sites, due to the temporal variation within each site (Fig. 2, lower panel). Comparison of percentages for each group within individual samples [(one group’s copies)/(total of MM+MST+MB copies)×100] more clearly showed that MM% was significantly higher than MST% (Tukey-Kramer HSD test, P<0.0001) in all the WCA-2A sites (Fig. 2). In contrast, site W3 exhibited that MST outnumbered MM in both the absolute number of mcrA copies (7.8×10⁷⋅g soil⁻¹ vs 1.9×10⁷⋅g soil⁻¹ on average) and the relative percentage (73.9±11.6% vs 14.2±9.0% [mean±sd]) in significant (P<0.0001 for both comparisons).

(ii) dsrB copy numbers. The dsrB copies estimated in WCA soils were from 5.1×10⁷ to 4.9×10⁹⋅g soil⁻¹ (Fig. S5 and Table S5). WCA-2A sites showed a significant variation in dsrB copies along seasons (one-way ANOVA, P<0.001 within each site), while site W3 in WCA-3A did not (one-way ANOVA, P=0.8855) (Fig. S5). In all WCA-2A sites, the highest dsrB copies were obtained in January, while the lowest copies were observed in
April.  Comparison of the pooled temporal data from each sites indicated that \textit{dsrB} copies were significantly different among sites (one-way ANOVA, \(P<0.001\)) (Fig. S5).  Site F1 had the highest number (1.88\(\times\)10\(^9\) g soil\(^{-1}\) on average), followed by F4 (1.52\(\times\)10\(^9\) g soil\(^{-1}\) > U3 (6.72\(\times\)10\(^8\) g soil\(^{-1}\) > W3 (8.55\(\times\)10\(^7\) g soil\(^{-1}\)).  

The \textit{dsrB} copy numbers were significantly higher than T-M \textit{mcrA} copies in WCA-2A sites (Student’s t test, \(P<0.05\)), but were not significantly different in W3 (Student’s t test, \(P=8.86\)) (Fig. 3).

Relatedness of \textit{mcrA/dsrB} copies with geochemical parameters.  Redundancy Analysis (RDA) was performed to evaluate the relationships between the abundances of methanogens and SRP with geochemical concentrations in the WCAs.  T-M copy numbers were positively correlated with TP concentrations (Fig. 4). \textit{dsrB} copy numbers were also positively correlated with SO\(_4^{2-}\) and TP concentrations.  The positive correlations of T-M and \textit{dsrB} copy numbers with those geochemical parameters were due to the elevated numbers at higher P and SO\(_4^{2-}\) concentrations, in the order of F1 > F4 > U3 > W3 as described above.

RDA was also applied to the relative proportions of MM, MST and MB within each sample to observe the relationships between the compositions of these methanogenic groups with geochemical factors.  MM\% was positively correlated with TP and SO\(_4^{2-}\) concentrations, while MST\% and MB\% were negatively correlated with those parameters.

Gene expression level of \textit{mcrA} and \textit{dsrB}.  In order to assess the extent to which the genes were transcribed, RT-qPCR for \textit{mcrA} and \textit{dsrB}, and potential activity for
methanogenesis and sulfate reduction (to indirectly measure the enzyme level of corresponding genes) were determined in WCA-2A site samples collected in August 2012. The mcrA mRNA copies averaged as follows: $1.7 \times 10^7 \cdot g$ soil$^{-1}$ in F1 > $1.1 \times 10^6$ in F4 > $9.3 \times 10^4 \cdot g$ soil$^{-1}$ in U3. dsrB mRNA copies were $1.6 \times 10^6$ in F1 > $9.0 \times 10^4$ in F4 > $5.9 \times 10^4 \cdot g$ soil$^{-1}$ in U3. The copies of mRNAs were 2 to 4 orders of magnitude lower than gene copies; however, they followed the order of the gene copy numbers according to the SO$_4^{2-}$ and P gradient, i.e., F1 > F4 > U3 (Fig. 5). Likewise, cDNA copy numbers from MM, MST and MB were consistent with the relative abundances of gene copy numbers within sites and among the sites.

