Composition of Methane-Oxidizing Bacterial Communities as a Function of Nutrient Loading in the Florida Everglades

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Abstract Agricultural runoff of phosphorus (P) in the northern Florida Everglades has resulted in several ecosystem level changes, including shifts in the microbial ecology of carbon cycling, with significantly higher methane being produced in the nutrient-enriched soils. Little is, however, known of the structure and activities of methane-oxidizing bacteria (MOB) in these environments. To address this, 0 to 10 cm plant-associated soil cores were collected from nutrient-impacted (F1), transition (F4), and unimpacted (U3) areas, sectioned in 2-cm increments, and methane oxidation rates were measured. F1 soils consumed approximately two-fold higher methane than U3 soils; additionally, most probable numbers of methanotrophs were 4-log higher in F1 than U3 soils. Metabolically active MOB containing *pmo*A sequences were characterized by stable-isotope probing using 10 % (v/v) $^{13}CH_4$. *pmo*A sequences, encoding the alpha subunit of methane monooxygenase and related to type I methanotrophs, were identified from both impacted and unimpacted soils. Additionally, impacted soils also harbored type II methanotrophs, which have been shown to exhibit preferences for high methane concentrations. Additionally, across all soils, novel *pmo*A-type sequences were also detected, indicating presence of MOB specific to the Everglades. Multivariate statistical analyses confirmed that eutrophic soils consisted of metabolically distinct MOB community that is likely driven by nutrient enrichment. This study enhances our understanding on the biological fate of methane being produced in productive wetland soils of the Florida Everglades and how nutrient-enrichment affects the composition of methanotroph bacterial communities.

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Introduction

According to the Intergovernmental Panel on Climate Change, natural wetlands release between 100 and 231 Tg of methane per year, amounting to approximately 20 to 39 % of global methane emissions [12]. Of particular interest are the pathways leading to the sources and sinks of biological methane because methane is 20- to 30-fold more potent as a greenhouse gas than CO_2.

A growing body of evidence has shown that the composition and activities of the resident microbial communities and processes such as respiration [21], methanogenesis [9], and sulfate reduction [6] are linked with nutrient status in the Everglades, a large nutrient-impacted wetland in South Florida, USA. Despite nutrient-rich wetland sediments being a rich source of microbially produced methane, relatively little of this methane may be released into the environment because of the activities of methane-oxidizing bacteria (MOB) in soil [16]. This has been previously demonstrated in a wide variety of habitats ranging from the floodplain sediments of southeastern Australia [3] to the North American peatlands and the Florida Everglades [30]. Methanotrophs consume methane as their sole source of carbon and energy; the first step in the biochemical pathway is initiated by the conversion of methane to methanol by methane monooxygenase (MMO). MMO is found as a membrane-associated form (pMMO) and as a soluble, cytoplasmic form (sMMO). Because pMMO is found in all methanotrophs except for members of the genera *Methylocella* and *Methyloferula* [13, 29], and sMMO is restricted to members of relatively few genera, *pmo*A encoding the alpha

subunit of pMMO is considered to be an appropriate functional gene marker to study methane-oxidizing bacteria [18]. Methanotrophs are a diverse group at the metabolic and phylogenetic levels [15] but are broadly divided into either type I, affiliated with *Gammaproteobacteria*, or type II, affiliated with *Alphaproteobacteria*. Among this division, the type II MOB are metabolically more efficient in oxidizing methane at lower O_2 concentrations [2].

Our previous findings on the microbial ecology of the Everglades have shown shifts in microbiota that cycle carbon, including fermentative bacteria, syntrophs and methanogens [6, 8, 9, 28]. Methane formation rates are significantly higher in eutrophic soils and dominated by hydrogenotrophic methanogens. King [17] has previously shown significant methane oxidation in the Everglades peat, consuming as much as 91 % of the potential methane diffusive flux [17]; however, little is known of the types of methane-oxidizing microbiota that may be metabolically active in these environments. This work extends our previous studies that document changes in the structure and function of microbial communities as a result of eutrophication in the Everglades, further facilitating the development of sensitive indicators of ecosystem response to nutrient loading and providing greater understanding of the mechanisms through which microbial communities in the Everglades respond to nutrient enrichment.

Materials and Methods

Site Description and Sample Collection

Soil samples were collected in spring 2005 along the P gradient in the northern Florida Everglades under flooded (0.2 to 0.3 m) conditions as previously described [6–9]. Dominant vegetation at the sampling sites were visibly different such that eutrophic (F1) areas were dominated by cattail (*Typha domingensis* Pers.), oligotrophic (U3) by sawgrass (*Cladium jamaicense* Crantz), and the transition region (F4) were predominated by a mix of cattail and sawgrass. From each site, three replicate cores within an area of approximately 25 m^2 were collected, containing soils directly in touch with the predominant plant communities. Cores were stored on ice and transported to the laboratory, where sectioning was performed at every 2 cm increments until 10 cm substrata of soil were reached. Subsamples for DNA analysis were frozen at −70°C until analyzed. Samples intended for measurement of rates were kept at 4°C until analysis, which was within a week after sampling.

