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RESEARCH ARTICLE

The relative contribution of methanotrophs to microbial communities and carbon cycling in soil overlying a coal-bed methane seep

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phospholipid fatty acids; ^{13}C; ^{14}C; coal-bed methane; methanotroph.

Abstract

Seepage of coal-bed methane (CBM) through soils is a potential source of atmospheric CH_4 and also a likely source of ancient (i.e. ^{14}C-dead) carbon to soil microbial communities. Natural abundance ^{13}C and ^{14}C compositions of bacterial membrane phospholipid fatty acids (PLFAs) and soil gas CO_2 and CH_4 were used to assess the incorporation of CBM-derived carbon into methanotrophs and other members of the soil microbial community. Concentrations of type I and type II methanotroph PLFA biomarkers (16:1ω8*c* and 18:1ω8*c*, respectively) were elevated in CBM-impacted soils compared with a control site. Comparison of PLFA and 16s rDNA data suggested type I and II methanotroph populations were well estimated and overestimated by their PLFA biomarkers, respectively. The $\delta^{13}C$ values of PLFAs common in type I and II methanotrophs were as negative as −67‰ and consistent with the assimilation of CBM. PLFAs more indicative of nonmethanotrophic bacteria had $\delta^{13}C$ values that were intermediate indicating assimilation of both plant- and CBM-derived carbon. $\Delta^{14}C$ values of select PLFAs (−351 to −936‰) indicated similar patterns of CBM assimilation by methanotrophs and nonmethanotrophs and were used to estimate that 35–91% of carbon assimilated by nonmethanotrophs was derived from CBM depending on time of sampling and soil depth.

Introduction

Methane is important in carbon cycling within many ecosystems. Methane produced by methanogenic archaea during decomposition of organic matter in the anoxic portions of the ecosystem is transported to oxic environments via advection or diffusion where it is oxidized by methanotrophic bacteria (Jones & Nedwell, 1993; Sundh *et al.*, 1995; Reeburgh, 1996; Sundh *et al.*, 1997; Macalady *et al.*, 2002). Biogenic CH_4 that escapes from terrestrial environments before it is oxidized is the dominant natural source of atmospheric CH_4 and also contributes to important anthropogenic sources (e.g. rice cultivation and landfills) (Solomon *et al.*, 2007).

Large reductions in net radiative forcing associated with CH_4 release are achieved by the oxidation of CH_4 to CO_2 in soils by methanotrophs because of the much greater global warming potential of CH_4 (Solomon *et al.*, 2007). However, not all CH_4 consumed by methanotrophs is oxidized to CO_2. Some is assimilated as formaldehyde, an oxidation intermediate, into methanotroph biomass (Anthony, 1982). This biomass can contribute to soil organic matter (SOM) (Kögel-Knabner, 2002; Kindler *et al.*, 2009; Miltner *et al.*, 2009) or be consumed by other members of the ecosystem (Bastviken *et al.*, 2003; Eller *et al.*, 2005; Jones & Grey, 2011). In this way, methanotrophic bacteria have been shown to provide CH_4-derived carbon to a variety of ecosystem organisms that are unable

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to utilize CH_4 directly, including deep-sea mussels (Childress *et al.*, 1986; Cavanaugh *et al.*, 1987; Jahnke *et al.*, 1995), *Sphagnum* mosses (Raghoebarsing *et al.*, 2005), and limnetic insect larvae and zooplankton (Bastviken *et al.*, 2003; Eller *et al.*, 2005; Jones & Grey, 2011).

Incorporation of CH_4-derived carbon into biomass and other organic carbon pools in most natural terrestrial environments simply represents the recycling of photosynthetically derived organic carbon generated within the ecosystem. However, at terrerstrial thermogenic CH_4 seeps, fossil CH_4 is a potential carbon and energy source for ecosystems that is decoupled from current photosynthetic primary production and methanotrophs may themselves become primary producers in the ecosystem. Recent studies suggest that geologic emissions, predominantly micro- and macro-seepage of thermogenic CH_4 produced in the subsurface, are important sources of atmospheric CH_4 (Etiope & Klusman, 2002; Etiope, 2009). Despite the potential importance of soil microbial communities in mitigating CH_4 release from these environments, very little work has been carried out to characterize the composition of methanotrophs relative to the 'background' nonmethanotrophic microbial community. Furthermore, few studies have attempted to assess the relative importance of methanotrophs with assimilating fossil CH_4-derived carbon into the soil microbial ecosystem relative to the assimilation of recent photosynthetically derived organic matter. Although methanotrophs may serve as a conduit of CBM-derived carbon into the microbial ecosystem, whether this translates into larger overall microbial populations could also depend on the availability of electron acceptors (e.g. O_2).

Because of its characteristically negative $\delta^{13}C$ value, the flow of CH_4-derived carbon through modern and ancient ecosystems can be easily traced (Childress *et al.*, 1986; Cavanaugh *et al.*, 1987; Jahnke *et al.*, 1995; Hinrichs *et al.*, 1999; Bastviken *et al.*, 2003; Eller *et al.*, 2005; Raghoebarsing *et al.*, 2005; Watzinger *et al.*, 2008; Mills *et al.*, 2010; Jones & Grey, 2011). However, potentially large and variable kinetic carbon isotope fractionation effects are associated with the oxidation and assimilation of CH_4 (Coleman *et al.*, 1981; Zyakun *et al.*, 1987; Jahnke *et al.*, 1999; Templeton *et al.*, 2006) and can complicate the interpretation of the $\delta^{13}C$ signatures of biomass that result. Natural abundance radiocarbon (^{14}C) can serve as a powerful inverse tracer for the incorporation of fossil carbon (e.g. CBM) into biomass and organic carbon pools. Carbon-14 values are readily corrected for kinetic isotope fractionation effects by a $\delta^{13}C$ value-based normalization (Stuiver & Polach, 1977) and have been successfully used to trace the incorporation of fossil carbon by microorganisms in a variety of environments (Petsch *et al.*, 2001; Pearson *et al.*, 2005; Slater *et al.*, 2005, 2006; Wakeham *et al.*, 2006; Ahad *et al.*, 2010; Cowie *et al.*, 2010; Mills *et al.*, 2010). We used a dual carbon isotope approach, in combination with compound-specific biomarker and 16s rDNA techniques, to characterize methanotroph and nonmethanotroph populations in soil overlying a natural coal-bed methane (CBM) seep. Our techniques included measurement of phosphoether lipids produced by methanogenic archaea in some of the deeper anoxic soils impacted most by CBM seepage. From these analyses, we also estimated the relative importance of CBM as a carbon source to the overall soil microbial community.

Despite their great utility in isotopic tracer studies (e.g. Cébron *et al.*, 2007; Murase & Frenzel, 2007), DNA and RNA stable isotope probing techniques cannot be applied to in-situ, natural abundance isotope investigations. Accurate natural abundance $\delta^{13}C$ and $\Delta^{14}C$ values are readily determined for microbial membrane phospholipid fatty acids (PLFAs), and PLFAs have proven especially useful for studies of methanotroph populations because both type I and type II methanotrophs produce unique monounsaturated PLFAs (Bowman *et al.*, 1991; Guckert *et al.*, 1991; Bodelier *et al.*, 2009). Previous studies have used concentrations of the PLFA biomarkers 16:1ω8*c* and 18:1ω8*c* to estimate populations of type I and type II methanotrophs, respectively, in a variety of environments (Sundh *et al.*, 1995; Costello *et al.*, 2002; Macalady *et al.*, 2002; Bastviken *et al.*, 2003; Sundh *et al.*, 2005; Watzinger *et al.*, 2008; Mills *et al.*, 2010). However, not all strains of type I and II methanotrophs produce these biomarkers (Bowman *et al.*, 1991; Guckert *et al.*, 1991; Bodelier *et al.*, 2009), and a type II methanotroph (*Methylocystis* strain) has recently been shown to produce substantial amounts of the type I biomarker 16:1ω8*c* (Dedysh *et al.*, 2007). This creates potential problems for determining methanotroph abundances based solely on PLFA biomarkers. As part of our study, we assessed the validity of using biomarker PLFAs to estimate methanotroph populations in CBM-impacted soils. We compared overall PLFA profiles for impacted and control soils and assessed the increased abundance of several different PLFAs common in methanotrophs. In addition, DNA pyrosequencing was used to create phylogenetic libraries for select soil samples to independently assess methanotrophic populations and the effect of CBM seepage on the overall microbial community composition.

Study area

Several areas of CBM seepage occur where the Fruitland Formation outcrops in southwest Colorado (Supporting Information, Fig. S1). The overall flux of the seepage for these combined areas has been estimated at 0.03–0.04 teragrams CH_4 per year (~0.01% of global CH_4 flux; Solomon *et al.*, 2007) over an area of ~150 hectares (LT

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Environmental, 2009). The Fruitland Formation is the dominant coal-bearing unit in the San Juan Basin (southwest Colorado/northwest New Mexico) and contains subituminous-A to medium volatile bituminous coal of late Cretaceous age (Rice, 1993). The Pine River CBM seep is located where the Pine River Valley cuts through a hogback outcrop of the Fruitland Formation and underlying Pictured Cliffs Sandstone (Fig. S1). The formation dips 35–45° to the south in the Pine River Valley and is overlain by Quaternary alluvium (0–12 m thick) (Fassett *et al.*, 1997; Martineau, 1997). The overall area affected by CH_4 seepage in this vicinity is approximately 1000 by 100 m (Martineau, 1997). There are two major and two minor East–West trending seeps that correlate with coal seams in the underlying Fruitland Formation (Martineau, 1997).

Historic observations suggest that CBM seepage was occurring in several areas in the San Juan Basin before extensive gas development in the area. (Fassett *et al.*, 1997; BLM, 1999). Video logs of area wells confirmed that CH_4 originates from coal beds that crop out below the alluvium (Fassett *et al.*, 1997). An unconfined aquifer in the valley floor alluvium, which is hydrologically associated with the Pine River, appears to control the nature of the CH_4 seepage (Martineau, 1997). Gas seepage from water-saturated soils is rapid (flows on the order of 20 moles CH_4 h^{-1} m^{-2} are typical) and prevents the diffusion of atmospheric gases into the soil (Martineau, 1997). In unsaturated areas, the seepage is more diffuse, and atmospheric O_2 is able to mix with CH_4 in the soils. This enables microbially mediated CH_4 oxidation as evidenced by high concentrations of soil gas CO_2 (Martineau, 1997).

A transect of 13 one-meter depth soil gas wells (labeled PR101 through PR113) was installed in the methane seep area in the mid-1990s and was monitored bimonthly by the United States Bureau of Land Management (BLM). The transect is approximately 120 m long and runs north–south, perpendicular to the seep trends. It crosses one of the minor and one of the major seeps delineated by Martineau (1997). Relatively high average concentrations of CH_4 (5–70% by volume) and low concentrations of O_2 (1–6% by volume) were measured in five out of the 13 wells from January 1999 to July 2001 (D. Swanson, BLM, pers. comm.). The highest average CH_4 concentration was measured in PR104 which was chosen as the focus of this study. Relatively low concentrations of CH_4 (< 110 ppmv) were detected in nearby well PR102, which was chosen as the control site.