Potential methane production rates were $3.1 \, \mu$mol$\cdot g$ soil$^{-1}$$\cdot$d$^{-1}$ in F1 soils > $0.9 \, \mu$mol$\cdot g$ soil$^{-1}$$\cdot$d$^{-1}$ in F4 soils > less than $0.1 \, \mu$mol$\cdot g$ soil$^{-1}$$\cdot$d$^{-1}$ in U3 soils (Table 1).

Potential SO$_4^{2-}$ reduction rates were $0.5 \, \mu$mol$\cdot g$ soil$^{-1}$$\cdot$d$^{-1}$ in F1 soils, which were higher than those measured for F4 and U3 soils ($\leq 0.03 \, \mu$mol$\cdot g$ soil$^{-1}$$\cdot$d$^{-1}$). These potential activities for CH$_4$ production and SO$_4^{2-}$ reduction were in good agreement with the order of the levels of genes mcrA and dsrB copies measured along nutrient gradients (F1 > F4 > U3); hence, the gene copies measured throughout this study are likely to predict the degree of gene expression level in each site.

**Impact of SRP activities on methanogenesis.** Soil incubation studies were conducted to evaluate potential influence of SRP activities on methanogenic pathways, and correspondingly on shaping of methanogen assemblage structure. The incubation was done using the relatively high SO$_4^{2-}$ WCA-2A soils in the presence or absence of MoO$_4^{2-}$, a specific inhibitor of SRP activity. Soil incubation studies showed higher methane production rates in the presence of MoO$_4^{2-}$ compared to the absence of the inhibitor. These findings support the hypothesis that SRP activities play a crucial role in regulating methanogenesis in these soils.
production rates in MoO$_4^{2-}$ treatments (specific inhibitor of SRP activity) for all sites (Fig. 6A): 1.9 vs 1.3 µmol g$^{-1}$ d$^{-1}$ in F1, 1.3 vs 0.6 µmol g$^{-1}$ d$^{-1}$ in F4, 0.5 vs 0.05 µmol g$^{-1}$ d$^{-1}$ in U3 (Fig. 6A), indicating that methanogens and SRP are in competition for common substrates.

MoO$_4^{2-}$ treatment resulted in increases of the $\delta^{13}$C of CH$_4$: -67‰ to -55‰ in F1 and -76‰ to -57‰ in F4. The small amount of CH$_4$ produced in U3 soil incubations without added MoO$_4^{2-}$ prohibited measurement of the $\delta^{13}$C, but in Mo treated incubations, U3 $\delta^{13}$C -CH$_4$ was -57‰ (Table 2, Fig. 6B). In contrast, there was no significant difference in $\delta^{13}$C-CO$_2$ observed between controls and MoO$_4^{2-}$ treatment. The apparent fractionation factor ($\alpha_{app}$) quantifies the isotopic difference between CH$_4$ and CO$_2$, and is a generally accepted index to estimate the contribution of a particular methane production pathway to a methane pool (56, 57). MoO$_4^{2-}$ treatment reduced $\alpha_{app}$ from 1.054 to 1.040 in F1 and 1.060 to 1.041 in F4, indicating a shift toward the acetoclastic pathway.

Inhibition of SRP activity using MoO$_4^{2-}$ resulted in an increase in the relative percentage of mcrA transcript copies of MST from 27 to 43% in F1 soils, and from 48% to 55% in F4 soils (Fig. 7), consequently reducing the relative percentage of MM mcrA transcripts. With MoO$_4^{2-}$ treatment, MB increased in relative percentage, from 3 to 9% in F1 soils, and from 1 to 2% in F4 soils, even though this group still appeared as a minor group. The changes in the mcrA transcript level in U3 soil incubations was not accurately determined due to low RNA recovery from the incubated soils.

DISCUSSION
The nutrient gradient in WCA-2A soils provides an excellent opportunity to study the impacts of nutrient additions to naturally P-poor wetlands. A very large body of work has been published on changes that the P additions have brought to the greater WCA-2A ecosystem, ranging from the distribution of endangered vertebrates to changes in biogeochemical cycling (49, 58). The present study builds on previous studies on the distribution and function of methanogenic and sulfidogenic guilds (10, 42, 51), and investigates controls on methanogenic pathways as a function of position along the nutrient gradient.