Potential Methane Oxidation Rates

Rates of potential methane oxidation from F1, F4, and U3 soils, in triplicate, were measured by adding 5 g soils to 25-ml serum bottles which were then immediately crimped using butyl rubber septa and aluminum seals (Bellco Glass Inc., Vineland, NJ). Microcosms were gently shaken for 2 h on an orbital shaker to release dissolved methane into the headspace. Serum headspace was then evacuated by using a syringe and then, using a gas tight syringe, 20 ml of ambient air was added back into the microcosms. To this, 3.75 ml (15 %) of CH_4 was added to initiate methanotrophy. Negative controls containing only air and methane mix were included to rule out leakage losses. Bottles were incubated horizontally on a roller at 25±1°C and CH_4 concentrations were monitored every 12 h by gas chromatography with a Shimadzu 8A GC as described previously [9]. Potential CH_4 oxidation rates were calculated after depletion of CH_4, which typically occurred within the first week. Methane oxidation rates increased linearly up to 4–5 days after which a steady state was reached most likely due to O_2 limitation. Analyses were carried out in triplicate, and average values with one standard deviation are reported. Moisture concentrations for optimum methane oxidation are known to be approximately 50 %; however, these wetland soils consisted of 60–80 % moisture (data not shown). Therefore, we also compared methane oxidation by air-dried but rewetted Everglades soils such that moisture was kept constant at 50 % with the addition of sterile pH-balanced distilled water made with diluted HCl or NaOH, respectively.

Methanotroph Enumeration

Triplicate soil samples from F1, F4, and U3 were used to establish microcosms for most probable number (MPN) determinations in 1:1 (*v/v*) mixture of air and methane as described previously [22]. A 1/10 diluted nitrate mineral salts (NMS) medium [31] was used to establish these serum bottle MPNs, consisting of 0.5 g of NH_4Cl per liter and 0.54 g of KH_2PO_4 per liter; the pH was adjusted to 7.0. Trace element solution SL10a (2 ml/L), $MgSO_4$ (final concentration, 0.2 g/L), and $CaCl_2$ (final concentration, 0.015 g/L) were added after autoclaving. These microcosms were gently shaken horizontally for 10 days. Turbidity in each serial dilution was estimated daily by measuring optical density at 600 nm. Biomass from serial dilutions that tested positive for growth were collected and again checked for growth in the presence of methane to confirm presence of methanotrophic bacteria.

Microcosms for Stable-Isotope Probing

Triplicate microcosms were established by placing 10 g soils in 60-ml serum bottles, from which the headspace was evacuated using a syringe. Water content for these samples was found to be within the same range and hence no adjustments in the moisture content were done prior to incubations. Using a gas tight syringe, 45 ml of ambient air was added back into the microcosms along with addition of 5 ml (approximately

0.5 mmol) of $^{13}CH_4$. Negative controls, with no soils, were also included to rule out leakage. All samples were incubated in the dark at 25±1°C. Headspace concentrations of CH_4 were determined daily; linear CH_4 removal rates were observed typically in the first week. The bottles were then flushed to remove $^{13}CO_2$ and spiked again with air and $^{13}CH_4$ to ensure that adequate labeling of functionally active methanotrophs had occurred.

Nucleic Acid Extraction and PCR Amplification

DNA was extracted from stable-isotope probing (SIP) microcosms using the Ultra Clean Soil DNA mega kit (MoBio, Solana Beach, CA) and evaluated by electrophoresis through a 0.7 % agarose gel with Tris–acetate–EDTA buffer; DNA concentration was also estimated by UV absorbance at 260 nm. PCR amplification of the alpha subunit of particulate methane monooxygenase gene (pmoA; approximately 470-bp fragment size), was performed by using the previously reported primer set A189 F (5′-GGNGACTGGGACTTCTGG-3′) (23) and Mb661 R (5′ GGTAARGACGTTGCNCCGG-3′) [11]. In a previous comparative analysis of three particulate methane monooxygenase primer sets (A189-A682, A189-A650, and A189-mb661), the primer set A189-mb661 demonstrated the largest retrieval of methanotroph diversity [5]. Therefore, we used the primer set A189-mb661 to obtain methanotroph diversity in this study.

The PCR mix was set up using the HotStarTaq Master Mix (Qiagen, Valencia, CA), extracted DNA (4–5 ng), and 0.5 pmol of each primer. Amplification was initiated by denaturation at 95°C for 15 min, followed by elongation steps of 92°C for 1 min, 55°C for 1.5 min, and 72°C for 1 min. This was followed by a final extension at 72°C for 5 min.

Separation of ^{13}C-DNA from ^{12}C-DNA

To avoid carryover of genomic DNA into the ^{13}C-DNA following ultracentrifugation, 300 ng of unlabeled Escherichia coli DNA (TOP10F' cells grown in Luria–Bertani medium) was mixed with environmental DNA prior to ultracentrifugation as described earlier [10, 23]. CsCl–ethidium bromide density gradient centrifugation was performed in a VTI 65.2 rotor at 55,000 rpm for 18 h at 20°C, as previously described [8, 10]; the lower band was extracted, purified, and concentrated. As shown in our previous reports, E. coli-specific primers were used to detect any traces of contamination; we did not detect E. coli DNA in any of the "heavier" bands, which confirmed purity of the ^{13}C DNA (data not shown).

Cloning and RFLP Analyses

Freshly amplified pmoA amplicons were ligated into pCRII-TOPO cloning vector and transformed into E. coli TOP10F'

cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Ninety-six positive clones containing the expected insert size of ~470 bp were analyzed by restriction fragment length polymorphism (RFLP) using HhaI and AluI enzymes in separate reactions and electrophoresed through a 2 % agarose gel. RFLPs were grouped according to their restriction patterns, and assigned to operational taxonomic units (OTU). Species abundance in the clone libraries were assessed by analytical rarefaction using aRarefactWin (version 1.3; S. Holland, Stratigraphy Lab, University of Georgia, Athens) (http://www.uga.edu/~strata/software), where sample sizes were represented by (n) and the expected number of species by (E) and the variance of the expected number of species by (Var) along with the upper and lower 95 and 99 % confidence limits.