Methods

Soil gas collection and analysis

Soil gas was collected from 1m-depth PVC soil gas wells installed by BLM and stainless steel probes installed adjacent to wells at sites PR104 and PR102. Three static volumes were purged before samples were collected in glass syringes with epoxied needles. Samples were sealed for transport by inserting needles into rubber stoppers. Analysis was by gas chromatography (Shimadzu GC-17A) within 36 h of collection. Carbon dioxide and O_2, N_2O, and CH_4 were measured by thermal conductivity, electron capture, and flame ionization detectors, respectively, after separation on a series of packed columns. Molecular oxygen and argon coeluted so that reported O_2 concentrations include interference from argon.

In October, 2002, soil gas samples were collected from PR102 and PR104 for $\delta^{13}C$ and $\Delta^{14}C$ analyses of CO_2 and CH_4. For CH_4, triplicate samples from PR104 (100 cm depth) were collected in evacuated 250 mL glass sampling bottles with airtight valves. For CO_2, samples from PR104 (20 and 100 cm depth) and PR102 (100 cm depth) were collected by syringe (see above). Additional samples for CO_2 from PR102 (20 and 100 cm depth) were collected in gas sampling bags. Within 48 h of collection, CO_2 was cryogenically purified. The valves on the gas sampling bags appeared to leak laboratory air as the sample was introduced onto the vacuum line. Carbon dioxide was analyzed for ^{14}C by accelerator mass spectrometry at the Tono Geoscience Center (Toki, Japan) as detailed by Mills *et al.* (2010). An aliquot of each CO_2 sample was also analyzed for its $\delta^{13}C$ value by dual-inlet-isotope ratio mass spectrometry (IRMS) in the same laboratory. Analytical error for ^{14}C measurements of PR104 CH_4 and CO_2 was reported to be < 5‰. Precision for the triplicate CH_4 samples from PR104 was 0.4‰ (1σ) for $\Delta^{14}C$ and 0.1‰ for $\delta^{13}C$. Values for the duplicate CO_2 samples from PR104 100 cm depth were within 4‰ for $\Delta^{14}C$ and 0.1‰ for $\delta^{13}C$. Both $\delta^{13}C$ and $\Delta^{14}C$ values for the PR102 CO_2 samples collected in gas sampling bags confirmed contamination by laboratory air.

In May 2004, additional soil gas samples were collected from PR102 and PR104 for $\delta^{13}C$ (but not $\Delta^{14}C$) analysis of CH_4 and CO_2. Samples were collected in either airtight gas sampling bottles (PR102) or inverted Vacutainer® tubes with an acidified water seal (PR104). $\delta^{13}C_{CO2}$ values were determined on a Micromass trace gas analyzer interfaced to a Micromass Isoprime isotope ratio mass spectrometer. Samples were measured in triplicate and standard deviations were $\leq$ 0.1‰ for PR104 samples and $\leq$ 0.5‰ for PR102 samples. The $\delta^{13}C$ values of CH_4 were determined by the isotope biogeochemistry laboratory at the University of Hawaii. The $\delta^{13}C$ values of duplicate analyses of one CH_4 sample were within 0.5‰.

Soil sampling and characterization

Soils were collected within 1 m of either PR104 or PR102 soil gas probes. PR104 soils were collected in April and

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October 2002 using a manual soil auger. Due to abundant cobbles in the vicinity of PR102, it was not possible to sample the control soil near this location with the hand augers/corers that we used for PR104 soils in April and October 2012. Given that the PR102 soil probe provided a long-term record of the soil gas composition at this control site, it was important to collect soils for PLFA analysis near this probe so that they could be directly compared to the gas composition and to be assured that they experienced minimum impact from CBM seepage. To penetrate the cobbles, we finally used a truck mounted hydraulic soil auger to collect control soil from the PR102 site in May 2004. PR104 soils were alternatively collected by an impact corer during this sampling. Due to technical issues with the auger, the depth resolution of PR102 samples was not as great as for PR104 samples. All soil samples were immediately placed in sterile polyethylene bags, purged with N_2 or Ar, sealed, and placed on ice at the field site. Samples were stored at −20 °C.

Visually similar soils from adjacent depths were combined for textural analysis. Samples that contained gravel were passed through 6.3 and 4.0 mm sieves. Soil texture and moisture curve determinations were performed at the Soil-Water-Plant Testing Laboratory (Colorado State University). Field capacities were determined at tensions of 0.33 and 0.10 bars for medium and coarse textured soils, respectively. Ambient water content was determined gravimetrically by drying at 105° C for 12 h.

Extraction and analysis of PLFAs and ether-linked lipids

Total lipids were isolated from soils by a modified Bligh–Dyer extraction as described in detail by White & Ringelberg (1998). Soils were extracted for 4 h in chloroform : methanol : 50 mM phosphate buffer (1 : 2 : 0.8 by volume). Solvent was decanted and added to additional chloroform and water to break the phase. Total lipids were recovered in the chloroform phase and fractionated on solid-phase extraction cartridges (0.5 g silica, Burdick and Jackson Inert II). For the April 2002 samples, the cartridges were eluted with 5 mL chloroform, 10 mL acetone and 5 mL methanol (White & Ringelberg, 1998). For all other samples, 10 mL of methanol was used for the final elution (Mills & Goldhaber, 2010). Mills & Goldhaber (2010) showed that not all classes of phospholipids (e.g. phosphocholine) are fully eluted from a typical 0.5 g silica solid-phase extraction cartridge with the 5 mL of methanol prescribed by White & Ringelberg (1998). Thus, PLFAs from the April 2002, samples may have been underestimated. Phospholipids recovered from the methanol were converted to fatty acid methyl esters (FAMEs) by mild-alkaline methanolysis (White & Ringelberg, 1998). FAMEs were quantified by GC-FID using an internal standard (13 : 0 FAME). Analysis was performed on a Perkin Elmer Autosystem GC equipped with a 60 m Supelco SP2380 column. Relative concentrations of more abundant FAMEs were within 5% for duplicate analyses. Coefficients of variation for replicate extractions of two select samples were also within 5% for both total and relative concentrations of individual FAMEs. Positive identification of FAMEs was by retention time comparison with authentic standards and by GC-mass spectrometry (MS) (Fig. S2). Positional isomers of monounsaturated FAMEs were quantified by comparing the relative intensities of specific cleavage mass peaks of their dimethyl disulfide derivatives (Fig. S2; Dunkelblum *et al.*, 1985).

Phospholipids with ether-linked hydrocarbon chains were isolated from methylated phospholipid samples by silica gel solid-phase extraction as previously described except that FAMEs were eluted with 5 mL chloroform and ether-linked phospholipid residues with 10 mL methanol (White & Ringelberg, 1998). Ether linkages were cleaved by hydrolysis in one mL 57% HI for 4 h at 110 °C (King *et al.*, 1998; Koga & Morii, 2006). The resulting alkyl iodides were washed with 1.5 mL 5% NaCl and extracted with 2 mL hexane (3×). Alkyl iodides were reduced in 1 mL tetrahydrofuran (THF) with 50 mg $LiAlH_4$ under a nitrogen atmosphere at 70 °C for 2 h (King *et al.*, 1998). The reaction was quenched with 0.5 mL ethyl acetate and 1.5 mL 5% NaCl and 0.5 mL 6 N HCl added. Hydrocarbons were extracted with 1 mL ethyl acetate (3×) and analyzed for phytane and biphytane by GC-MS (J&W Scientific DB-1 column).

$\delta^{13}C$ measurements of FAMEs and phytane/biphytane

Gas chromatography–combustion–IRMS was performed on a Europa GEO20/20 isotope ratio mass spectrometer interfaced through a combustion furnace to an Agilent 6890 GC operated with a Supelco SP2380 column for FAMEs and a J&W Scientific DB-1 column for phytane/biphytanethe. The $^{13}C/^{12}C$ ratios of unknowns were determined by comparison with an internal working standard (12 : 0 FAME) and reference CO_2. A majority of samples were analyzed in duplicate or triplicate and analytical errors were typically $\leq 2‰$. Exceptions are noted in Table S4. Baseline separation during gas chromatography is required for accurate determination of $\delta^{13}C$ values using GC-C-IRMS because of differences in retention time of isotopologues during gas chromatography (Sessions, 2006). Baseline peak resolution of the positional isomers of monounsaturated FAMEs can be particularly

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difficult depending on the number of isomers in a given sample. As a result, we report a single $\delta^{13}C$ value (e.g. $\delta^{13}C_{\Sigma18:1}$) for all combined positional isomers of a given monounsaturated PLFA.

$\Delta^{14}C$ measurements of PLFAs

Individual PLFAs were separated and collected by preparatory GC (Eglinton *et al.*, 1996). Column bleed contamination was removed on silica gel columns and FAMEs combusted to CO_2 in evacuated quartz tubes containing CuO. The CO_2 was cryogenically purified and submitted to the National Ocean Sciences Accelerator Mass Spectrometry Facility (NOSAMS), Woods Hole, MA for ^{14}C analysis. Two samples (Table S5) contained < 25 μg carbon, the lower limit for ^{14}C measurements. These samples were diluted with modern CO_2 by NOSAMS, and measured values were corrected for the added CO_2. Error for a standard sample measured using this method was 44‰. Reported analytical errors for all other samples were < 20‰ (Table S1). When possible, NOSAMS also measured $\delta^{13}C$ values of the submitted samples which were compared with $\delta^{13}C_{PLFA}$ values determined by GC-IRMS as a consistency check on sample preparation and analysis. The absolute difference between NOSAMS and GC-IRMS $\delta^{13}C$ measurements was $\leq$ 2.1‰ for all samples except one (Table S1).

Principal components analysis

Principal components analysis (using a correlation matrix) was performed on square root transformed data using the statistical software package MINITAB (version 15).

Profile comparison method for estimating concentrations of methanotroph PLFAs

An approach was developed as an alternative to estimating type I and type II methanotroph abundances using only the respective PLFA biomarkers 16:1ω8*c* and 18:1ω8*c*. The relative amounts of 18:1ω7*c* (mean± 1σ=29 ± 28 mol%) and 18:1ω8*c* (51 ± 33 mol%) PLFAs produced by type II methanotrophs vary considerably, but when these two PLFAs are considered together the variation is much lower (18:1ω7*c* + 18:1ω8*c* = 79 ± 15 mol%) (Bodelier *et al.*, 2009). Likewise, the sum of the concentrations of the PLFAs 14:0, 16:0, 16:1ω7*c*, 16:1ω8*c*, 16:1ω6*c*, and 16:1ω5*t* (88 ± 13 mol%) in type I isolates is much more consistent than the concentrations of the individual PLFAs (Bodelier *et al.*, 2009). Therefore, we attempted to estimate methanotroph populations using these more comprehensive sets of PLFA structures.