Methanogenic assemblage structure based on mcrA sequences revealed distinct features reflecting the nutrient status of WCA-2A. One of the key features is the numerical dominance and diversity of the hydrogenotrophic MM. MM sequences accounted for >49% of total sequences retrieved from WCA-2A soils, and broadly distributed phylogeny across 7 distinct clades, MM-I to MMV-II. MM has been shown to dominate in at least some acidic bogs or rice fields (30, 59), however, WCA-MM members are distinct from MM sequences referred to as to the “Fen Cluster” (60) or "Rice Cluster” (55) (Fig. 1).

Another feature is that the acetotrophic order MS was dominated by MST (≥95% of MS sequences). In general, MST exhibits a low threshold for acetate (61); WCA-2A porewaters harbor low concentrations of acetate (<0.03 mM) (Table 1), as would be expected for a system dominated by MST. The low concentrations of acetate might result from SRP activities competing for acetate, thereby selecting for this type of acetotrophic methanogen in WCA-2A. Along with MM (49% to 65%), MST (22% to
is a dominant methanogenic group in WCA-2A, such that these two groups play a significant role in determining the pathways of methanogenesis in WCA-2A.

Recently, Holmes and colleagues (14) reported that AM is the dominant pathway (50% to 75%) over HM (25% to 50%) based on the differences in CH₄ production rates in soils with and without incubation under methyl fluoride (an inhibitor of AM) and the δ¹³C-CH₄ and δ¹³C-CO₂ values in pore waters collected from the same sites used in this study. Those results appear not to be consistent with our observation that MST was outnumbered by MM in all sites of WCA-2A. This paradoxical result might be explained by the higher free energy of formation in HM (4 H₂ + CO₂ → CH₄ + 2H₂O: ΔG°′ = -135 kJ·mol CH₄⁻¹) compared with AM (CH₃COOH → CH₄ + CO₂: ΔG°′ = -33 kJ·mol CH₄⁻¹) (62); which allow HM to produce higher biomass even if less CH₄ is produced by this pathway. We did not calculate ΔG for these reactions in situ, however, and there may be alternative explanations for these observations. Most hydrogenotrophs are able to grow with additional substrates (e.g., formate, methyl amines, methanol) other than H₂ and CO₂. For example, the numerically dominant group in WCA-2A, MM, utilizes acetate as a carbon source even though it does not use it for methanogenesis (63).

Even though AM is the dominant methanogenic process overall in sites of WCA-2A, Holmes et al. (14) reported that HM became relatively more important at site F1, amounting to almost 50% of the total methane produced. These results are relatively consistent with the group specific qPCR results reported here, where MM accounted for 60.3% in F1 > 58.6% in F4 > 55.0% in U3, consequently decreasing MST%, F1 (35.7%) < F4 (38.6%) < U3 (42.1%).
One of primary aims of this study is to evaluate the influence of SRP activities on the methanogenic pathways and the methanogen community as controlling forces in response to nutrient gradients within the WCAs. The qPCR results for MM and MST across the SO$_4^{2-}$ gradients provide evidence that SRP activity is likely involved in shaping methanogen assemblage structure and activity. In our long-term monitoring, WCA-2A sites representing SO$_4^{2-}$ rich environments consistently showed a dominance of hydrogenotrophic MM (58% on average) over the acetotrophic MST (39%), while site W3, representing a SO$_4^{2-}$ poor environment, revealed the opposite relationship (MM [14% on average], MST [74%]) (Fig. 2 and Table S5). An RDA plot shows the positive correlation of MM%, while MST% correlated negatively with SO$_4^{2-}$ concentration (Fig. 4). High SO$_4^{2-}$ concentrations might cause an enrichment of sulfidogenic SRP which typically is thought to outcompete methanogens for acetate (26, 64), consequently decreasing MST% while increasing MM%.