DNA Sequencing and Phylogenetic Analysis

Ninety-six clones each from F1, F4, and U3 clone library were sequenced at the DNA Sequencing Core Laboratory at the University of Florida with pmoA forward primer. Sequences were compared with previously identified sequences in the National Center for Biotechnology Information database using BLAST [1], and sequences were aligned by ClustalX v. 1.8 [27]. The pmoA sequences were grouped by using a threshold of 90 % sequence identity, taking into account the current concept that defines 16 S rRNA gene sequences as belonging to the same species if they show >97 % sequence identity [25] and the 3.5-times-higher nucleotide substitution rate of the pmoA gene [16]. Phylogenetic trees were generated using deduced pmoA amino acid sequences with PAUP v. 4.0b8 using maximum parsimony algorithm with default settings (D. L. Swofford, Sinauer Associates, Sunderland, MA). Bootstrap resampling analysis for 100 replicates was performed to estimate the confidence of tree topologies. Additionally, phylogenetic trees were also constructed by the neighbor joining and maximum-likelihood algorithms which showed almost identical topology (data not shown).

Statistical Analyses

Methane oxidation rates and methanotroph cell numbers were analyzed by multivariate analyses using ANOVA (one way and two way), Tukey–Kramer multiple comparisons test, Bonferroni corrections, chi-square differences, Pearson r, and Spearman r correlation assuming data are sampled from a Gaussian population and tested for significance between F1, F4, and U3 soils with GraphPad Prism 5.0 (La Jolla, CA). Using an Excel-based program, methanotroph (pmoA) richness (H), evenness (E) and abundances were calculated according to the formula: $H' = -\sum pi\ln(pi)$, where H' = Shannon–Weaver diversity index and p_i=the

relative abundance of each group of organisms. Additionally, *pmoA* gene sequence differences between F1, F4, and U3 soils were analyzed using EstimateS (Version 8.2.0), (http://viceroy.eeb.uconn.edu/estimates). These included the abundance and incidence-based coverage estimator of species richness, Simpson's diversity index (using reciprocal logarithms), Chao 1 and 2 richness at log-linear 95 % confidence intervals, first and second-order Jackknife richness, bootstrap richness, Michaelis–Menten richness, species accumulation curves, and Fisher's alpha diversity index (the alpha parameter of a fitted logarithmic series distribution).

To further test for differences between F1, F4, and U3 soils, UniFrac [19] was applied on the *pmoA* sequences retrieved from the SIP microcosms. Analyses included *P* test, UniFrac metric test, Friedman test (non-parametric repeated measures ANOVA), and principal coordinate analyses with the scatter plot option under non-normalized conditions. These analyses were based on lineage-specific and *G* test of significance was applied to each lineage separately to determine whether species had different distributions over the nutrient gradients.

Nucleotide Sequence Accession Numbers

The partial *pmoA* sequences obtained from F1, F4, and U3 soils were deposited in GenBank under accession numbers GU735492 to GU735570.

Results and Discussion

Methane Oxidation Rates and Methanotroph Cell Numbers

Potential methane oxidation rates in eutrophic (F1) soils were at least two-fold higher compared with U3 soils. Additionally, across all soils, the highest methane oxidation was observed from the top 0–2 cm soil fraction and declined rapidly from the 8–10 cm depths (Fig. 1). In accordance with methane oxidation, time series MPNs showed that methanotroph cell numbers peaked in the F1 soils on day 3 with approximately 4-log higher (10^{11} MPN/g) cell numbers than the U3 soils (10^9 MPNs/g), which responded much slower and peaked at the ninth day (Fig. 2). Transition (F4) soil methanotrophic bacterial numbers mirrored those observed in the eutrophic soils. Methanotroph bacterial MPN in our study are higher than those reported for other systems. It is very likely that the experimental setup promoted other microorganisms to also grow in the 1/10 diluted NMS medium. In fact, Escoffier et al. [14] have shown that NMS media, found to be most suitable for growth of methanotrophs, can also support the growth of non-methanotrophic bacteria, actinomycetes, and even certain fungi.

Pearson *r* squared and Spearman r correlation analyzed on methane oxidation rates indicated a slightly better correlation

of methane oxidation rates between F4 and U3 [0.96 (upper and lower confidence limits ranged between 0.99 and 0.78)] than those measured between F1 and U3 [0.95 (upper and lower confidence limits ranged between 0.99 and 0.68)]. Typically, *R* values between 0 and 1.0 suggest that the measured variables correlate such that they tend to increase or decrease together. In other words, this indicated that if methane oxidation rates increase in F1 and F4 soils, rates will tend to increase in U3 soils as well; if rates decline in these sites, then U3 will also show reduced methane oxidation rates.

ANOVA values on methane oxidation rates between F1 and U3 were <0.001 (upper and lower confidence limits ranged between 5.4 and 3.2); similarly F4 vs. U3 values were <0.001 (upper and lower confidence limits ranged between 4.3 and 2.1), respectively. ANOVA conducted separately on the MPN values of F1, F4, and U3 soils were consistent at the *p* value of <0.0001. Additionally, the chi-square *p* values (<0.0001) also showed significant differences in cell numbers and methane oxidation rates along the nutrient gradient of the Florida Everglades soils.

Therefore, both, methane oxidation rates and methanotroph cell numbers indicated that the 0–10 cm Everglades soils in close vicinity of the predominant plant communities have the propensity to rapidly sequester methane produced and released by the lower anoxic soils. Previously, we showed that composition and activities of resident microbial communities and processes such as respiration [21], methanogenesis [9], and sulfate reduction [6, 7] are heightened in the nutrient-enriched areas of the Everglades. Similar to these processes, we find that methanotrophy is also significantly enhanced as a function of nutrient loading in the Florida Everglades soils.