An algorithm written as an Excel Visual Basic macro iteratively subtracted abundances of the PLFAs 14:0, 16:0, 16:1ω5*t*, 16:1ω6*c*, 16:1ω7*c*, 16:1ω8*c*, 18:1ω7*c*, and 18:1ω8*c* from a PR104 sample until the best least squares fit between the resulting PR104 PLFA profile and the PR102 PLFA profile for a sample most similar in depth was obtained (Fig. S2). This approach was based on the assumption that the PLFA profiles of type I and type II methanotrophic bacteria in PR104 soils were simply overprinted on the PLFA profile of nonmethanotrophic bacteria, which was identical to the PLFA profile for the PR102 soil of corresponding depth. The abundance of type I methanotroph PLFAs at the PR104 site was determined by summing the excess abundances of the 14:0, 16:0, 16:1ω5*t*, 16:1ω6*c*, 16:1ω7*c*, 16:1ω8*c* PLFAs in the PR 104 sample vs. PR102 sample. Analogously, the sum of the 18:1ω7*c* and 18:1ω8*c* PLFAs was attributed to type II methanotrophs. The sampling difficulties at PR102 resulted in less resolved depth sampling, so that each PR104 sample had to be compared with the PR102 sample most similar in depth (e.g. PR104 0–5 cm depth compared with PR102 0–31 cm depth). Because we were not able to sample PR102 soils in April and October 2002, all PR104 samples, including those collected in 2002, were compared with the PR102 samples collected in May 2004.

16s rDNA and functional gene analyses

Four PR104 and one PR102 samples were chosen for 16S rRNA sequencing and functional gene (mcrA) analyses. DNA was extracted from 0.2 g soil using the Powersoil extraction kit (MoBio, Inc). 16S rRNA universal primers were chosen to flank the V4 and V5 hypervariable regions (*Escherichia coli* positions 515F and 926R) (Baker *et al.*, 2003). A unique forward primer was designed for each sample and included the 454 life Science A adapter, a distinct 8 bp barcode, a two bp linker, and the 515F primer (5′-GCCTCCCTCGCGCCATCAG-XXXXXXXX-CA-GTG CCAGCMGCCGCGGTAA-3′). The same reverse primer was used for all samples and included the 454 life science B sequencing adapter, a two bp linker, and the 926R primer (5′-GCCTTGCCAGCCCGCTCAG-TC- CCGTCA ATTCMTTTRAGTTT-3′). PCR reactions were conducted using Promega Master Mix (12.5 μL Promega Master Mix, 7.5 μL H_2O, 2.5 μL of each primer at 50 μM and 2 μL of template DNA per reaction). The thermal cycler program ran at 94 °C (30 s), 53.8 °C (30 s), and 72 °C (45 s) for 30 cycles, with a final extension of 72 °C for 6 min. Amplicons were normalized using invitrogen's SequalPrep Normalization Plate (96) Kit. The normalized amplicons from each sample were pooled together and sequenced on a GS FLX platform at the University of Colorado Health Sciences Center (Denver, CO).

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Pyrosequencing reads were analyzed using the QIIME software pipeline (Caporaso *et al.*, 2010). Reads outside 200–300 nt in length, with a minimum quality score lower than 27, or incorrect barcodes, were discarded from further analysis. In total, 8,366 pyrosequencing reads qualified for further processing. Sequences were clustered into OTUs at 97% similarity using the UCLUST furthest neighbor algorithm. Taxonomy was assigned by comparing OTUs against the Silva database using BLAST. The mcrA gene was amplified from the same DNA extractions using the primers and PCR program described by Steinberg & Regan (2008).

Results

Soil characteristics and moisture content

Both the PR104 and PR102 (control) near-surface soils (0–30 cm) were sandy loams or sandy clay loams (Table S2). Mid depth soils (30–75 cm) at both sites had loam to sandy loam textures. At depths > 75 cm, the PR104 soil became progressively sandier with substantial contributions of gravel (> 2 mm diameter). The PR102 soils became sandier with depth but lacked a substantial gravel fraction. Cobbles were present throughout the PR102 soil but absent in the PR104 soil.

Surface soils (0–10 cm) at PR104 were wettest in April 2002 (47–65% of FC) (Fig. 1). Surface soils in October 2002 were only slightly drier than in April 2002, likely due to a rain event that occurred just before collection in October. Much drier conditions in the deeper soils in October vs. April 2002 are indicative of typically hot, dry summers in the area. The PR104 surface soils were drier in May 2004 (32–50% of FC), but deeper soils had similar moisture content as in April 2002 (Fig. 1). A direct comparison of soil moistures for May 2004 PR104 and PR102 soils is difficult due to different sampling resolution, but, given their proximity, both sites received the same precipitation.

Soil gas concentrations and carbon isotopes

Site PR102

Soil gas was collected at several depths to 1 m from PR102 in October 2002 and May 2004. Concentrations of gasses were generally unchanged at depth and always $\leq$ 110 ppmv for CH_4, ~21% for O_2, < 2% for CO_2, and < 1 ppmv for N_2O (Fig. 1b and c). Stable carbon isotope compositions of CH_4 and CO_2 were determined for select PR102 samples collected in October 2002 and May 2004. The $\delta^{13}C_{CO2}$ values measured at 20 and 100 cm depths in May 2004 were -23.2 and -23.6‰, respectively (Fig. 1c). One sample of PR102 soil gas collected by syringe from 100 cm depth in October 2002 had a similar $\delta^{13}C_{CO2}$ value of -21.4‰ and a $\Delta^{14}C_{CO2}$ value of -46‰ (Fig. 1b). Although accurate determination of a $\Delta^{14}C$ value for the CO_2 collected at 20 cm depth at PR102 in October 2002 in gas sampling bags was hindered by contamination with atmospheric CO_2, an estimate based on a contamination-mixing curve indicated that it was relatively modern (between 0 and -115‰). The $\delta^{13}C_{CH4}$ values determined for site PR102 at 20 and 60 cm depths were -62.3 and -64.9‰, respectively (Fig. 1c). Due to low abundance of CH_4, no $\Delta^{14}C_{CH4}$ values were determined for PR102 samples.

Site PR104

Soil gasses were collected from depths up to 1.2 m at PR104 for all three sampling dates, but clogged probes prevented complete profiles in April and October 2002. In general, CH_4 concentrations were highest in the deep soils and decreased toward the soil surface (Fig. 1). Methane concentrations at 100 cm depth were higher in April 2002 (93%) and May 2004 (55%) than in October 2002 (25%). Oxygen concentrations decreased with soil depth during all three collections (Fig. 1). Lowest measured concentrations of O_2 in soil gas were ~2%, but this was likely an artifact of argon coeluting with O_2 during analysis. Measurements of NO_3^- , SO_4^{2-} and acid volatile sulfide in April 2002 indicated that the deepest PR104 soils were anoxic (Fig. S3). Up to 13% by volume soil gas CO_2 was measured in the PR104 soils (Fig. 1). Elevated concentrations of N_2O were observed in the near-surface soils during April and October 2002 and in the deeper soils during May 2004 (Fig. 1).

The $\delta^{13}C_{CH4}$ values for May PR104 samples increased from -50‰ at 100 cm to -22‰ at 20 cm (Fig. 1c). The $\delta^{13}C_{CO2}$ values ranged from -39 to -44‰. Two very negative $\Delta^{14}C_{CO2}$ values were measured from 20 and 100 cm depth at PR 104 in October 2002 (-754 and -830‰, respectively; Fig. 1b). A $\Delta^{14}C$ value of -930 ± 1‰ (1σ) and $\delta^{13}C$ value of -42.1 ± 0.1‰ was determined for triplicate samples of CH_4 collected at PR104 (100 cm) in October 2002.

Total PLFA abundance

Total concentrations of PLFAs in both PR104 and PR102 soils displayed similar trends of lower concentrations with increasing depth (Table S3). Concentrations of total PLFAs in the deepest, saturated soils from all three PR104 collections were very similar (between 4 and 6 nmol PLFA g^{-1}) and were more than tenfold less than their respective concentrations in the surface soils. Total PLFA concentrations in the 0–5 cm interval of the PR104 soil were greater in April 2002 and May 2004 (247 and

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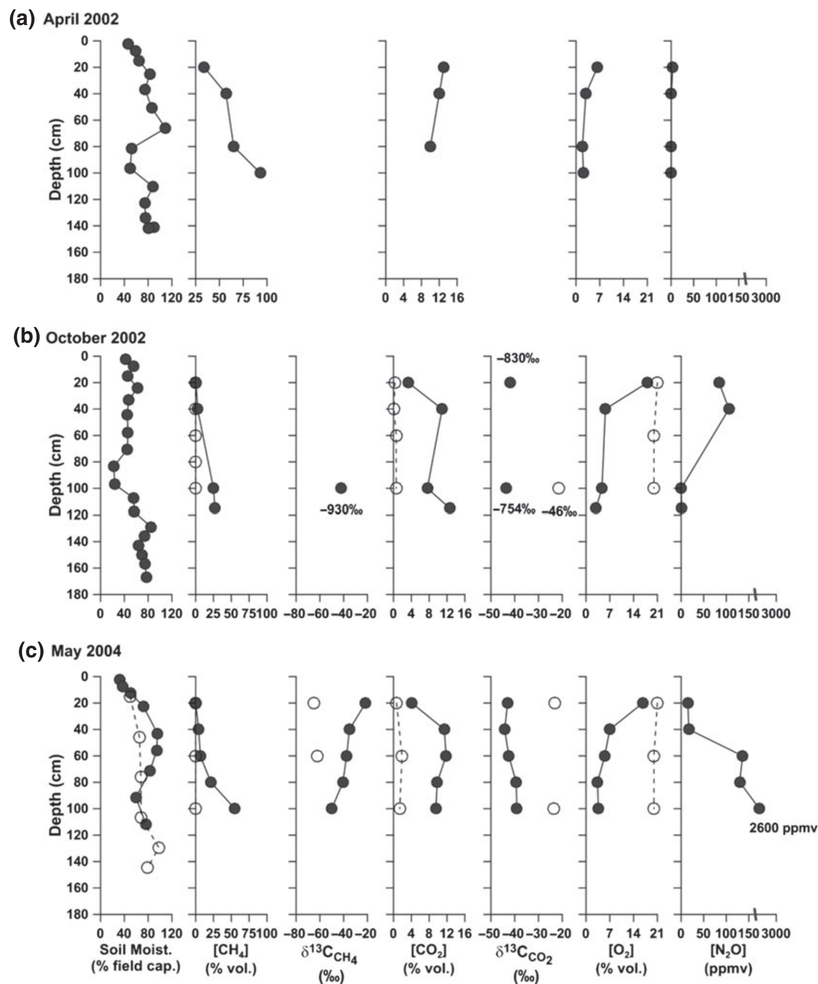

Fig. 1. Depth profiles of soil moisture; soil gas concentrations of CH_4, CO_2, O_2, and N_2O; and $\delta^{13}C$ values of soil gas CH_4 and CO_2 for three different sample collection dates. Data from both the high-CBM PR104 site (closed circles) and control PR102 site (open circles) are presented. Values next to October 2002 data points indicate the measured $\Delta^{14}C$ values of these samples. N_2O concentrations were always below detection in PR102 samples from both October 2002 and May 2004. Note the broken N_2O concentration axis. The saturated zone, as determined by the observation of free water, was reached during all three PR 104 soil collections. This occurred at approximately 142 and 171 cm depth for the April 2002, October 2002 collections, respectively, and between 142 and 188 cm in May 2004.

