# A Microbial Link between Elevated $CO_2$ and Methane Emissions that is Plant Species-Specific

**Jenny Kao-Kniffin · Biao Zhu**

**Abstract** Rising atmospheric $CO_2$ levels alter the physiology of many plant species, but little is known of changes to root dynamics that may impact soil microbial mediation of greenhouse gas emissions from wetlands. We grew co-occurring wetland plant species that included an invasive reed canary grass (*Phalaris arundinacea* L.) and a native woolgrass (*Scirpus cyperinus* L.) in a controlled greenhouse facility under ambient (380 ppm) and elevated atmospheric $CO_2$ (700 ppm). We hypothesized that elevated atmospheric $CO_2$ would increase the abundance of both archaeal methanogen and bacterial methanotroph populations through stimulation of plant root and shoot biomass. We found that methane levels emitted from *S. cyperinus* shoots increased 1.5-fold under elevated $CO_2$, while no changes in methane levels were detected from *P. arundincea*. The increase in methane emissions was not explained by enhanced root or shoot growth of *S. cyperinus*. Principal components analysis of the total phospholipid fatty acid (PLFA) recovered from microbial cell membranes revealed that elevated $CO_2$ levels shifted the composition of the microbial community under *S. cyperinus*, while no changes were detected under *P. arundinacea*. More detailed analysis of microbial abundance showed no impact of elevated $CO_2$ on a fatty acid indicative of methanotrophic bacteria ($18:2\omega6c$), and no changes were detected in the terminal restriction fragment length polymorphism (T-RFLP) relative abundance profiles of acetate-utilizing archaeal methanogens. Plant carbon depleted in $^{13}C$ was traced into the PLFAs of soil microorganisms as a measure of the plant contribution to microbial PLFA. The relative contribution of plant-derived carbon to PLFA carbon was larger in *S. cyperinus* compared with *P. arundinacea* in four PLFAs (i14:0, i15:0, a15:0, and $18:1\omega9t$). The $\delta^{13}C$ isotopic values indicate that the contribution of plant-derived carbon to microbial lipids could differ in rhizospheres of $CO_2$-responsive plant species, such as *S. cyperinus* in this study. The results from this study show that the $CO_2$–methane link found in *S. cyperinus* can occur without a corresponding change in methanogen and methanotroph relative abundances, but PLFA analysis indicated shifts in the community profile of bacteria and fungi that were unique to rhizospheres under elevated $CO_2$.

## Introduction

Concerns over the impacts of climate change on greenhouse gas emissions have peaked interest in examining microbial responses to elevated atmospheric levels of $CO_2$ [1–3]. In wetlands, methane ($CH_4$) is a major greenhouse gas that is partly mediated by soil microorganisms. Plant rhizodeposits (root cells and exudates), microbial necromass, plant litter, and soil organic matter are organic C substrates mineralized by a subset of archaeal methanogens that may be impacted by elevated $CO_2$ levels. The $CO_2$ stimulation of C-fixation in plants can lead to increased inputs of organic C into sediments and enhanced emissions of methane through methanogenesis. However, plant species respond differently to changes in atmospheric $CO_2$ levels. Differential growth responses of plant species to elevated atmospheric $CO_2$ have been found [4, 5], yet few studies examine how methane dynamics are altered with rising $CO_2$ levels. Given the importance of natural wetlands in comprising 20–39 % of total global methane emissions [6], it is important to understand how changing $CO_2$ levels stimulate shifts in microbial community structure relevant to methane dynamics.

Methane emissions from natural wetlands are determined in large part by the differences in methane production from

J. Kao-Kniffin (✉) · B. Zhu
Department of Horticulture, Cornell University,
134A Plant Sciences Building, Ithaca, NY 14853, USA
e-mail: jtk57@cornell.edu

B. Zhu
Earth Sciences Division, Lawrence Berkeley National Laboratory,
Berkeley, CA 94720, USA

methanogenic archaea and methane consumption by methanotrophic bacteria [7]. Wetland plants play a critical role in modifying the net emission of methane by altering the quantity of the organic C source for methanogenic archaea and determining the oxidation levels in the rhizosphere for methanotrophic bacteria. Additionally, wetland plants alter net emissions of methane by providing a gas pathway allowing the transfer of methane bound in sediment porewater to the atmosphere through interconnected gas-filled intercellular spaces. Thus, wetland plants that respond positively to elevated $CO_2$ could enhance methane emissions through three important routes: (1) altering the quantity of the organic C source utilized by methanogenic archaea, (2) modifying the level of oxygen in the rhizosphere for methane consumption by methanotrophic bacteria, and (3) increasing the biomass of plants containing interconnected intercellular spaces for enhanced methane transfer from sediment porewater to the atmosphere.