The soil incubation study using MoO$_4^{2-}$ as a SRP inhibitor provides evidence that SRP control, at least to some extent, the methanogenic pathways and drives an enrichment of hydrogenotrophs, specifically the MM group in WCA-2A. The addition of MoO$_4^{2-}$ resulted in increasing values of $\delta^{13}$C-CH$_4$ and lower $\alpha_{app}$ (Fig. 6B and Table 2) consistent with competition between SRP and acetotrophic methanogens for acetate. Since the production of CH$_4$ by AM is generally associated with lower $\alpha_{app}$ values and often with less negative $\delta^{13}$C-CH$_4$ values than HM (56, 65, 66), those increments imply that AM was enriched by blocking SRP activity; in other words, SRP activity most likely inhibited MST activities in these soils. The increased proportion of MST mcrA mRNA
observed in the MoO$_4^{2-}$ treatments (Fig. 7) supports this contention, which is in good agreement with the aforementioned increase in AM caused by SRP inhibition.

The specific interactions between SRP and methanogens can be quite complex. It is possible that some syntrophic fermentation of primary fermentation products such as short chain fatty acids or alcohols occurred in our incubations with MoO$_4^{2-}$, which may have contributed to the acetate used by MST. Wu et al. (67) reported that MoO$_4^{2-}$ inhibited syntrophic fermentation by SRP to varying degrees (97% for propionate; 24% for ethanol) in a wastewater bioreactor.

An additional sink for acetate and corresponding source of H$_2$ might have been via syntrophic acetate oxidation to H$_2$; however, we determined in separate experiments without MoO$_4^{2-}$ that SAO was not significant in our samples (data not shown).

It should be noted that other factors, such as differences in organic carbon quality, may also impact the relative proportions of AM and HM (68). We also expected that P concentration may be an important factor governing methanogen composition responding AM and HM, likely through increased primary productivity and carbon input to the soil. Increases in P concentrations correlated with increases in the population size of methanogens, such that a positive correlation between mcrA copies with P concentrations was observed (Fig. 4). However, the P gradient was not related to the relative compositions of hydrogenotrophs and acetotrophs to the degree as was observed for the SO$_4^{2-}$ gradient (e.g., between WCA-2A and WCA-3A). Thus, sulfate concentrations and the activities of SRP appear to be the most dominant controllers of methanogenic pathway in the WCAs of Everglades.
SRP are important co-inhabitants with methanogens (12, 42, 52). Our qPCR results indicated that SRP outnumbered methanogens in WCA-2A, and revealed that their numbers were similar even in WCA-3A (Fig. 2). Even though WCA-2A has higher concentrations of SO$_4^{2-}$ compared with many other freshwater marshes, such as WCA-3A, (Table 1), SO$_4^{2-}$ may not be high enough to support such high numbers of SRP relative to the numbers of methanogens in WCAs. In a recent study, we found that the syntrophic SRP belonging to *Syntrophobacterales* comprised $\geq 75\%$ of total *dsrB* transcripts found in soils of F1, U3 and W3 (52). F4 soils absent from that previous study also showed similar proportions of syntrophs in the present work ($76\%$) (Fig. S6). The high proportion of syntrophic SRPs likely explains the relatively high number of SRPs that were observed in our studies.

In conclusion, the numbers and structures of *dsrB* and *mcrA*, and their respective activities, vary with nutrient status in the Water Conservation Areas of the Florida Everglades. Depending on available SO$_4^{2-}$ concentration, SRPs are involved in controlling the methanogenic pathways, shaping methanogen assemblage structure, and in controlling the CH$_4$ emission rate and pathway.

**ACKNOWLEDGEMENTS**

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mercury methylation-related gene hgcA in the water conservation areas of the Florida


FIGURE LEGENDS

Figure 1. Maximum Likelihood [ML] tree representing the phylogeny of deduced amino acid sequences from mcrA retrieved from soils taken from sites F1, F4 and U3 in October 2009. Bootstrap values of ≥50% from 1000 re-assemblages were placed at branch points. Gray colored clades were targeted by group specific primers designed in present study. The relative percentage of clades generated from the ML tree and the clades targeted by the currently designed primers are presented in the stacked column graphs.