196 nmol PLFA g^{-1}, respectively) than in October 2002 (52 nmol PLFA g^{-1}). The 0–31 cm interval of the PR102 soil collected in May 2004 had a lower PLFA concentration (139 nmol PLFA g^{-1}) than the PR104 surface soil in both April 2002 and May 2004. However, at greater depths, PLFA concentrations in the PR102 soils were larger than those at comparable depths at PR104 for any collection date.

PLFA-based estimation of methanotroph abundance by two different methods

The PLFA profiles of all the May 2004 PR102 samples were relatively similar and contained < 1 mol% of the PLFA biomarkers for type I (16:1ω8*c*) and type II (18:1ω8*c*) methanotrophs (Table S3, Fig. 2). In contrast, some PR104 soil samples had relative abundances of 16:1ω8*c* and 18:1ω8*c* as great as 9.5 and 36.9 mol%, respectively. Principal components analysis was performed on the PLFA profiles of all PR102 and PR104 samples. The first two principal components (PC1 and PC2) explained 46 and 20% of the variation in the profiles, respectively (Fig. 2). Score and loading plots of PC2 vs. PC1 show that PR104 samples are differentiated from PR102 samples by two different groups of PLFAs that are common in type II methanotrophs (18:1ω7*c*, 18:1ω8*c*) and type I methanotrophs (14:0, 16:1ω7*c*, 16:1ω8*c*, 16:1ω6*c*, 16:1ω5*t*) (Fig. 2b). The 16:0 PLFA did not group as closely in the PC loading plot with other PLFAs common in type I methanotrophs, likely because it is ubiquitous in a wide variety of microorganisms. Figure 3 illustrates (for the May 2004 PR 104 0–5 cm sample) the greater total abundance of PLFAs in the surface soil at PR104 vs. PR102 can be attributed solely to the presence of type I methanotrophs.

The differences in PLFA profiles of the PR102 and PR104 samples were clearly dominated by variable concentrations of PLFAs common in type I and II methanotrophs (Figs. 2 and 3). These differences were used to estimate population sizes of type I methanotrophs, type II

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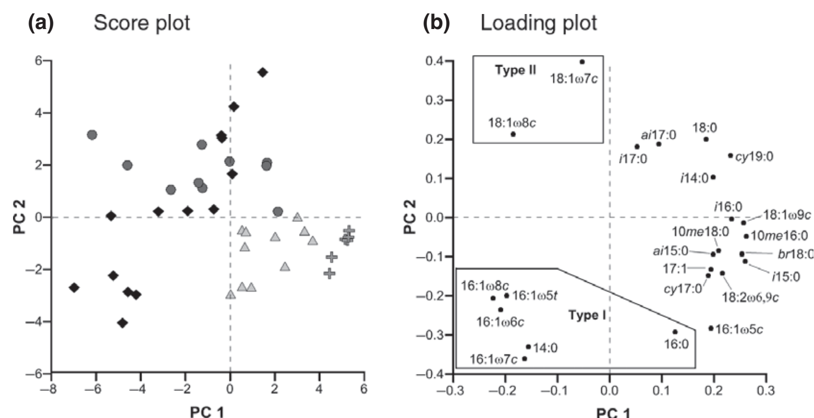

Fig. 2. Principal components analysis score (a) and loading (b) plots for PLFA profiles from all PR 104 and PR 102 samples. April 2002 PR 104 (diamonds), October 2002 PR 104 (circles), May 2004 PR 104 (triangles), May 2004 PR 102 (crosses). All May 2004 PR102 samples had relatively similar PLFA profiles compared with PR104 samples. Polygons indicate PLFAs commonly found in type I and type II methanotrophs.

methanotrophs, and nonmethanotrophs as detailed in section 3.7. This approach allowed PLFAs which are abundant in, but not exclusive to, methanotrophs to be partitioned between methanotrophic and nonmethanotrophic bacteria. It also allowed methanotroph populations to be quantified by PLFAs other than 16:1ω8 and 18:1ω8, which are not produced in consistent amounts by all type I and type II methanotrophs (Bodelier *et al.*, 2009).

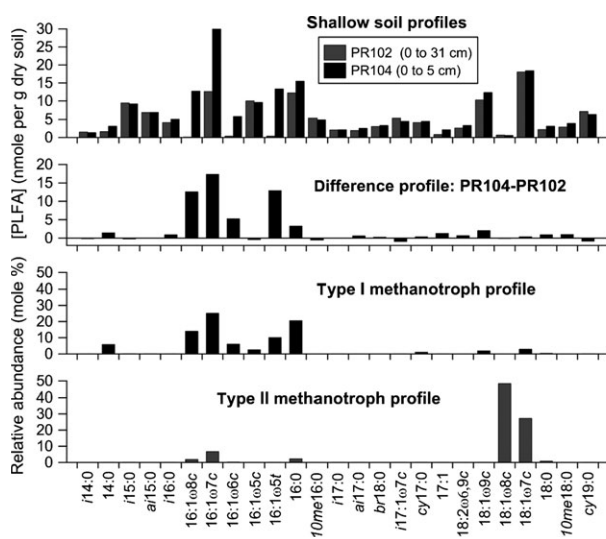

Fig. 3. Comparisons of the absolute concentrations of PLFAs in near-surface soils samples from PR 104 and PR 102 and relative PLFA profiles of type I and type II methanotrophs. A difference profile was generated by subtracting the absolute concentration of PLFAs in the PR 102, 0–31 cm sample from the absolute concentrations of PLFAs in the PR 104, 0–5 cm sample. The result is compared with the average relative abundance of PLFAs reported for type I and type II methanotrophs reported by Bodelier *et al.* (2009) and suggests that the differences in PLFA abundances in the PR 102 and PR104 samples can be explained by the presence of a large type I methanotroph population in the PR 104 soil.

The abundances of type I methanotroph PLFAs estimated from comparison of the PR102 and PR104 PLFA profiles correlate well with concentrations of the 16:1ω8*c* biomarker (Fig. 4a). In contrast, the abundances of type II methanotroph PLFAs estimated from soil PLFA profile comparisons were only moderately correlated with concentrations of the 18:1ω8*c* biomarker (Fig. 4b). From the soil PLFA profile comparisons, depth profiles of type I and type II methanotroph populations at PR 104 were generated for all three collections (Fig. 5). Concentrations of type I PLFAs were greatest in the April 2002 and May 2004 surface soils (51 and 55 nmol PLFA g^{-1}, respectively) and decreased almost tenfold within the uppermost 20 cm of soil. Maximum abundance of type II methanotrophs in these spring soils occurred at approximately 20 cm depth just as type I populations were declining. However, the type II maximum was much greater in April 2002 (103 nmol PLFA g^{-1}) than May 2004 (8 nmol PLFA g^{-1}) (Fig. 5). In October 2002, type I populations were relatively small and constant with depth ($\leq$ 6 nmol PLFA g^{-1}) while type II populations were greater and varied (7–21 nmol PLFA g^{-1}) unsystematically with depth.

PLFAs attributed to nonmethanotrophic bacteria

Concentrations of PLFAs attributed to nonmethanotrophic bacteria in the PR 104 soils were determined by subtracting estimates of type I and type II methanotroph PLFAs from total PLFA concentrations. Concentrations of nonmethanotroph PLFAs in the PR102 soils are assumed

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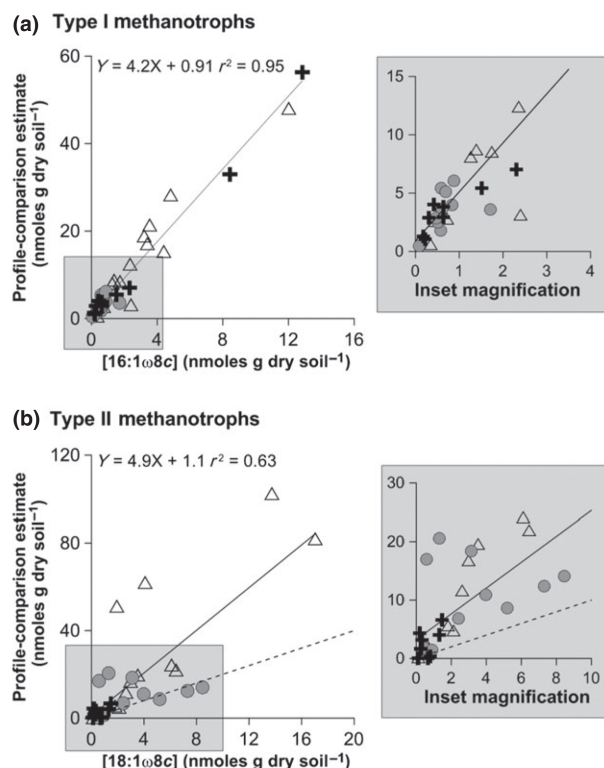

Fig. 4. Correlations between the methanotroph biomarker PLFAs and profile comparison estimates of the concentrations of (a) Type I Methanotroph and (b) Type II Methanotroph PLFAs in PR 104 soils. April 2002 (triangles); October 2002 (circles); May 2004 (crosses).

to be equivalent to the total PLFA concentrations. Concentrations of nonmethanotroph PLFAs in the uppermost surface soils in the April 2002 (PR104) and May 2004 (PR104 and PR102) collections were similar (144, 139, and 139 nmol PLFA g^{-1}, respectively) (Fig. 5). Nonmethanotroph PLFA concentrations decreased with depth in all three soils but much more steeply than in the May 2004 PR102 soils. In October 2002, concentrations of nonmethanotroph PLFAs in soils <50 cm depth (27–83 nmol PLFA g^{-1}) were much lower than at similar depths in April 2002 and May 2004 (Fig. 5). Concentrations of nonmethanotroph PLFAs for soils below 50 cm at PR104 were relatively similar for all three dates.

Concentrations of phospholipid-derived phytane and biphytane

Concentrations of both phospholipid-derived phytane and biphytane increased with depth in the May 2004 PR104 soil but did not show any consistent trends with depth in the PR102 soils (Fig. 5). Likewise concentrations of these lipids were quite variable with depth in the October 2002 PR104 soils (Fig. 5). Based on our particular field site location, as well as the low $\delta^{13}C$ values of these lipids (see section 4.8), it was assumed that the ether lipids from the deepest soil depths, where concentrations were generally higher, were derived from methanogenic archaea.

Lipid-based estimates of depth-integrated cell numbers

Depth-integrated estimates of cell numbers for type I and II methanotrophic bacteria, nonmethanotrophic bacteria, and methanogens were made using conversion factors from the literature (Fig. 6). Both methanotrophs and nonmethanotrophs were more abundant at PR104 in April 2002 than in May 2004 or October 2002. Total bacterial cell numbers were similar for May 2004 and October 2002, but type II methanotrophs were more abundant in October 2002. In May 2004, the depth-integrated abundance of nonmethanotrophs was approximately three times greater for PR102 than PR104. Methanogen cell numbers were only estimated from data for the deepest two samples from the May 2004 PR104 collection where $\delta^{13}C$ values of phytane and biphytane suggested that they were derived from methanogens, at which time they may have been comparable with methanotroph cell numbers.