Several field studies conducted in wetlands and rice paddy fields showed enhanced emissions of methane with $CO_2$ fumigation [8–13]. However, no linkages of elevated $CO_2$ to soil methanogen or methanotroph population changes have been published from these field-based $CO_2$ fumigation studies. In a European grassland FACE site with a seasonally fluctuating water table, methanogenic archaeal gene copy numbers did not change after 10 years of $CO_2$ fumigation [14]. Several controlled environment studies have reported a change in microbial community structure under elevated $CO_2$ levels linked to methane dynamics [9, 15, 16]. The lack of field-based evidence for a microbially mediated $CO_2$–methane link suggests that methanogen and methanotroph communities become acclimated to high $CO_2$ levels over time, and instead, the increase in methane is a plant-driven response. Another alternative explanation is that the set of techniques used to study microbial communities do not capture changes in community structure or function relevant to methane dynamics.

The objective of this study is to examine microbially mediated links between elevated atmospheric $CO_2$ and rising methane emissions for wetland plant species grown in controlled environment greenhouses. A previous study indicated that the use of terminal restriction fragment length polymorphism (T-RFLP) for characterizing changes in the relative abundance of different methanogenic archaeal groups was limiting, yet it still revealed a positive relationship between plant biomass and the relative abundance of a group of acetoclastic methanogens found in the rhizosphere of an invasive cattail (*Typha angustifolia* L.) [17]. In this study, we examine if the microbial contribution to the $CO_2$–methane link is highly dependent on the composition of the plant community. We focus on two co-occurring wetland plant species that are dominant in many temperate wetlands of North America. We used a multitude of microbial analyses to characterize changes in community structure with the

intention of understanding both nucleic acid-based and fatty acid-based changes. These include T-RFLP of archaeal methanogen community structure and phospholipid fatty acid (PLFA) of bacterial community structure. Additionally, we measured the contribution of plant-derived C to microbial lipids using the $^{13}C$ isotopic shift of the phospholipid fatty acids from the plant–soil systems grown under depleted $^{13}CO_2$ versus ambient $^{13}CO_2$. The $\delta^{13}C$ isotopic signal of depleted $CO_2$ sources is traceable using isotope ratio mass spectrometry.

## Materials and Methods

### Experimental Design

The greenhouses used in the labeling experiment were located on the University of Wisconsin-Madison campus Biotron facility. The $CO_2$ enrichment was replicated in three greenhouse rooms, and three additional rooms provided ambient $CO_2$ levels. Individual seedling transplants of reed canary grass (*Phalaris arundinacea* L.) and woolgrass (*Scirpus cyperinus* L. Kunth) were transferred into separate pots, with three replicate potted plants per greenhouse room. The wetland plant *P. arundinacea* is considered a noxious invader of wetlands [18], whereas *S. cyperinus* is native to North American wetlands and does not show invasive traits. These plant species were chosen in this study for their potential to respond to $CO_2$ fumigation with increased biomass accumulation, as was found in many $C_3$ fast-growing plant species [4]. There is concern that *P. arundinacea* is increasing in distribution across temperate wetlands, resulting in reduced biodiversity in invaded wetlands [19]. The potential for $CO_2$ fertilization of *P. arundinacea* invasion could further impact biodiversity losses in wetlands.