These results, however, should be interpreted with caution because in freshwater sediments, the activity of methanotrophs is largely restricted to a narrow layer at the oxic–anoxic interface, where methane and oxygen gradients overlap. These studies were conducted in the 0–10 cm soils of the Florida Everglades, which are thought to be permanently anoxic. It is highly likely that these soils are exposed to fluctuating environmental conditions such as diurnal and annual cycles of light and temperature that also influence other environmental variables, e.g., oxygen status and methane production. Paradigm dictates that methanotrophic bacteria cannot be metabolically active under such anoxic conditions; however, a previous study has clearly shown a large potential for aerobic methane oxidation to occur in the anoxic littoral sediments of [4].

Additionally, methane generated in the vegetated sediments can circumvent oxidation by convective flow and/or diffusion through the lacunar system (intercellular airspaces) of aquatic macrophytes [24]. Likewise, these aquatic plants also transport oxygen from the surface to the soils in their vicinity, allowing for microaerophilic "hotspots" of methane oxidation as shown in this and other studies [26, 30]. In fact, previously it has been conclusively shown that significant methane oxidation

Figure 1 Methane oxidation rates from eutrophic (*diamond*), transition (*pyramid*) and oligotrophic (*circle*) soils after incubation with 10 % (*v/v*) $^{13}CH_4$. Analyses were conducted in triplicate and mean values are presented; *error bars* represent ±1 standard deviation
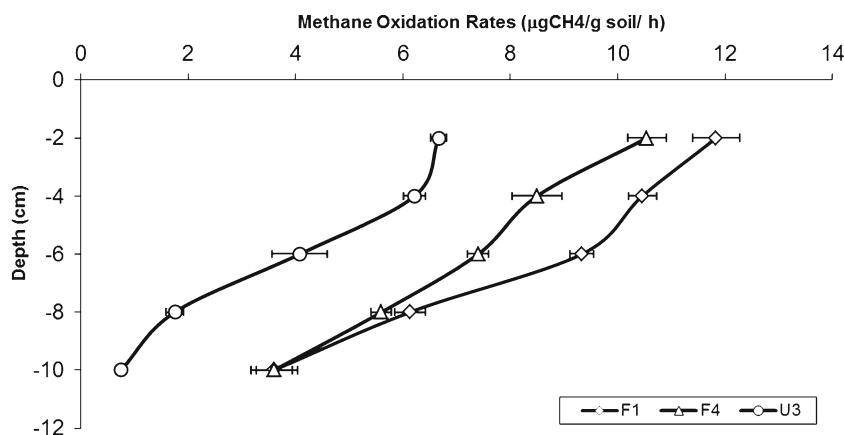
occurred in Everglades peats, consuming as much as 91 % of the potential diffusive flux and accounting for about 14–20 % of the sediment oxygen uptake [17]. Therefore, at least some portion of the MOB community in dynamic environments, such as wetlands, may be exposed to shifts from anoxic to oxic conditions on time scales of hours between darkness and daylight conditions. This explains the dramatic metabolic response of methane oxidation that we observed from the 0–10 cm soils of the Florida Everglades.

Taxonomic Affiliations of Methanotroph *pmo*A Gene Sequences

The ability to link the structure of metabolically active MOB to their function along the nutrient gradient in the Florida Everglades was provided by stable-isotope probing analysis using $^{13}CH_4$ followed by PCR cloning of the alpha subunit of particulate methane monooxygenase (*pmo*A) gene. We found that *pmo*A sequences related to type I methanotrophs were present in both eutrophic and oligotrophic soil microcosms suggesting that majority of methane is oxidized by type I methanotrophs in

these environments. Relative abundance and taxonomic analysis of *pmo*A gene sequences revealed that the most dominant sequences (63 %), retrieved from the eutrophic (F1) SIP microcosms, affiliated approximately at 86–90 % homology with *Methylobacter pmo*A sequences (Figs. 3 and 4), followed by *Methylomonas* spp. (31 %). In the transition (F4) microcosms, *Methylococcus* spp. dominated at 44 % followed by *Methylobacter* (27 %) and *Methylomonas* spp. (21 %). As shown in Fig. 4, the *Methylobacter*-like OTUs identified from this study grouped into two clades (groups I and II). Group I sequences from F1 soils clustered with *pmo*A sequence of *Methylobacter* sp. SV96, and the F4 soil group II was related to *pmo*A from *Methylomonas methanica*. A minor fraction of the F1 clone library also contained *pmo*A gene sequences from *Methylococcus* (2 %) to *Methylocaldum* (1 %). *Methylocystis* spp. were also identified from F1 soils (1 %) and F4 soils (4 %), respectively. In the oligotrophic (U3) SIP microcosms, four additional *pmo*A gene clades were identified that clustered between 86 and 90 % with *Methylocaldum szegediense* (Fig. 4). Additionally, *Methylococcus* (26 %) and *Methylobacter* sp. (3 %) were also identified in U3 SIP microcosms.