Carbon isotopes of PLFAs and isoprenoid lipids

The $\delta^{13}C_{PLFA}$ values for PR102 were between −20.6 and −32.5‰, except for 14:0 from the 0–31 cm depth (−14.5‰) and displayed no depth trends (Fig. 7, Table S4). The $\delta^{13}C_{PLFA}$ values at PR104 were more negative and varied (−25.2 to −66.9‰), ranging from the most negative to positive values as follows: Σ16:1, 16:0, 14:0, Σ18:1, *cy*17:0, *cy*19:0, i15:0, *ai*15:0 = 10*me*16:0, *i*16:0, *i*17:1ω7. Generally, the $\delta^{13}C_{PLFA}$ values at PR104 were more negative with depth, with two exceptions: (1) $\delta^{13}C_{PLFA}$ values for the deepest sample of each collection were typically more positive than for the depth just above and (2) in May 2004, $\delta^{13}C$ values of 14:0 and Σ16:1 PLFAs trended toward more negative values with decreasing depth near the surface.

The $\Delta^{14}C$ values of PLFAs from three October 2002 and two May 2004 soil depth intervals at PR104 ranged from −351 to −936‰ (Fig. 8 and Table S1). For both collection dates, the $\Delta^{14}C$ values of the PLFAs extracted from deeper soils (−682 to −936‰) were more negative (reflecting the assimilation of older carbon) than those from the shallow soils (−351 to −672‰). For the shallow soils, the $\Delta^{14}C_{PLFA}$ values of corresponding PLFAs were very similar in the October 2002 and May 2004 collections, with the $\Delta^{14}C$ values of Σ16:1 and Σ18:1 consistently being 150‰ depleted relative to the other PLFAs

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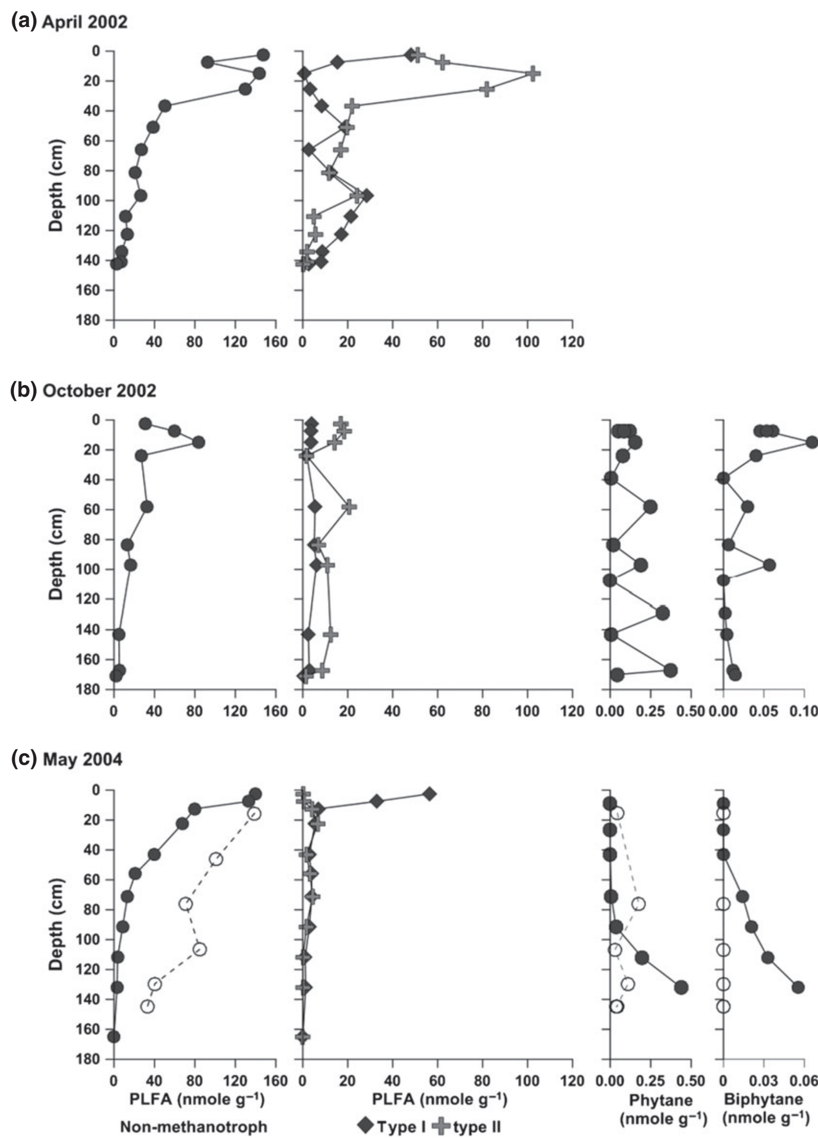

Fig. 5. Depth profiles of concentrations of PLFAs attributed nonmethanotroph, type I methanotrophs, and type II methanotrophs; phytane; and biphytane in PR104 (closed circles) and PR102 (open circles) soil samples. Type I (diamonds) and type II (crosses) methanotroph PLFA concentrations are for PR104 only. All PLFA concentrations were determined by the profile-fitting method described in the text except for nonmethanotroph PLFAs at PR 102 which are simply total PLFA concentrations.

(16:0, *i*15:0, *ai*15:0). For the deeper soils, there was much less compound-specific difference in $\Delta^{14}C_{PLFA}$ values.

In October 2002, the $\delta^{13}C$ values of phytane and biyphytane from PR104 samples ranged between −44 and −31‰, with similar values for each at corresponding depths (Fig. 7). The exception was the deepest sample (163–171 cm) which had $\delta^{13}C$ values of phytane and biphytane of −69 and −48‰, respectively. The $\delta^{13}C$ values of phytane and biphytane were even more negative (−49 to −86‰) from the two depths measured (102–122 cm, 122–142 cm) at PR 104 in May 2004 (Fig. 7). As in the October 2002 collection, the $\delta^{13}C$ values for phytane and biphytane were distinctly different in the deepest sample, which in May 2004 was very close to the standing water table.

16s rDNA and functional gene analyses

Figure 9 summarizes the 16s rDNA gene libraries created for one PR102 sample and four PR104 samples. General trends are a substantial decrease in the relative abundance of Firmicutes OTUs and increases in Alpha, Beta and Gammaproteobacteria OTUs in the CBM-impacted soils. Increases in Alphaproteobacteria were dominated by rhizobiales and increases in γ-Proteobacteria by both Methylococcales and Xanthomonadales. Increases within β-Proteobacteria were dominated by *Burkholderiales* and decreases in the Firmicutes by *Bacilli* and *Clostridia* (Fig. 9). No potential type I methanotroph and relatively few potential type II methanotroph OTUs were detected in the PR102 sample (Table 1). All four PR104 samples

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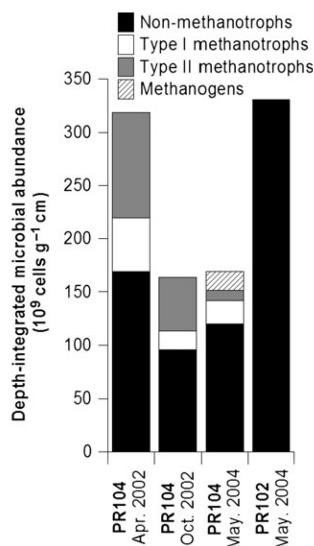

Fig. 6. Depth-integrated cell numbers estimated from concentrations of PLFAs and phytane/biphytane. Integrated abundance = ∑([PLFA] × conversion factor × span of depth interval). For methanogens, phytane plus biphytane concentrations were used instead of PLFA. Conversion factor for PLFAs was 4.0×10^{-17} moles PLFA per bacterial cell (Green & Scow, 2000). For phytane plus biphytane, it was 8.5×10^{-19} mole of phospholipid-derived isoprenoidal side chain per bacterial cell (Mancuso *et al.*, 1990). Methanogen abundance was only estimated for the deepest two depths of the May, 2004 PR104 collection. Phytane plus biphytane concentrations in other samples were not utilized for estimates in any other depths or collections.

contained OTUs belonging to the order Methylococcales, which hosts all type I methanotrophs. OTUs belonging to several families in the order Rhizobiales, known to host type II methanotrophs, were detected in the PR104 samples. Relatively large copy numbers of functional gene mcrA, which codes for the terminal enzyme complex in methanogens, were found only in the two deep PR104 soil samples (Fig. S5).

Discussion

Comparison of estimates of methanotroph abundance

The two different PLFA-based estimates of type I methanotroph abundance were well-correlated while the estimates of type II methanotrophs were not (Fig. 4a). The slope of the linear regression (4.2) for the type I comparison is within the range of conversion factors (2.4–6.7) previously used to estimate the abundance of total PLFAs associated with type I methanotrophs based on concentrations of 16:1ω8c (Sundh *et al.*, 1995; Costello *et al.*, 2002; Macalady *et al.*, 2002; Bastviken *et al.*, 2003; Sundh *et al.*, 2005). In four out of the five samples for which 16s rDNA was analyzed, the estimates of type I abundance based on PLFAs are consistent with type I methanotroph OTU abundances (Fig. 10a and b). This is surprisingly good agreement between the two different biochemical measures because phospholipids are thought to provide a better real-time measure of microbial abundance (White, 1994).

The correlation observed between the two PLFA-based estimates for type II methanotrophs was far less robust than that observed for type I methanotrophs (Fig. 4b). Furthermore, the slope of 4.9 for the linear regression (Fig. 4b) was much larger than the conversion factor of 2.0 previously used to estimate type II abundance from 18:1ω8*c* concentrations (Bastviken *et al.*, 2003; Sundh *et al.*, 2005). Some very large discrepancies (> 10 fold) were observed between the two estimates, especially in April and October 2002 surface soils and one intermediate depth (51–65 cm) in October 2002. These discrepancies likely resulted from populations of type II methanotroph strains that produce substantial amounts of 18:1ω7*c* but very little or no 18:1ω8*c* (e.g. *Methylocella* sp.; Bodelier *et al.*, 2009). The abundance of OTUs belonging to families in the order Rhizobiales, which host type II

Table 1. Relative abundance of operational taxonomic units that are potentially derived from type I or type II methanotrophs

Site	PR 102	PR 104			
Date	May 2004	May 2004		October 2002	
Depth Interval (cm)	122–137	36–51	122–143	51–65	163–171
Type I methanotrophs					
Gammaproteobacteria/Methylococcales	0.00	1.04	0.28	1.11	2.47
Type II methanotrophs					
Alphaproteobacteria/Rhizobiales/Methylobacteriaceae	0.00	0.26	0.06	0.00	0.00
Alphaproteobacteria/Rhizobiales/Methylocystaceae	0.25	0.20	0.06	0.93	1.51
Alphaproteobacteria/Rhizobiales/Methylocystaceae/Methylocystis	0.00	0.00	0.17	2.22	7.66
Alphaproteobacteria/Rhizobiales/Beijerinckiaceae	0.16	0.39	0.56	5.61	0.30

Values are percent of total OTUs detected in each sample.