A total of nine replicates of plants were examined for each species and each $CO_2$ treatment. The seedlings were grown in pots measuring 38.1 × 15.2 cm lined with thick plastic liners and 5 cm of sand. The soil medium consisted of a 1:1 mixture of wetland soil located near Dickeyville, Wisconsin, USA (42˚39′21″N, 90˚34′39″W) and from various wetlands located in the University of Wisconsin-Madison Arboretum (43˚01′40″N, 89˚26′15″W) and topsoil (Liesener Soils Inc., Jackson, WI) that was continuously flooded to 2.5 cm above the soil surface with 10 % Hoagland's solution (Ca, Cl, Fe, Mg, N, P, S, B, Cu, Mn, Mo, and Zn) using an automatic drip system. The plants were grown for 4 months starting from seeds. Germination occurred within 1 week of difference between the two species. The greenhouse temperatures were set at 24 °C for 14 h and 18 °C for 10 h. The daily photosynthetic photon flux density in the rooms measured 490 μmol m$^{-2}$ s$^{-1}$ for 14 h, followed by 10 h of darkness. The $CO_2$ concentrations in the greenhouses averaged 380 ppm in the ambient rooms and 700 ppm in the elevated rooms. The

gas used to augment the CO$_2$ concentrations in the greenhouses was derived from commercial sources as C from petroleum byproducts depleted in $^{13}$C. The mean $\delta^{13}$ of commercially sourced CO$_2$ averages between −27 and −45‰ and ambient atmospheric CO$_2$ averages −8‰ [20].

## Methane Analysis

We measured methane flux from plants through PVC chambers encased over the plant leaves and stems. The chambers were erected at the soil surface and sealed with standing water to minimize methane diffusion and ebullition from the soil. The chambers remained open 45 min prior to sampling to allow for gas equilibration. When the chambers were sealed, gases were circulated inside the chamber using a 12-volt fan. Atmospheric pressure was maintained within the chamber through an opening created by a 20-gauge needle inserted into a gray butyl septa. Methane was sampled using a 30 mL polyurethane syringe fastened with a stop cock valve. Gases were injected into vacuumed Wheaton serum bottles sealed with gray butyl septa and aluminum collars. Methane samples were collected at times 0, 15, and 30 min to calculate a linear regression curve for flux estimations. Samples were injected into the Shimadzu 14B GC (Shimadzu Corporation, Kyoto, Japan) with flame ionization detector (FID).

## Soil and Plant Samples

Plant biomass and soil were collected 4 months after seed germination. Loose soil was removed from the roots, and sterilized spatulas were then used to collect rhizosphere soil from the surface of roots. The soil was homogenized and frozen at −20 °C prior to microbial analysis. The soils for microbial lipid analysis were additionally lyophilized prior to analysis. Plant biomass was determined after washing and then drying at 60 °C until constant weight.

## Microbial Community Analysis

*Microbial lipid analysis* Lipid analysis, known as PLFA analysis is commonly used to characterize microbial biomass. The technique allows researchers to characterize a snapshot of recently living microorganisms because phospholipids breakdown rapidly in soils [21, 22]. We used a modified procedure of phospholipid fatty acid based on the extraction of signature lipid biomarkers from soil organisms [23, 24]. Details of the extraction method are found in Kao-Kniffin and Balser [25].

The parameters for the analytical work consisted of a Hewlett-Packard gas chromatography (GC) 6890 and FID with a split/splitless inlet and an Ultra 2 (5 %-phenyl/95 % methyl; 25 m length, 0.2 mm ID, 0.33 μm film thickness) capillary column (Agilent Technologies). Hydrogen was used as the carrier gas, N as the makeup gas, and air supported the flame. Gas chromatograph conditions are set by the MIDI Sherlock program (MIDI, Inc. Newark, DE). Bacterial fatty acid standards were used to identify peaks using the Sherlock peak identification software (MIDI, Inc. Newark, DE). PLFAs were quantified by comparisons of peak areas from the sample compared to peak areas of two internal standards, 9:0 (nonanoic methyl ester) and 19:0 (nonadeconoic methyl ester), of known concentration. The fatty acids are described as 'A:BωC' where 'A' indicates the total number of carbon (C) atoms, 'B' the number of double bonds (unsaturations), and 'ω' indicates the position of the double bond from the methyl end of the molecule. The prefixes 'i' and 'a' refer to iso and anti-iso methyl branching. Hydroxy groups are indicated by "OH." Cyclopropyl groups are denoted by "cy." The structure of the PLFAs is used to indicate broad associations of microorganisms and is not commonly used to identify one specific organism for a given PLFA [26–28]. For example, monounsaturated PLFAs having double bonds are recognized as Gram-negative bacteria, while iso-, anteiso-, and mid-chain branched PLFAs indicate Gram-positive bacteria [29, 30].