Figure 2 Most probable numbers (*MPNs*) of methane-oxidizing bacteria (MOB) in microcosms established with soils collected from *F1* (eutrophic), *F4* (transition), and *U3* (oligotrophic) soils; *error bars* represent ±1 standard deviation
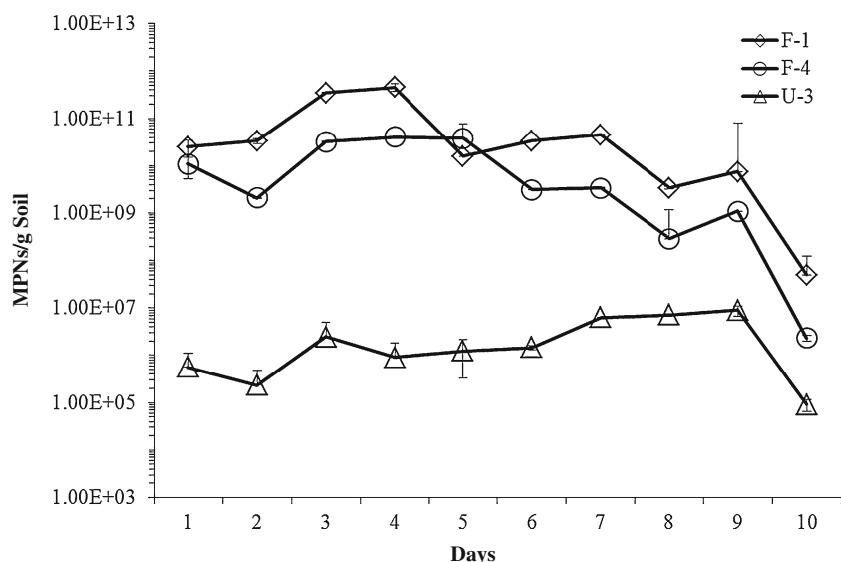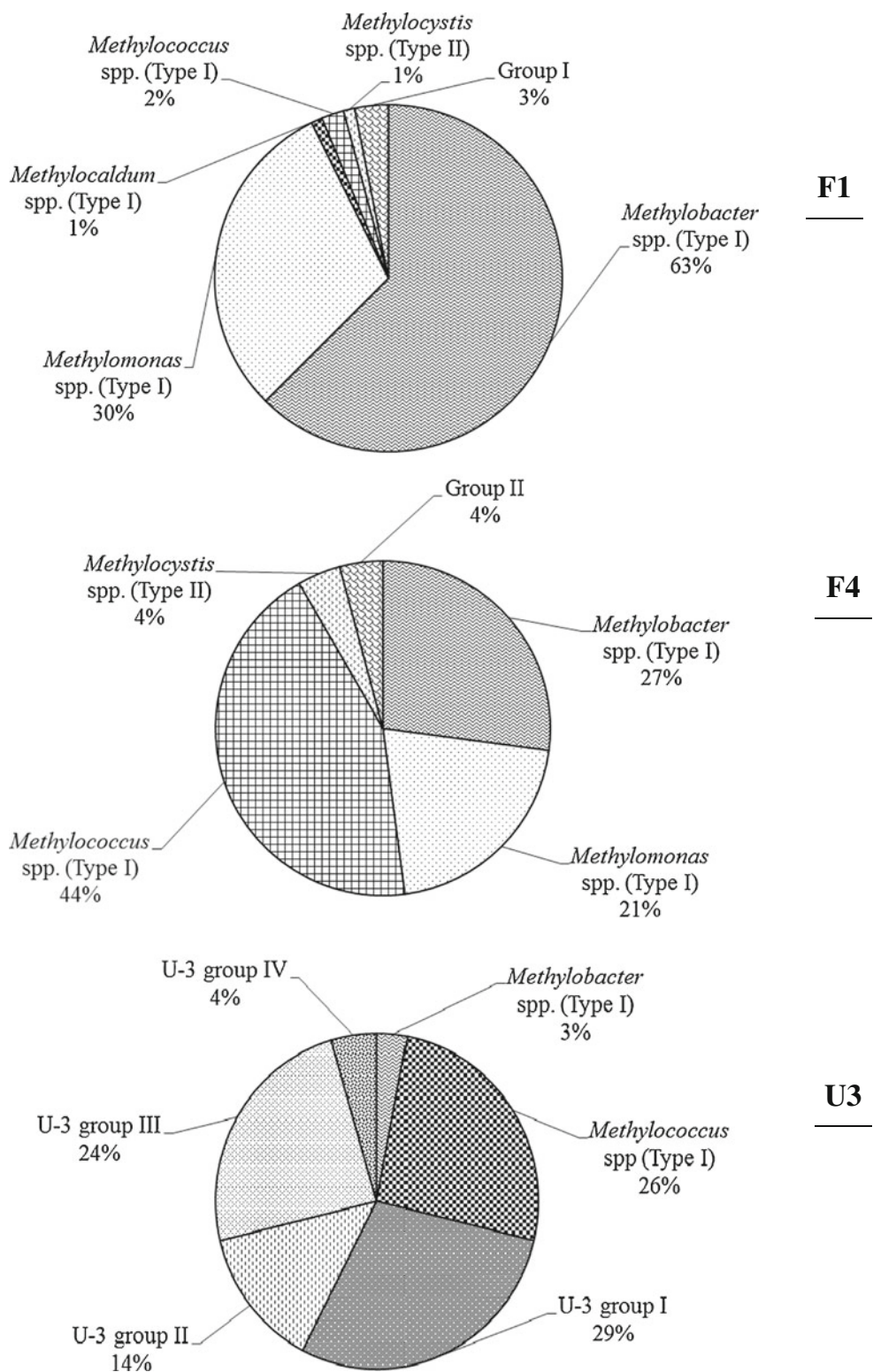
Figure 3 Relative abundance of functionally active methane-oxidizing bacteria (MOB) in $^{13}CH_4$ microcosms established with soils collected from F1 (eutrophic), F4 (transition), and U3 (oligotrophic) soils, based on frequencies of *pmoA* genes in clone libraries constructed from the [^{13}C]-DNA fractions