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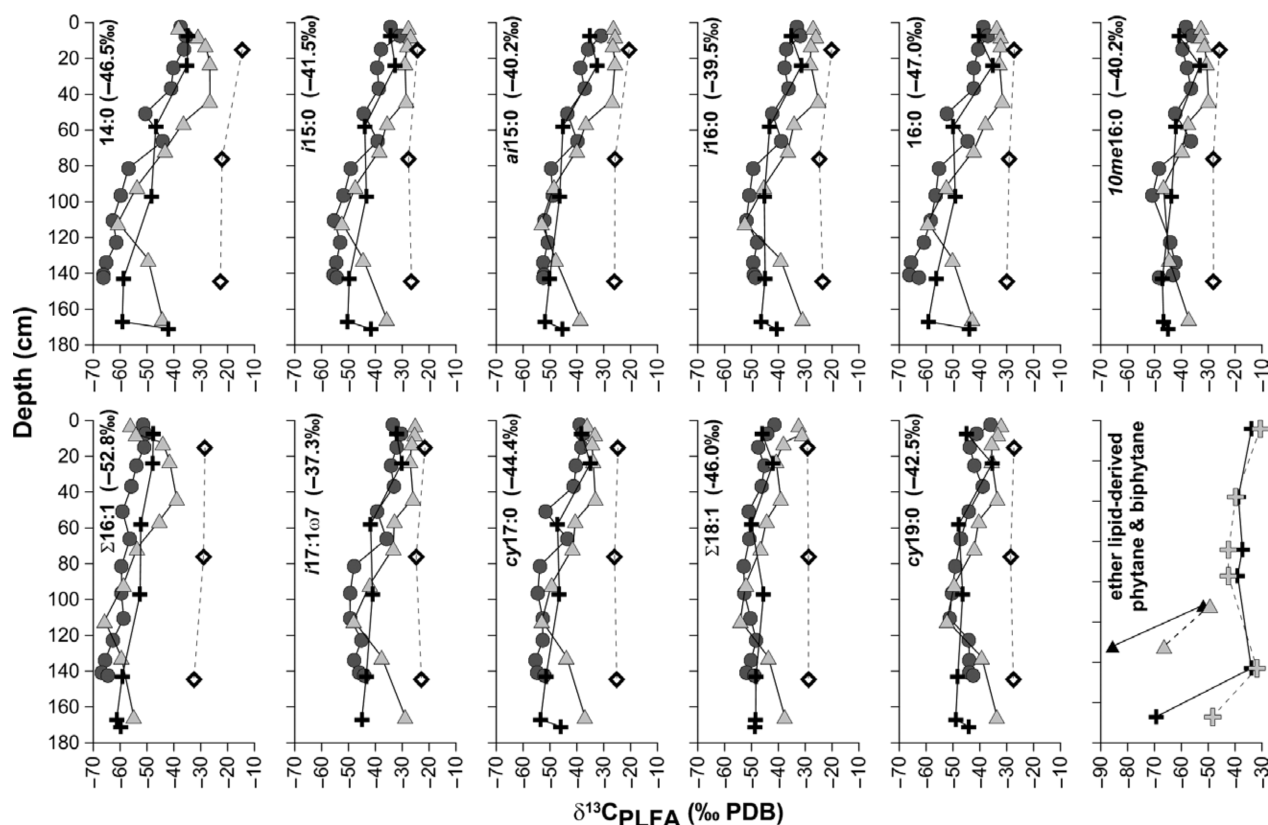

Fig. 7. δ^{13}C values of PR102 and PR104 soil PLFAs and ether lipid derivatives. PR 104 April 2002 (circles), October 2002 (crosses), May 2004 (triangles), PR 102 May 2004 (diamonds). For ether derived lipids, black and gray symbols indicated phytane and biphytane, respectively. Average value for all PR 104 samples collected is in parentheses. Values and associated analytical errors are available in Table S4 in the online supplementary data.

methanotrophs, was highly correlated with type II PLFA abundance as determined by soil profile comparison (Fig. 10c). A similar correlation between potential type II OTUs and concentrations of the 18:1ω8*c* biomarker was not as strong due primarily to the October 2002 PR 104 (51–65 cm) sample (Fig. 10d). This inconsistency is likely due to the large percentage of OTUs belonging to the family Beijerinckiaceae in this sample. Bodelier *et al.*, 2009 showed that strains of type II methanotrophs belonging to Beijernckiaceae produce most of their PLFAs as 18:1ω7*c* and produce no 18:1ω8*c*. From $^{13}CH_4$ labeling experiments, Crossman *et al.* (2004) similarly observed that only 18:1ω7*c* was labeled in shallow landfill soils, but both 18:1ω7*c* and 18:1ω8*c* were labeled in deeper soils.

A critical examination of PLFA profiles in combination with 16s rDNA analyses provided a better assessment of methanotroph populations than PLFA biomarkers alone. However, the profile-fitting approach is based on assumptions that are not fully met and has some inherent errors. The PLFA profile-fitting approach could have been improved with more extensive sampling of the control soils and PLFA profiles that spanned greater time, depth, and areal extent. More 16s rDNA analyses would also have allowed for more comprehensive evaluation of discrepancies between biomarker and profile-fitting estimates of methanotroph populations. Furthermore, laboratory- or field-based ^{13}C-labelling studies on the PR soils could have provided more direct evidence of PLFAs produced by methanotrophs (Crossman *et al.*, 2004; Maxfield *et al.*, 2012).

Despite some of the deficiencies in this current study, the initial results presented here suggest that PLFA profile fitting might serve as an alternative approach to track low-affinity methanotroph populations. Future investigations might include 16s rDNA analyses and ^{13}C-labelling studies to provide more detailed information about the methanotroph community and the PLFAs that they produce in a given ecosystem. Once established, the profile-fitting approach could be used to track methanotroph populations spatially and temporally. This approach could potentially be less costly and less biased than ^{13}C-labelling methods which, depending on laboratory- or field-based studies, require varying degrees of manipulation. However, to ensure a complete characterization of the background (nonmethanotrophic) soil microbial

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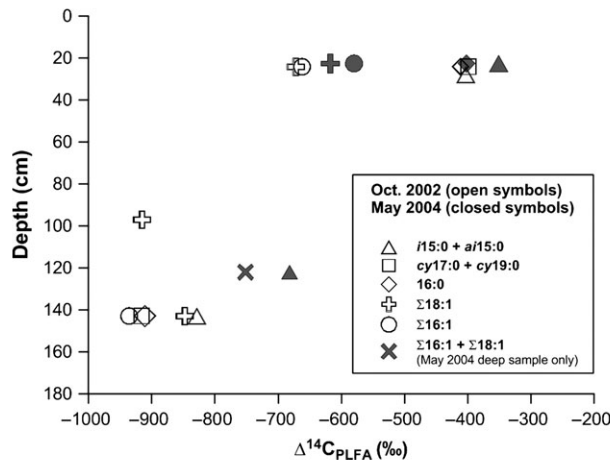

Fig. 8. Δ^{14}C values of PLFAs from select PR104 samples. The shallow October 2002 *i*15:0+ *ai*15:0 sample (open triangle) is from the same depth as other PLFAs shown – symbol is offset vertically for clarity.

community, use of the profile-fitting approach in future studies should be accompanied by a thorough spatial and temporal analysis of controls soils.

CBM oxidation and modern methanogenesis

Extensive oxidation of CBM in the PR104 soils is indicated by the high concentrations of ^{13}C-depleted CO_2 ($\delta^{13}C_{CO2}$ = −39 to −44‰). These fall within the range of $\delta^{13}C_{CO2}$ values predicted (−27 to −80‰) by applying stable carbon isotope fractionation effects expressed during microbial CH_4 oxidation (Barker and Fritz, 1981; Templeton *et al.*, 2006) to $\delta^{13}C_{CH4}$ values at PR104. Relatively high concentrations of CO_2 are not uncommon in coal-bed gasses, but the $\delta^{13}C_{CO2}$ values reported for CO_2 in coal-bed gasses in the San Juan Basin are ^{13}C-enriched (7–19‰; Rice, 1993). Convex depth profiles of both CH_4 and O_2, decreasing concentrations and increasing δ^{13}C values of CH_4 with decreasing soil depth, and elevated concentrations of N_2O have all been linked with aerobic CH_4 oxidation in other environments and provide additional evidence of extensive CBM oxidation in the PR104 soils (Coleman *et al.*, 1981; Bergamaschi *et al.*, 1998; Mandernack *et al.*, 2000, 2009).

Soil gas measurements at PR102 indicate that the soil is not impacted by CBM seepage. Concentrations and δ^{13}C values of CO_2 are consistent with CO_2 production by heterotrophic respiration of SOM (Ehleringer *et al.*, 2000). The relatively negative $\delta^{13}C_{CH4}$ values at PR102 (−62 to −65‰) are indicative of biogenic CH_4 (Whiticar, 1999) and consistent with similarly negative $\delta^{13}C_{CH4}$ values (−53 to −65‰) measured for CH_4 produced during anaerobic incubations of deep soils collected from both PR102 and 104 (Fig. S6, Table S5). In contrast, very high CH_4

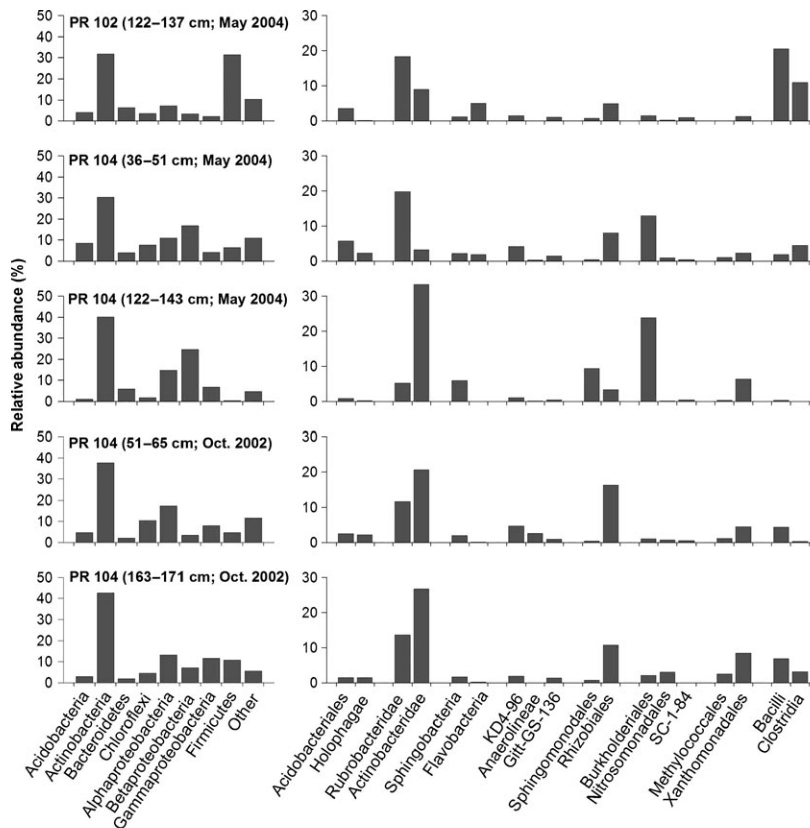

Fig. 9. Relative abundance of operational taxonomical units associated with selected bacterial phyla, orders, and families as determined by nearest neighbor analysis in the Greengenes (greengenes.lbl.gov) database for PR102 and PR 104 soil libraries.