*T-RFLP analysis* One of the disadvantages of using PLFA to characterize the soil microbial community is the inability to identify archaea. PLFAs were thought to be absent in the complex polar lipids of archaea, but non-ester-linked PLFAs were detected in several Euryarchaeota I strains [31]. In spite of the recent discovery, PLFAs are still considered to be inadequate biomarkers for archaeal abundance and composition. Instead, we used T-RFLP to characterize the community structure of archaeal methanogens. This polymerase chain reaction (PCR)-based method provides a fingerprint of the unidentified microbial community based on enzymatic digestion at restriction sites nearest the terminal fluorescent end of the amplified DNA. The signal intensity of a single T-RF in proportion to all T-RFs in a sample gives a relative gene frequency of different fragments with the microbial community fingerprint.

Total community DNA was extracted from soil using the UltraSoil DNA extraction kit (MoBio Laboratories, Solana Beach, CA). The universal archaeal primers Ar109f (5'-ACG/TGCTCAGTAACACGT-3') and Ar912r (5'-CTCCCCCGCCAATTCCTTTA-3') were used to amplify a section of the 16S rRNA gene [32]. We used the primer Ar109f to add the 6-carboxy-flourescein (FAM) label at the 5' end. Details of the PCR reaction and settings are found in Kao-Kniffin et al. [17]. An aliquot (16.8 μL) of the PCR products were digested with 5 U *Taq*I (Promega, Madison, WI), 1× buffer, and 1 μg bovine serum albumin for 2 h at 65 °C. A 625 bp ROX-labeled internal size standard (CHIMERx, Madison, WI) was added to the samples containing 1 μL of the digestion and 10 μL of formamide. The

samples were sent to the University of Wisconsin-Madison Sequencing Facility for analysis using denaturing capillary electrophoresis on an ABI 3700 genetic analyzer (Applied Biosystems). The resulting T-RFLP patterns were analyzed using GeneMarker v1.50 (SoftGenetics LLC, State College, PA).

### Stable Isotope Analysis

Soil and plant tissue samples were dried at 60 °C until constant weight. The samples were then ground to powder in a stainless steel ball mill. These plant and soil samples were then analyzed for C%, N%, $\delta^{13}C$, and $\delta^{15}N$ using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer at the UC Davis Stable Isotope Facility.

The extracted fatty acid methyl esters (FAME) were analyzed at the UC Davis Stable Isotope Facility using a GC-combustion-isotope ratio mass spectrometer (IRMS) system comprised of a Trace GC Ultra (Thermo Electron) interfaced with a Finnigan Delta Plus IRMS (Thermo Electron) and installed with a BPX70 column (30.0 m length, 0.25 mm ID, 0.25 μm film thickness). The samples were injected in splitless mode (inlet temperature 260 °C; carrier gas, helium; constant flow rate of 0.8 ml min–1; oven temperature rise from 100 to 190 °C at 4 °C min–1 with 10 min hold, then 190 to 250 °C at 8 °C min–1 with 5 min hold). The $\delta^{13}C$ values were corrected using working standards consisting of FAMEs calibrated against standard reference materials. Conversion of the $\delta^{13}C$ values of FAMEs to fatty acids are based on the correction of the one C methyl group addition during derivatization [33].

Based on the calculation, the relative amount of plant-derived C in microbial PLFAs is represented in samples grown in the elevated $CO_2$ rooms. The relative amount of plant-derived C ($F_{plant}$) used as microbial C source in the elevated $CO_2$ treatment was calculated using the $^{13}C$ isotopic shift of both PLFA and plant input materials between the ambient $CO_2$ treatment and the elevated $CO_2$ treatment (Eq. 1, [34]. These calculations were conducted only for the PLFAs whose $\delta^{13}C$ values differed significantly ($P < 0.05$) between the two $CO_2$ treatments (Fig. 2).

$$F_{plant} = \left(\delta^{13}C_{PLFA-elevated} - \delta^{13}C_{PLFA-ambient}\right)/ \qquad (1)$$

$$\left(\delta^{13}C_{plant-elevated} - \delta^{13}C_{plant-ambient}\right)$$

where $F_{plant}$ is the relative contribution of plant C to each individual PLFA, $\delta^{13}C_{PLFA-elevated}$ and $\delta^{13}C_{PLFA-ambient}$ are the $\delta^{13}C$ values of PLFA in the elevated and ambient $CO_2$ treatments, respectively, and $\delta^{13}C_{plant-elevated}$ and $\delta^{13}C_{plant-ambient}$

are the $\delta^{13}C$ values of shoots in the elevated and ambient $CO_2$ treatments, respectively.