Analysis of derived amino acid sequences of *pmoA* genes obtained by PCR, using DNA isolated directly from the F1, F4, and U3 soils, also revealed the presence of highly divergent *pmoA* sequences that did not cluster tightly with previously characterized methane-oxidizing bacteria (data not shown). It remains unclear at this time if the group I–VI *pmoA* sequences represent novel lineages specific to the Florida Everglades. It is however
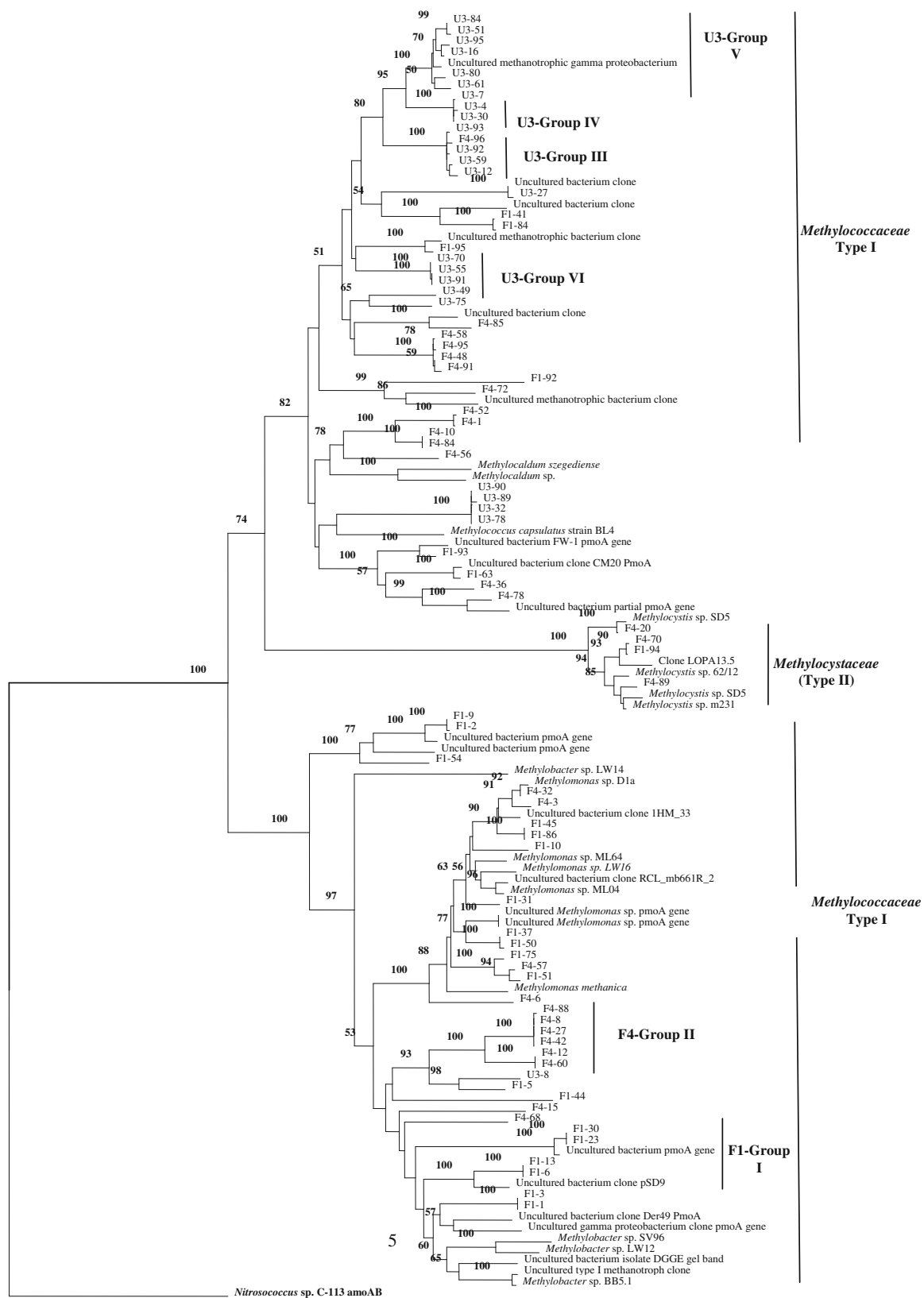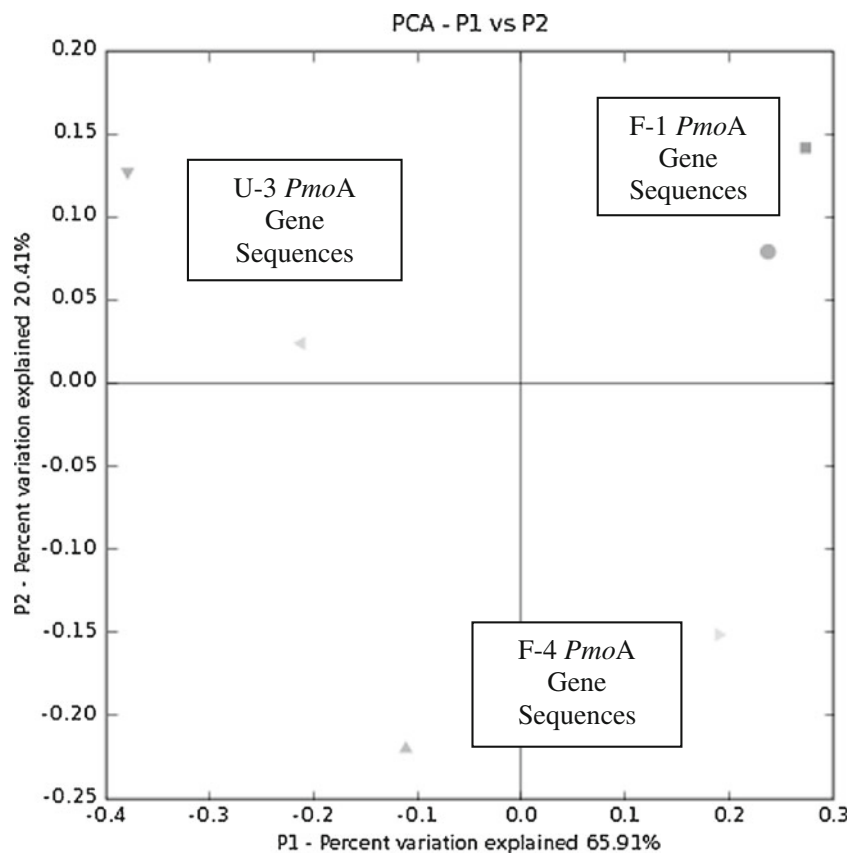
Figure 4 Phylogeny of functionally active methane-oxidizing bacteria (MOB) from the ¹³C-DNA fractions. Phylogenetic tree of *pmoA* gene sequences was constructed with PAUP v. 4.0b8 using maximum parsimony. Sequences were obtained from F1 (eutrophic), F4 (transition), and U3 (oligotrophic) microcosms spiked with ¹³CH₄. Numbers at nodes represent bootstrap values (100 times resampling); only values >50 are presented. *Nitrosococcus* sp. C-113 *amoAB* gene sequence was used as out-group