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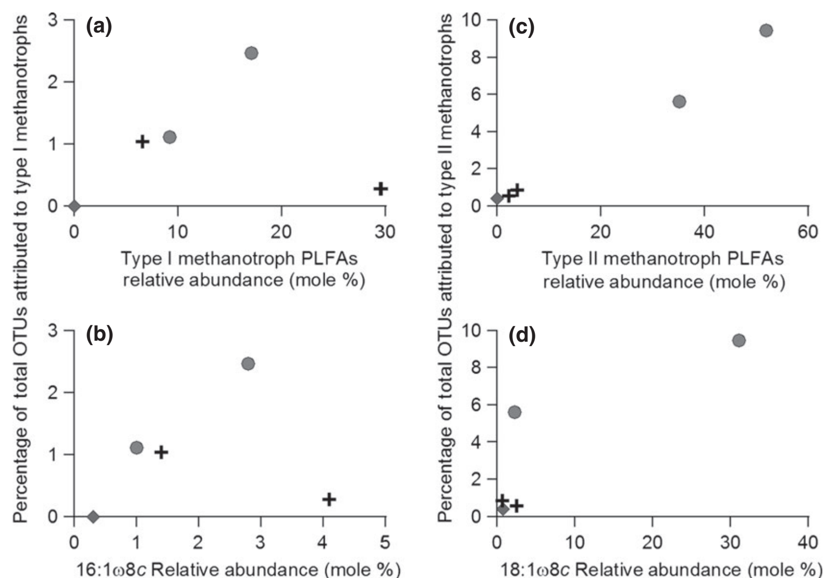

Fig. 10. Comparison of potential methanotroph OTUs and estimated methanotroph PLFAs. Potential type I methanotroph OTUs are compared with type I PLFAs estimated by profile comparisons (a) and the 16:1ω8*c* biomarker (b). Potential type II methanotroph OTUs are compared to type II PLFAs estimated by soil PLFA profile comparisons (c) and the 18:1ω8*c* biomarker (d). PR 102 May 2004 (diamond), PR 104 May 2004 (crosses), PR 104 October 2002 (circles).

concentrations at PR104 can only be explained by CBM seepage. However, the $\delta^{13}C_{CH4}$ value at depth (−50‰) in May 2004, which is more negative than the range (−38.5 to −46.6‰) reported for CBM in the San Juan Basin (Rice, 1993), suggests that the anoxic conditions at depth resulting from an upward flux of CBM are favorable to biogenic methanogenesis. This conclusion is consistent with the incubation experiments (Fig. S6) and further supported by the $\Delta^{14}C$ value (−930‰) of CH_4 at depth. Because CBM is expected to be ^{14}C-dead, a value of −930‰ suggests that at least 7% of the CH_4 in this sample was generated from more modern carbon via microbial methanogenesis.

Further evidence for enhanced methanogenesis at depth in the PR104 samples is the much greater abundance of mcrA genes in the deep vs. shallower PR104 soils (Fig. S5). Phytane derived from the phospholipid fraction of total lipids (Fig. 5b and c) could have originated from either archaeol or hydroxyarchaeol core lipids with a phosphate ester polar head group. Biphytane likely originated from caldarchaeol core lipids with similar polar head groups. Although archaeol and caldarchaeol are archaeal biomarkers that are not specific for methanogens, the very negative $\delta^{13}C$ values of phytanyl chains derived from these core lipids, particularly in the deeper PR 104 soils, are indicative of methanogens active in CH_4 cycling (Pancost *et al.*, 2000; Londry *et al.*, 2008; Alperin & Hoehler, 2009).

Controls on methanotroph populations

Type I and II methanotroph populations were very dynamic in the CBM-impacted soils (Fig. 5). The maximum absolute concentrations of 16:1ω8*c* and 18:1ω8*c* detected in PR104 soils (13 and 18 nmol g^{-1}, respectively) are on the same order as those detected in a variety of landfill cover soils (< 20 and < 50 nmol g^{-1} for 16:1ω8*c* and 18:1ω8*c*, respectively) (Börjesson *et al.*, 1998, 2004; Watzinger *et al.*, 2008) and an order of magnitude greater than measured in rice paddy soils and peat bogs (Sundh *et al.*, 1995; Macalady *et al.*, 2002). The relative depth distributions of type I and type II methanotrophs observed for the April 2002 and May 2004 soils were also similar to those previously observed in landfill soils where type II methanotrophs dominated at the lower depths exposed to higher rates of CH_4 seepage (Crossman *et al.*, 2004). These field observations are consistent with laboratory studies showing type I and II methanotrophs grow optimally under low and high CH_4 conditions, respectively (Graham *et al.*, 1993; Amaral *et al.*, 1995; Amaral & Knowles, 1995; Henckel *et al.*, 2000). Type I methanotrophs may also be favored at lower temperatures (Börjesson *et al.*, 2004) possibly explaining their higher abundance in the first few cm of soil during spring samplings (Fig. 5a and c). Because type II methanotrophs can fix N_2, they are able to out-compete type I methanotrophs when fixed nitrogen is limiting as might be expected in deeper soils (Graham *et al.*, 1993; Hanson & Hanson, 1996; Buckley *et al.*, 2008), and also observed in deep anoxic groundwater of sedimentary and granitic formations (Mills *et al.*, 2010). This competitive advantage is best expressed at low O_2 concentrations due to the O_2 sensitivity of nitrogenase enzymes and giving type II methanotrophs an advantage in deeper, more anoxic soils (Graham *et al.*, 1993; Hanson & Hanson, 1996).

Soil moisture content can be a major influence on methanotroph populations resulting in saturated pore spaces that inhibit the transport of CH_4 and O_2 or dry conditions that desiccate cells (Whalen *et al.*, 1990; Adamsen

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& King, 1993; Bender & Conrad, 1995; Börjesson *et al.*, 1998). Relatively dry conditions throughout the summer likely limited methanotroph populations in the October 2002 surface soils, but may also have allowed for greater O_2 penetration into deeper soils and a more broadly distributed methanotroph population. Higher depth-integrated populations of methanotrophs at PR104 in April 2002 vs. May 2004 may have resulted from a greater flux and availability of CH_4 as inferred from soil gas profiles (Figs 1 and 6).

CBM as a microbial carbon source

$\delta^{13}C_{PLFA}$ values

Given that the PR102 soils are not impacted by CBM seepage, the $\delta^{13}C_{PLFA}$ values for these soils can be assumed representative of heterotrophic bacteria assimilating SOM-derived carbon. The presence of large amounts of coal fragments and dust within the soil matrix, which was shed from a nearby exposure of the Fruitland Formation, precluded the accurate measurement of the $\delta^{13}C$ values for bulk SOM at either PR102 or PR104, but the $\delta^{13}C_{CO2}$ values measured at PR102 (−21.4 to −23.6‰) provide a good estimate of the $\delta^{13}C$ value of SOM being processed by soil microorganisms. The average $\delta^{13}C_{PLFA}$ value for the PR102 soils of −25.7‰ is consistent with this estimated range of $\delta^{13}C_{SOM}$ because pure culture and field studies have shown $\delta^{13}C_{PLFA}$ values of aerobic heterotrophs are up to 3‰ more negative than their carbon source (Monson & Hayes, 1982; Blair *et al.*, 1985; Teece *et al.*, 1999; Burke *et al.*, 2003).

More negative $\delta^{13}C_{PLFA}$ values for the PR104 soils reveal that CBM provides a substantial secondary carbon and energy source for the soil microbial community (Fig. 7). Our results showed that PLFAs common in type I methanotrophs (Σ16:1, 16:0, 14:0) were the most ^{13}C-depleted followed by PLFAs common in type II methanotrophs (Σ18:1) (Fig. 7). These measured values are consistent with literature studies of carbon isotope fractionation by type I and II methanotrophs (Zyakun *et al.*, 1987; Summons *et al.*, 1994; Jahnke *et al.*, 1999; Templeton *et al.*, 2006). Although the overall fractionation effects associated with CH_4 oxidation and assimilation are variable, the $\delta^{13}C_{PLFA}$ values of type I methanotrophs can be as much as 30‰ depleted in ^{13}C relative to their CH_4 source (Jahnke *et al.*, 1999). Because type II methanotrophs assimilate some of their carbon from CO_2, they typically have more positive $\delta^{13}C_{PLFA}$ values than type I methanotrophs (Jahnke *et al.*, 1999; Watzinger *et al.*, 2008).

The $\delta^{13}C$ values of PLFAs more typical of 'nonmethanotrophs' are also much more negative for PR104 than PR102 samples (Fig. 7). These include PLFAs that are often associated with Gram-positive (e.g. *i*15:0, *ai*15:0; *10me*16:0) and Gram-negative (e.g. *cy*17:0, *cy*19:0) soil bacteria (Zelles, 1997). Watzinger *et al.* (2008) observed similar ^{13}C depletion of nonmethanotroph PLFAs in soils fumigated with landfill gas. While some of these PLFAs (e.g. *i*15:0, *cy*17:0) are produced in small amounts by some methanotrophs (Bowman *et al.*, 1991; Bodelier *et al.*, 2009), it is unlikely that this is the sole explanation for the ^{13}C-depletion observed in these PLFAs. Several studies that analyzed the incorporation of either ^{13}C- or ^{14}C-labelled CH_4 into soil or sediment PLFAs showed significant CH_4-carbon incorporation into 16:0, 16:1 and 18:1 PLFAs but not into branched or cyclopropane structures (Boschker *et al.*, 1998; Bodelier *et al.*, 2000; Bull *et al.*, 2000; Macalady *et al.*, 2002; Knief *et al.*, 2003). Two different laboratory isotope labeling studies of landfill soils showed significant but delayed incorporation of CH_4-derived carbon into PLFAs such as *i*15:0, *ai*15:0, *cy*17:0, and *ai*17:0, indicating consumption of CH_4-derived carbon by secondary consumers. In contrast, labeled CH_4 carbon was immediately incorporated into 16:1 and 18:1 PLFAs common in methanotrophs (Crossman *et al.*, 2004; Maxfield *et al.*, 2012). The PLFAs 10*me*16:0 and *i*17:1ω7, which are also ^{13}C-depleted in the PR104 soils, have not been shown to be produced by methanotrophs in pure culture (Nichols *et al.*, 1985; Bowman *et al.*, 1991; Guckert *et al.*, 1991; Bodelier *et al.*, 2009). Thus, it appears from the $\delta^{13}C_{PLFA}$ values that CBM is not only a carbon source for the methanotrophic community, but is also incorporated into the nonmethanotrophic bacterial community at PR104.