### Statistical Analysis

The data were analyzed using plant species and $CO_2$ as the fixed factors and greenhouse rooms as the random factor in the ANOVA analysis. Data were analyzed using SPSS version 18.0. The effects of plant species and $CO_2$ on microbial lipid biomass, microbial lipid relative abundance (mol %), archaeal relative gene frequencies (also known as the relative signal intensity of each T-RFLP fragment), plant biomass, and methane flux was determined using post hoc Tukey's test at the $P < 0.05$ level. We tested each variable used in our analysis for normality using the Shapiro-Wilk's statistic and used arcsine transformations of microbial mol % data and log-transformations of plant biomass data. For multivariate analysis of microbial lipid data, we performed principal component analysis on the arcsine-transformed mol fractions of individual lipids.

## Results

### Plant–microbial physiological responses to $CO_2$ enrichment

We found that the increase in $CO_2$ levels in the greenhouse led to a 1.5-fold increase in methane emissions from plant leaves of *S. cyperinus* (Table 1). The increased methane flux did not correspond to enhanced shoot and root biomass of *S. cyperinus* (Table 1). Similarly for *P. arundinacea*, we found no changes to the biomass or methane emission levels when exposed to an elevated $CO_2$ atmosphere.

Despite the lack of a significant $CO_2$ fertilization effect for both plant species, the higher $CO_2$ levels led to distinct changes in the structure of the rhizosphere microbial community for *S. cyperinus*. Principal components analysis revealed a shift in the total microbial PLFA profile of *S. cyperinus* when exposed to higher $CO_2$ levels (Fig. 1). There was no corresponding shift detected for *P. arundinacea* PLFA profiles, which suggests that the $CO_2$-induced shift in microbial community structure is plant species-specific. Further analysis of PLFAs showed no significant differences in relative abundances or biomass of individual and ecologically grouped fatty acids for either plant species under the contrasting $CO_2$ levels. Unexpectedly, the lipid indicative of methanotrophic bacteria (18:1ω7c) showed no changes with $CO_2$ increases for either plant species. The PLFA data represent information only pertaining to bacterial and fungal fatty acids. Archaeal relative abundances were measured using a nucleic–acid-based method. Using the different method, no changes occurred in methanogen community structure or

**Table 1** Plant biomass (grams per pot), methane emission (milligrams CH$_4$-C per square meter per hour), total PLFA abundance of bacteria and fungi (nanomoles per gram soil), T-RFLP of methanogenic archaea (total fluorescence intensity), fungi/bacteria ratio, and δ$^{13}$C values (‰) of plant (shoots) and soil organic carbon in the four treatments at the end of the experiment

| Treatment | Shoot biomass | Root biomass | CH$_4$ emission | PLFA | T-RFLP | Fungi/bacteria ratio | δ$^{13}$C$_{plant}$ | δ$^{13}$C$_{soil}$ |
|---|---|---|---|---|---|---|---|---|
| *P. arundinacea* (ambient CO$_2$) | 51.7 (6.1)a | 32.7 (3.3)a | 1.33 (0.24)c | 0.23 (0.01)a | 19,177 (2,193)a | 0.30 (0.02)a | −28.2 (1.3)a | −22.6 (0.3)a |
| *P. arundinacea* (elevated CO$_2$) | 52.1 (2.6)a | 27.9 (1.6)ab | 1.50 (0.30)c | 0.20 (0.02)a | 28,293 (2,887)ab | 0.30 (0.01)a | −41.4 (0.4)c | |
| *S. cyperinus* (ambient CO$_2$) | 18.8 (1.5)b | 20.6 (2.2)b | 12.31 (1.52)b | 0.19 (0.02)a | 25,999 (3,052)ab | 0.28 (0.02)a | −31.2 (0.1)b | −23.0 (0.2)a |
| *S. cyperinus* (elevated CO$_2$) | 20.9 (2.1)b | 24.8 (1.8)ab | 19.01 (2.00)a | 0.22 (0.02)a | 34,074 (3,310)b | 0.29 (0.02)a | −42.7 (0.5)c | |
| ANOVA *P* value | | | | | | | | |
| Plant | <0.001 | 0.002 | <0.001 | 0.650 | 0.042 | 0.523 | 0.012 | 0.296 |
| CO$_2$ | 0.707 | 0.871 | 0.007 | 0.957 | 0.007 | 0.673 | <0.001 | |
| Plant X CO$_2$ | 0.793 | 0.054 | 0.010 | 0.045 | 0.862 | 0.907 | 0.220 | |