Figure 5 First two coordinates from a principal coordinate analysis (*PCA*) of *pmo*A gene sequences belonging to F1 (eutrophic), F4 (transition), and U3 (oligotrophic) microcosms spiked with $^{13}CH_4$. Percentages shown in the axis are variations that are explained by the principal coordinates

PCA - P1 vs P2

U-3 *Pmo*A Gene Sequences

F-1 *Pmo*A Gene Sequences

F-4 *Pmo*A Gene Sequences

P2 - Percent variation explained 20.41%

P1 - Percent variation explained 65.91%

interesting to speculate that these unique *pmoA* gene sequences belong to specialized methane-oxidizing bacteria that are ecologically adapted to the unique Florida Everglades habitat.

Additionally, *pmo*A sequence diversity was shown to be statistically different between F1, F4, and U3 soils (Fig. 5; Table 1). Specifically, both Shannon's species diversity (*H*) and evenness (*E*) declined in F1 and F4 soils when compared with U3 soils. Other statistical analyses performed included Simpson's diversity index and Chao 1 and 2 richness estimates at log-linear 95 % confidence intervals, abundance and incidence-based coverage estimator of species richness, first- and second-order Jackknife richness estimator, Bootstrap richness estimator, Michaelis–Menten richness estimator, and Fisher's alpha diversity index; these analyses confirmed that methanotroph diversity declined in the nutrient-enriched soils of the Florida Everglades (Table 1).

Ecological Significance

The Florida Everglades is unique with variable landscape consisting of ridges, sloughs, marshes, and tree islands. Each environmental compartment harbors unique microbial communities that are integral to the development and functioning of the greater Everglades ecosystem. Central to these issues are the specific roles played by microbial communities in the decomposition of organic matter coupled with the biogeochemical cycling of phosphorus, sulfur, and mercury. Characterization of the compositions and activities of microbial communities in the Everglades therefore provides important information regarding fundamental processes impacted by anthropogenic activities, as well as information required for development of restoration strategies.

Increased biological productivity as a function of nutrient loading has been reported from the Florida Everglades

Table 1 Statistical analyses on the methanotroph *pmo*A gene sequence diversity in the ^{13}C microcosms constructed with soils from F1, F4, and U3

[a]Upper and lower confidence limits are shown in parentheses

Site	Shannon's diversity (*H*)	Shannon's evenness (*E*)	Simpson's diversity	Chao1 estimate	Chao2 estimate	α-Diversity index
F1	0.39	0.5	3.7	6.5 (13;5.8)[a]	14 (48; 7.7)	1.3
F4	0.3	0.44	5.6	9.4 (15; 8.8)	19.4 (66; 10)	1.8
U3	0.63	0.81	6.3	11 (11; 110)	23.2 (79; 13)	2.2

including formation of significantly higher concentrations of methane [9]. Thus, nutrient-rich wetland sediments can be a rich source of microbially produced methane, which is released into the environment as a potentially strong greenhouse gas. However, variable concentrations of this greenhouse gas fraction is microbially sequestered and oxidized by MOB. This study provides a comprehensive understanding of the metabolically active methane-oxidizing bacterial guilds and the impacts of eutrophication on methanotrophic processes in natural wetlands such as the Everglades.

It is generally accepted that a reduction of species richness occurs as a consequence of pollution, whereby, unperturbed species-rich assemblages are typically evenly distributed. Subsequent to perturbation, species-rich guilds are replaced by species-poor ones exhibiting high dominance [20]. Correspondingly, the diversity and evenness analyses of the Everglades soil MOB indicated that competition resulted in domination by certain methanotrophs that are able to survive in the highly productive, nutrient-rich soils; conversely, oligotrophic soils consisted of a more even distribution of methane-oxidizing bacteria with less degree of competition between species.

Synthesis of our findings on the Florida Everglades suggests that changes occur in carbon cycling microbial and archaeal groups as a result of nutrient loading. With this study, we also show that benthic methanotrophic bacteria have the potential to intercept and oxidize methane produced and released by the lower anoxic methanogen archaeal communities of the Florida Everglades.