$^{14}C_{PLFA}$ values

The $\Delta^{14}C_{PLFA}$ values have greater power for mass balance estimates of CBM incorporation than $\delta^{13}C$ values. It is standard that reported $\Delta^{14}C$ values are normalized to their respective $\delta^{13}C$ values to account for kinetic isotope fractionation effects (Stuiver & Polach, 1977). Variations in the carbon isotope fractionation effects associated with CH_4 metabolism (ε_{CBM}) have contributed to difficulties with using $\delta^{13}C_{CH4}$ values to quantify CH_4 oxidation in landfill cover soils (De Visscher & De Poureq, 2004; Templeton *et al.*, 2006). The ability to ignore such variable fractionation effects makes ^{14}C-based calculations of the fraction of CBM incorporated into a particular PLFA (F_{CBM}) straight forward using the equation:

$$F_{CBM} = \frac{\Delta^{14}C_{PLFA} - \Delta^{14}C_{SOM}}{\Delta^{14}C_{CBM} - \Delta^{14}C_{SOM}} \quad (1)$$

The $\Delta^{14}C$ value of soil gas CO_2 (−46‰) collected from the 100 cm depth at PR102 is a good estimate of the

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$\Delta^{14}C$ value of labile SOM that is available to soil microorganisms (Trumbore, 2000; Fierer *et al.*, 2005). The $\Delta^{14}C$ value of soil CH_4 is likely variable depending on the relative contribution of biogenic CH_4 produced from more modern carbon as previously discussed. However, because of its ancient origin, CBM itself is assumed to have a $\Delta^{14}C$ value of $-1000‰$.

Values of F_{CBM} calculated using Eqn. 1 indicate that CBM contributed between 32 and 93% of the carbon incorporated into PLFAs (Table S1). A plot of $\delta^{13}C_{PLFA}$ vs. F_{CBM} values (Fig. 11) illustrates that both stable and radiocarbon isotopes consistently indicate a dual carbon source for PLFAs in the PR 104 soils: modern carbon derived from dead plant tissue ($\delta^{13}C$ value $\sim-23‰$) and ancient CBM ($\delta^{13}C$ value $\sim-40‰$). The linear regression in this mixing plot intersects the *y*-axis at $-22.5‰$ which is within 3‰ of the average $\delta^{13}C$ value of PR102 PLFAs ($-25.7‰$). The regression indicates that the other end-member, PLFAs derived entirely from CBM, has a $\delta^{13}C$ value of $-57‰$ which is 10‰ more negative than the most depleted $\delta^{13}C$ value reported for San Juan Basin CBM ($-46.6‰$) (Rice, 1993). This is expected given that methanotrophs preferentially assimilate $^{12}CH_4$ (Summons *et al.*, 1994; Jahnke *et al.*, 1999; Templeton *et al.*, 2006). The increased vertical scatter in the data for larger F_{CBM} values (Fig. 11) is likely due to isotope fractionation effects associated with the assimilation of CH_4 or methane oxidation intermediates (e.g. methanol), which are more varied than those associated with assimilation of SOM, and also to large variations in $\delta^{13}C_{CH4}$ with depth as observed in the PR104 soils (Fig. 1).

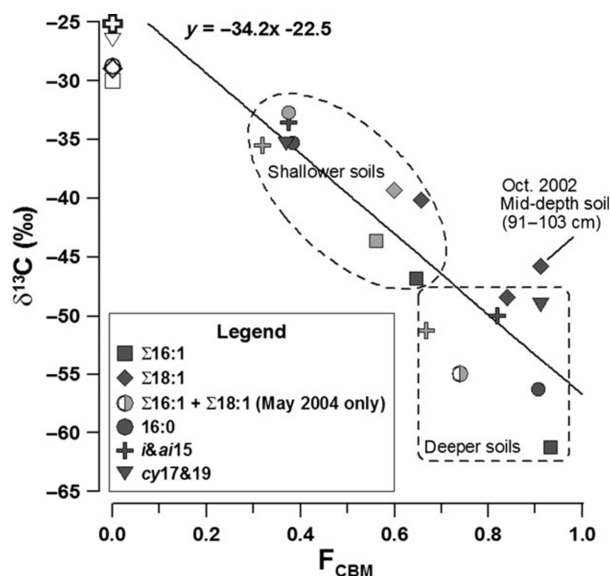

Fig. 11. Plot of the $\delta^{13}C$ vs. F_{CBM} Values for PR104 and PR102 Soil PLFAs. PR102 May 2004 PLFAs are indicated by open symbols, PR104 October 2002 dark gray symbols, and PR104 May 2004 by light gray. Values from shallower and deeper soil are indicated by dotted lines. The $\delta^{13}C$ values for the PR102 PLFAs are averages for all depths and a F_{CBM} value of 0.0 for all of the PR102 PLFAs was assumed. The regression is fit through the PR104 data points only.

The F_{CBM} value of 56% for the Σ16:1 PLFAs from the shallow soil collection in May 2004 indicates a greater contribution from CBM than can be accounted for by methanotroph PLFAs alone. From the PLFA profile comparison, it was estimated that type I methanotroph PLFAs only contributed 32% of the total 16:1 PLFAs in this sample. Assuming that type I methanotroph PLFAs were derived entirely from CBM (i.e. they have a F_{CBM} value of 100%); mass balance requires that the nonmethanotrophic portion of the Σ16:1 has a F_{CBM} value of 35% (Table S1). Similarly, the F_{CBM} values for the nonmethanotrophic portion of the 16:0 and Σ18:1 in this sample were 36 and 41%, respectively (Table S1). These values are consistent with the F_{CBM} value of 32% for the *i*15:0/*ai*15:0 PLFAs in this shallow sample. The *i*15:0 and *ai*15:0 PLFAs are not common in methanotrophs and thus serve as a measure of the contribution of CBM to the nonmethanotrophic community. A similar, but wider range of F_{CBM} values (37–54%) was determined for nonmethanotroph PLFAs in the October 2002 shallow soil. Conversely, higher estimates of F_{CBM} were calculated for nonmethanotroph PLFAs in the deeper soils of October 2002 than in May 2004 (70–91% and 51–67%, respectively). The greater contribution of CBM to the deeper soil microbial community in October 2002 may have resulted from greater CH_4 oxidation due to more O_2 penetration through a drier soil column (Fig. 1).

Despite the additional carbon and energy source that CBM is evidently providing to the soil microbial community at PR104, the net result of CBM seepage is lower bacterial abundance compared with PR102 (Figs 5 and 6). This is likely due to the decreased availability of O_2 throughout the soil column at PR104 (Fig. 1). Although the anoxic conditions that develop in the deeper CBM-impacted soils result in increased methanogen populations, estimates suggest that, on a cell number basis, this enhanced population does not compensate for the decrease in bacteria.

Potential Pathways for cycling of CBM

Although several attempts to measure the $\delta^{13}C$ $_{values}$ of SOM in the PR soils were hindered by the presence of coal particles, it is likely that carbon derived from CBM has become integrated into the SOM pool through the repeated growth and death of methanotrophic cells (Kindler *et al.*, 2006; Maxfield *et al.*, 2012). Thus, a host of soil microorganisms likely incorporate CBM-derived carbon indirectly.

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However, transfer of CBM-derived carbon to nonmethanotrophic microbial communities could also be occurring by more direct mechanisms. These include the assimilation of CO_2 produced from oxidation of CBM by heterotrophic bacteria through anaplerotic reactions (Miltner *et al.*, 2004) and the predation of methanotrophic bacteria (Bastviken *et al.*, 2003; Eller *et al.*, 2005; Murase & Frenzel, 2007; Jones & Grey, 2011).

It is possible that a substantial amount of CBM-derived carbon is transferred to the nonmethanotrophic community through the consumption of exuded CH_4 oxidation intermediates or polysaccharides (Hilger *et al.*, 2000; Hršak & Begonja, 2000). *Methylophilales* are methylotrophs that can use methanol but are incapable of utilizing CH_4 (Lidstrom, 2006). However, $\leq$ 1% of OTUs in PR104 samples were related to *Methylophilales*. The recognized diversity of bacteria capable of methylotrophy has recently been expanded and includes both Gram-positive and Gram-negative bacteria (e.g. members of Sphingomonadales Burkholderiales and *Arthrobacter*) (Borodina *et al.*, 2000; Lidstrom, 2006; Boden *et al.*, 2008; Kalyuzhnaya *et al.*, 2008). Consumption of CBM-derived compounds by diverse methylotrophic bacteria could account for a variety of PLFAs, including branched chain PLFAs (Zelles *et al.*, 1997), being synthesized from CBM-derived carbon.

Conclusions

The microbial community in the CBM-impacted soil column at PR104 was substantially different in abundance and composition than the community at the PR102 control site. Elevated concentrations of the PLFA biomarkers 16:1ω8*c* and 18:1ω8*c* in PR104 soils indicated type I and II methanotroph communities that are similar in size to those previously observed in landfill cover soils. Estimates of the abundance of type I methanotrophs made by comparing CBM-impacted, and control soil PLFA profiles were similar to estimates made using just the 16:1ω8c biomarker. In contrast, abundances of type II methanotrophs estimated by PLFA profile comparison were typically much greater than estimates made using the 18:1ω8*c* biomarker. Results of 16S rDNA analysis on select soil samples indicate that estimates of type II methanotrophs made using the soil PLFA profile comparison were more accurate because of the presence of type II methanotrophs that do not produce any 18:1ω8*c*. These methanotroph populations were dynamic, likely responding to environmental conditions such as moisture, temperature, and availability of CH_4, O_2 and fixed N.

Consumption of soil gas O_2 during CH_4-oxidation in the shallow soils likely contributed, in part to more anoxic conditions in the deeper PR104 soils resulting in decreased abundances of bacteria and increased populations of anaerobic methanogens. Although the CBM seepage dominates the overall CH_4 load in the PR104 soils, these methanogens appear to generate substantial amounts of CH_4 from relatively modern carbon as evidenced by the $\Delta^{14}C_{CH4}$ value that was not ^{14}C dead.

Both $\delta^{13}C$ and $\Delta^{14}C$ values of soil PLFAs indicate that methanotrophs in the PR104 soil act as primary producers in a trophic system fed mostly by fossil CBM. Calculations using $\Delta^{14}C_{PLFA}$ values show that CBM contributes between 32 and 93% of the carbon incorporated into PR104 PLFAs. Carbon derived from CBM is incorporated into PLFAs that can be attributed to both methanotrophic and nonmethanotrophic bacteria. Increased numbers of OTUs were detected in PR104 samples that are related to genera which contain strains capable of using methanol as a carbon and energy source. Methanol may serve as an important direct intermediate for the transfer of CBM-derived carbon from methanotrophs to the nonmethanotrophic microbial community.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Location of several areas of coal-bed methane seepage along an outcrop of the Fruitland Formation near Durango, Colorado.
Fig. S2. Example chromatograms of PLFAs (as FAMEs) from PR102 and PR104 soils.
Fig. S3. Concentrations of acid volatile sulfide (AVS), NO_3^- , and SO_4^{2-} measured in PR104 soil samples collected April 2002.
Fig. S4. Correlation of PLFAs attributed to type I (a) and type II (b) methanotrophs by profile comparison.
Fig. S5. Abundance of amplified mcrA genes in PR102 and PR104 samples.
Fig. S6. Methane and CO_2 production in anaerobic incubations of PR104 and PR102 soils that were collected in May 2004.
Table S1. Δ^{14}C and calculated F_{CBM} values of PLFAs from PR 104 soils.
Table S2. Soil characteristics of Pine River soils.
Table S3. Relative (mole %) and total concentrations of PLFA in PR 102 and PR 104 soils.
Table S4. $\delta^{13}C_{PLFA}$ values for PR 102 and PR 104 soils.
Table S5. δ^{13}C values of headspace CH_4 collected at the end of anaerobic incubations of Pine River soils.

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