Standard errors are shown in parenthesis followed by means. The number of replicates is 3 for plant and soil isotopes and 9 for the other variables. Different letters within each column represent significant differences in mean value between the treatments (post hoc Tukey's test, *P*<0.05)

total fluorescence levels of the fragments under the contrasting CO$_2$ levels for either plant species (Table 1).

### $^{13}$C Depletion in Plant tissues and Microbial PLFAs

Continuous labeling of plant tissues with depleted $^{13}$CO$_2$ resulted in plant tissues highly depleted in $^{13}$C for both species. The concentration of CO$_2$ in the greenhouses averaged 380 ppm in the ambient rooms and 700 ppm in the elevated rooms, ensuring a strong $^{13}$C label in the CO$_2$ source. The mean δ$^{13}$C values (‰) in plant tissues of *P. arundinacea* were lower in the elevated CO$_2$ rooms compared with the ambient CO$_2$ rooms (−41.4‰ versus −28.2‰) (Table 1). A similar difference in tissue isotopic signatures was found in *S.*



**Fig. 1** Principal component analysis of microbial lipids from *P. arundinacea* v versus *S. cyperinus* and elevated CO$_2$ versus ambient CO$_2$ treatments. The four different symbols differentiate four different treatments, as accompanied by text. Error *bars* are ±SE of the mean (*n*=9). The first and second principal components account for 53 % of the variability

*cyperinus*, where lower $^{13}$C values were obtained in elevated CO$_2$ rooms (−42.7‰ versus −31.2‰).

Plant C depleted in $^{13}$C was traced into the PLFAs of rhizosphere microorganisms as a measure of plant C assimilation. The $^{13}$C values of 16 common PLFAs indicative of four main groups of soil microorganisms are listed in Fig. 2. Seven PLFAs (i14:0, i15:0, a15:0, i16:0, i17:0, a17:0, and 18:1ω9t) have branched-chain fatty acid structures that represent Gram-positive bacteria. Another seven PLFAs (16:1ω7c, 17:1ω7c, 17:1ω8c, 18:1ω7c, 16:0 2OH, cy17:0, and cy19:0) have monosaturated, hydroxyl, and cyclopropyl fatty acid structures that represent Gram-negative bacteria. Two PLFAs (18:1ω9c and 18:2ω6c,9c) indicate saprotrophic fungi, and 16:1ω5c indicates arbuscular mycorrhizal fungi (AMF) [30]. In general, most of the lipids included in Fig. 2 are $^{13}$C-depleted compared with the C sources (plant C or soil organic C). It is consistent with the general pattern that $^{13}$C fractionation occurs during the biosynthesis of lipids. The two-way ANOVA (species and CO$_2$) results indicated that rhizosphere microorganisms from the elevated CO$_2$ rooms showed significant reductions in the δ$^{13}$C values of most PLFAs (except i16:0, 18:1ω7c, and cy19:0) compared with the ambient CO$_2$ rooms. The lower δ$^{13}$C values of the PLFAs in the elevated CO$_2$ rooms are likely due to greater depletion in the δ$^{13}$C values of the microorganism's C source.