References

1. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410
2. Amaral JA, Knowles R (1995) Growth of methanotrophs in methane and oxygen counter gradients. FEMS Microbiol Lett 126:215–220
3. Boon P, Lee K (1997) Methane oxidation in sediments of a floodplain wetland in south-eastern Australia. Lett Appl Microbiol 25:138–142
4. Bosse U, Frenzel P, Conrad R (1993) Inhibition of methane oxidation by ammonium in the surface layer of littoral sediment. FEMS Microbiol Ecol 13:123–134
5. Bourne DG, McDonald IR, Murrell JC (2001) Comparison of *pmoA* PCR primer sets as tools for investigating methanotroph diversity in three Danish soils. Appl Environ Microbiol 67:3802–3809

6. Castro H, Reddy KR, Ogram A (2002) Composition and function of sulfate-reducing prokaryotes in eutrophic and pristine areas of the Florida Everglades. Appl Environ Microbiol 68:6129–6137
7. Castro H, Newman S, Reddy KR, Ogram A (2005) Distribution and stability of sulfate reducing prokaryotic and hydrogenotrophic methanogenic assemblages in nutrient impacted regions of the Florida Everglades. Appl Environ Microbiol 71:2695–2704
8. Chauhan A, Ogram A (2006) Phylogeny of acetate-utilizing microorganisms in soils along a nutrient gradient in the Florida Everglades. Appl Environ Microbiol 72:6837–40
9. Chauhan A, Ogram AV, Reddy KR (2004) Novel syntrophic-methanogenic associations along a nutrient gradient in the Florida Everglades. Appl Environ Microbiol 70:3475–3484
10. Chauhan A, Cherrier J, Williams HN (2009) Impact of sideways and bottom up control factors on bacterial community succession over a tidal cycle. Proc Natl Acad Sci USA 106:4301–4306
11. Costello AM, Lidstrom ME (1999) Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. FEMS Microbiol Ecol 65:5066–5074
12. Denman KL, Brasseur G, Chidthaisong A, Ciais P, Cox PM, Dickinson RE, Hauglustaine D, Heinze C, Holland E, Jacob D, Lohmann U, Ramachandran S, da Silva Dias PL, Wofsy SC, Zhang X (2007) Couplings between changes in the climate system and biogeochemistry. In: Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M, Miller HL (eds) Climate change 2007: the physical science basis. contribution of working group I to the fourth assessment report of the intergovernmental panel on climate change. Cambridge University Press, Cambridge, pp 499–588
13. Dunfield PF, Khmelenina VN, Suzina NE, Trotsenko YA, Dedysh SN (2003) *Methylocella silvestris* sp. nov., a novel methanotroph isolated from an acidic forest cambisol. Int J Syst Evol Microbiol 53:1231–1239
14. Escoffier S, Le Mer J, Roger PA (1997) Enumeration of methanotrophic bacteria in ricefield soils by plating and MPN techniques: a critical approach. Europ J Soil Biol 33:41–51
15. Hanson RS, Hanson TE (1996) Methanotrophic bacteria. Microbiol Rev 60:439–71
16. Heyer J, Galchenko VF, Dunfield PF (2002) Molecular phylogeny of type II methane-oxidizing bacteria isolated from various environments. Microbiol 148:2831–2846
17. King GM (1994) Associations of methanotrophs with the roots and rhizomes of aquatic vegetation. Appl Environ Microbiol 60:3220–3227
18. Kolb S, Knief C, Stubner S, Conrad R (2003) Quantitative detection of methanotrophs in soil by novel *pmoA*-targeted real-time PCR assays. Appl Environ Microbiol 69:2423–2429
19. Lozupone C, Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol 71:8228–35
20. Magurran AE, Phillip DAT (2001) Implications of species loss in freshwater fish assemblages. Ecography 24:645–650
21. Reddy KR, White JR, Wright A, Chua T (1999) Influence of phosphorus loading on microbial processes in the soil and water column of wetlands. In: Reddy KR, O'Connor GA, Schelske CL (eds) Phosphorus biogeochemistry in subtropical ecosystems. Lewis, New York, pp 249–273
22. Saitoh KI, Yagi O (2002) Development of a new most-probable-number method for enumerating methanotrophs using 48-well microtitre plates. Environ Microbiol 17:191–196
23. Singleton DR, Powell SN, Sangaiah R, Gold A, Ball LM, Aitken MD (2005) Stable-isotope probing of bacteria capable of degrading salicylate, naphthalene, or phenanthrene in a bioreactor treating contaminated soil. Appl Environ Microbiol 71:1202–1209

24. Sorrell BK, Boon PI (2002) Convective gas flow in *Eleocharis sphacelata* R. Br.: methane transport and release from wetlands. Aq Bot 47:197–212

25. Stackebrandt E, Goebel BM (1994) Taxonomic note: a place for DNA–DNA reassociation and 16 S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44:846–849

26. Stephen KD, Arah JRM, Daulat W, Clymo RS (1998) Root-mediated gas transport in peat determined by argon diffusion. Soil Biol Biochem 30:501–508

27. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882

28. Uz I, Chauhan A, Ogram AV (2007) Cellulolytic, fermentative, and methanogenic guilds in benthic periphyton mats from the Florida Everglades. FEMS Microbiol Ecol 61:337–347

29. Vorobev AV, Baani M, Doronina NV, Brady AL, Liesack W, Dunfield PF, Dedysh SN (2011) *Methyloferula stellata* gen. nov., sp. nov., an acidophilic, obligately methanotrophic bacterium possessing only a soluble methane monooxygenase. Int J Syst Evol Microbiol 61:2456–63

30. Westerman P (1993) Wetland and swamp microbiology. In: Ford TE (ed) Aquatic *microbiology*: An ecological approach. Blackwell Scientific, Cambridge, pp 215–238

31. Whittenbury R, Phillips KC, Wilkinson JF (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. J Gen Microbiol 61:205–218