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Figure 3. Box and Whisker plot (upper) and temporal profile (lower) of dsrB copy numbers compared with T-M mcrA copy numbers estimated at the same sample. Data in the temporal profile was presented in order (from left) of Apr2010, Aug2011, Jan Aug and Dec2012 in WCA-2A sites; Feb2012, and Mar and Apr2013 in site W3. Error bars in bar graph represent +/- 1 SE
(n=3). Boxes and Whisker plot was constructed from the pooled data of temporal profile. Boxes indicate the medians (horizontal lines) and the lower and upper quartiles (bottoms and tops of boxes), while the whiskers show the highest and the lowest values, excluding outliers. The different letters indicate a significant difference between \( dsrB \) and T-M \( mcrA \) copy numbers (P<0.05 in Student’s t-test).

**Figure 4.** Redundancy analysis (RDA) representing the correlation among \( mcrA \) and \( dsrB \) copy numbers with geochemical parameters obtained from the samples of F1, F4 and U3 collected April 2010, and August and December 2012 and the W3 samples taken February 2012 and April 2013. Arrows pointing in the same direction indicate positive correlations and arrows pointing in opposite directions indicate negative correlations. Arrow length corresponds to variance explained by the environmental variable. The first two axes explain 88.3% of the total canonical eigenvalues, with a significant Monte-Carlo test value (P<0.05).

**Figure 5.** Copies of genes \( mcrA \) and \( dsrB \), and their transcripts measured from surface soils (0 to 4 cm depth) sampled from WCA-2A in August 2012. Error bars represent +/- 1 SE (n=3).

**Figure 6.** (A) The CH\(_4\) production rate, and (B) the composition of \( \delta^{13}\text{C}-\text{CH}_4 \) and \( \delta^{13}\text{C}-\text{CO}_2 \) produced during the incubation of soils sampled in August 2012. Error bars represent +/- 1 SD (n=3, note: the control incubation of U3 soils did not produce a detectable amount of \( \delta^{13}\text{C}-\text{CH}_4 \)). CT, control without MoO\(_4^2\); Mo, addition of MoO\(_4^2\) (20 mM).

**Figure 7.** The \( mcrA \) transcript copy numbers estimated using RT-qPCR from the incubation of F1 and F4 soils sampled in August 2012. For this RT-qPCR analysis, the RNA was isolated the
soils sampled on the same date that the gas samples were collected for the $\delta^{13}$C-CH$_4$ and $\delta^{13}$C-
CO$_2$ analysis. CT, control without MoO$_4^{2-}$; Mo, addition of MoO$_4^{2-}$ (20 mM). Error bars
represent +/- 1 SE (n=3).
TABLE 1. Summary of geochemical characteristics of soils and porewaters, and the rates of CH$_4$ production and SO$_4^{2-}$ reduction measured in the soils of WCAs.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>F1</th>
<th>F4</th>
<th>U3</th>
<th>W3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coordinates</td>
<td>26°21′39″N, 80°22′10″W</td>
<td>26°18′59″N, 80°23′01″W</td>
<td>26°17′15″N, 80°24′41″W</td>
<td>26°02′35″N, 80°49′39″W</td>
</tr>
<tr>
<td>Soil chemistry$^a$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP (g/kg)</td>
<td>1.28±0.24$^b$</td>
<td>0.60±0.26</td>
<td>0.25±0.04</td>
<td>0.38±0.02</td>
</tr>
<tr>
<td>TN (g/kg)</td>
<td>31±3</td>
<td>34±3</td>
<td>27±5</td>
<td>36±1</td>
</tr>
<tr>
<td>TC (g/kg)</td>
<td>442±31</td>
<td>424±29</td>
<td>385±69</td>
<td>424±4</td>
</tr>
<tr>
<td>Porewater chemistry$^c$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate (µM)</td>
<td>19.5±9.9</td>
<td>25.9±33.8</td>
<td>17.1±16.4</td>
<td>ND</td>
</tr>
<tr>
<td>H$_2$ (µM)</td>
<td>0.33±0.10</td>
<td>0.28±0.20</td>
<td>0.28±0.14</td>
<td>ND</td>
</tr>
<tr>
<td>SO$_4^{2-}$ (µM)$^d$</td>
<td>56±86</td>
<td>74±110</td>
<td>39±35</td>
<td>≤4</td>
</tr>
<tr>
<td>CH$_4$ production rate (µmol·g soil$^{-1}$·d$^{-1}$)$^e$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact soils</td>
<td>3.1±3.2</td>
<td>0.9±0.9</td>
<td>&lt;0.1</td>
<td>ND</td>
</tr>
<tr>
<td>Acetate</td>
<td>5.3±1.4</td>
<td>2.7±0.5</td>
<td>0.4±1.2</td>
<td>ND</td>
</tr>
<tr>
<td>H$_2$/CO$_2$</td>
<td>11.6±3.5</td>
<td>5.6±2.9</td>
<td>2.1±1.0</td>
<td>ND</td>
</tr>
<tr>
<td>SO$_4^{2-}$ reduction rate (µmol·g soil$^{-1}$·d$^{-1}$)$^e$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact soils</td>
<td>0.5±0.4</td>
<td>&lt;0.01</td>
<td>0.03±0.04</td>
<td>ND</td>
</tr>
<tr>
<td>Acetate</td>
<td>1.4±0.3</td>
<td>0.1±0.1</td>
<td>0.07±0.07</td>
<td>ND</td>
</tr>
<tr>
<td>H$_2$/CO$_2$</td>
<td>1.2±1.0</td>
<td>&lt;0.01</td>
<td>0.14±0.17</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$Soil chemistry was assayed in top soils (0 to 4 cm) sampled in April 2010, August and December 2012 from WCA-2A sites (n=9 from triplicate of each sample), and February 2012 and April 2013 from site W3 (n=6).