Plant species had significant influence on the δ$^{13}$C values of most of the PLFAs, except i16:0, i17:0, a17:0, 17:1ω8c, 18:1ω7c, and cy17:0. The PLFAs of soils planted with *S. cyperinus* showed greater depletion in δ$^{13}$C values compared with those with *P. arundinacea*. This is likely due to the more $^{13}$C-depleted plant C inputs of *S. cyperinus* and the larger relative contribution of *S. cyperinus* plant-derived C to microbial PLFAs. We observed no significant interaction
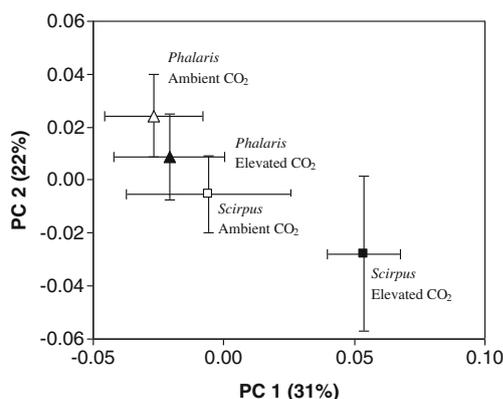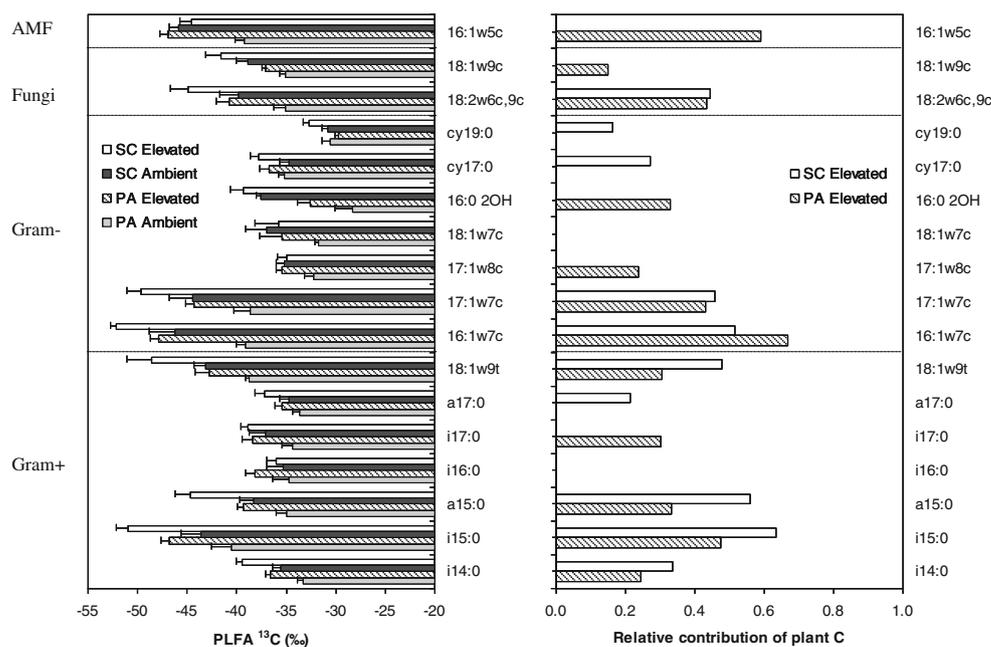
**Fig. 2** The $\delta^{13}C$ value
(mean+SE, $n=6$) and the
relative contribution of plant
carbon to microbial carbon
source of 16 common PLFAs
that indicate four main groups
of soil microorganism (AMF,
Fungi, Gram-negative bacteria,
and Gram-positive bacteria). *SC*
stands for *S. cyperinus*, and *PA*
stands for *P. arundinacea*



between plant species and $CO_2$ concentration on the $\delta^{13}C$
values of most PLFAs, except for cy19:0 and 16:1ω5c. We
also measured the $\delta^{13}C$ values of 11 saturated PLFAs, from
11:0 to 20:0. Similar to the pattern of the PLFAs shown
above, the $\delta^{13}C$ values of these saturated PLFAs (except
10:0) ranged from −47.0‰ to −28.5‰ and were more de-
pleted in *S. cyperinus* and under elevated $CO_2$ conditions
(data not shown).