$^b$Mean±SD

$^c$Acetate and H$_2$ concentrations were measured in porewaters (0 to 4 cm) sampled in April 2010 and August 2011 (n=6).

$^d$SO$_4^{2-}$ concentrations for F1, U3 and W3 were adapted from our previous study (52) with additional data for site F4 analyzed from samples of April 2010, August, and December 2012 (n=9) in this study.

$^e$The rates of methane production and SO$_4^{2-}$ reduction were measured in incubations with soils (0 to 4 cm depth) sampled in August 2012. Acetate (4 mM) and H$_2$/CO$_2$ (50 kPa) were added in triplicate.

ND, not determined.
TABLE 2. $\delta^{13}$C-$\text{CH}_4$ (‰), $\delta^{13}$C-$\text{CO}_2$ (‰), and apparent fractionation factors for the incubated Everglades soils.

<table>
<thead>
<tr>
<th>Site</th>
<th>Treatment</th>
<th>$\delta^{13}$C-$\text{CH}_4$</th>
<th>$\delta^{13}$C-$\text{CO}_2$</th>
<th>$\alpha$ apparent fractionation$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>CT</td>
<td>-67.00±2.16$^c$</td>
<td>-17.02±1.20</td>
<td>1.054</td>
</tr>
<tr>
<td></td>
<td>Mo</td>
<td>-55.02±1.17</td>
<td>-17.16±0.84</td>
<td>1.040</td>
</tr>
<tr>
<td>F4</td>
<td>CT</td>
<td>-75.92±1.00</td>
<td>-20.12±0.88</td>
<td>1.060</td>
</tr>
<tr>
<td></td>
<td>Mo</td>
<td>-57.35±2.81</td>
<td>-18.92±2.28</td>
<td>1.041</td>
</tr>
<tr>
<td>U3</td>
<td>CT</td>
<td>ND</td>
<td>-17.46±0.58</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Mo</td>
<td>-56.88±1.67</td>
<td>-15.87±0.53</td>
<td>1.043</td>
</tr>
</tbody>
</table>

$^a$CT, control without MoO$_4^{2-}$; Mo, addition of MoO$_4^{2-}$ (20 mM)

$^b$%app is defined as $(\delta^{13}$C-$\text{CO}_2 + 1000)/(\delta^{13}$C-$\text{CH}_4 + 1000)$.

$^c$Mean±SD (n=3).

ND, not determined; NA, not applicable.
FIG 1. Maximum Likelihood [ML] tree representing the phylogeny of deduced amino acid sequences from \textit{mcrA} retrieved from soils taken from sites F1, F4 and U3 in October 2009. Bootstrap values of ≥50% from 1000 re-assemblages were placed at branch points. Gray colored clades were targeted by group specific primers designed in present study. The relative percentage of clades generated from the ML tree and the clades targeted by the currently designed primers are presented in the stacked column graphs.
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