We used the $\delta^{13}C$ values of the PLFAs in the elevated and
ambient $CO_2$ rooms to calculate the relative contribution of
plant-derived C to microbial PLFAs for the two plant spe-
cies. Most of the PLFAs showed a shift in the $^{13}C$ isotopic
signal between the two $CO_2$ treatments, suggesting incorpo-
ration of plant-derived C into microbial lipids. Overall, the
relative contribution of plant-derived C to PLFA C is larger
in *S. cyperinus* than in *P. arundinacea*. Particularly, the
relative amount of plant-derived C in the four PLFAs of
Gram-positive bacteria (i14:0, i:15:0, a15:0, and 18:1ω9t)
was higher in *S. cyperinus* (34–63 %) than in *P. arundinacea*
(24–48 %). Moreover, three PLFAs (a17:0, *cy*17:0, and
*cy*19:0) showed plant-derived C signals in *S. cyperinus* but
not in *P. arundinacea*, while five PLFAs (i17:0, 17:1ω8c,
16:0 2OH, 18:1ω9c, and 16:1ω5c) showed plant-derived C
signals in *P. arundinacea* but not in *S. cyperinus*.

## Discussion

Microbial mediation of the $CO_2$–methane link is highly
dependent on plant species. Methane emissions increased
1.5-fold from *S. cyperinus*, but no changes were detected in
*P. arundinacea*. The enhanced methane levels from *S.
cyperinus* did not show a corresponding increase in plant

shoot or root biomass. Other studies indicating a link be-
tween elevated $CO_2$ and methane emissions have found in-
creases in root biomass for both wetlands and rice paddy
ecosystems [2]. In a previous study, we detected a positive
relationship between T-RFLP relative abundance of a group
of acetoclastic methanogens with plant biomass of an inva-
sive cattail *Typha anugustifolia* [15]. No similar relation-
ships were found with *S. cyperinus* despite the significant
increase in methane emissions. While increased plant bio-
mass could be impacting methane emissions, other $CO_2$-
induced plant physiological changes could be driving
changes in microbial activity. In an earlier study, we found
changes in the relative abundances of rhizosphere microor-
ganisms, despite the lack of a $CO_2$ fertilization effect on *P.
arundinacea* biomass [25]. Elevated $CO_2$ can impact C flux
without a corresponding change in plant biomass [35]. Plant
physiological changes other than biomass influenced by
elevated $CO_2$ include rhizodeposition and root exudation.
Rhizodeposits are composed of plant exudates, lysates, mu-
cilage, secretions, and dead cell materials, as well as gases
[36], and recent evidence suggests that root exudates are
dominated by low-molecular-weight organic compounds
such as sugars, amino acids, and organic acids [37, 38].
Enhanced root exudation and rhizodeposition have been
hypothesized by several researchers [2, 39], but only recently
has a field-based study showed a measurable increase in root
exudation of organic C compounds with elevated $CO_2$ [40].

Other studies have shown the importance of plant species
in determining the composition and function of the soil
microbial community [41–43], but few studies are able to
link the mechanism to rhizodeposition [44]. There are many
challenges to isolating rhizodeposits and examining their
impacts on ecosystems [45]. While rhizodeposition and root

exudation were not quantified in this study, we used a different measure of root C influence on soil microorganisms. The plants grown in elevated CO$_2$ rooms (receiving a CO$_2$ source highly depleted in $\delta^{13}$C) showed more negative $\delta^{13}$C values in microbial lipids corresponding with more negative $\delta^{13}$C values in plant tissues. The results show that C from rhizodeposits can be tracked into the PLFA C of soil microorganisms, providing additional information on plant species effects on soil microorganisms. There was a larger relative contribution of plant-derived C to microbial lipids in *S. cyperinus* compared with *P. arundinacea*. In addition to the relative contribution of plant-derived C to microbial lipid C being higher overall in the native plant, three PLFAs (a17:0, cy17:0, and cy19:0) indicated plant-derived C signals in *S. cyperinus* that were absent in *P. arundinacea*. The data suggest that the microbial community in the *S. cyperinus* rhizospheres shows greater incorporation of recently fixed plant C (mainly rhizodeposits) into microbial cell membrane lipids, in comparison with the invasive plant *P. arundinacea*. Some PLFAs did not show a $^{13}$C isotopic shift between ambient and elevated CO$_2$, indicating no or little plant contribution to microbial PLFAs. Overall, these results indicate the need for $^{13}$C labeling in elevated CO$_2$ studies to assess the impact of plant physiological changes to soil microorganisms via changes in plant C inputs.

It is important to note that, in this study, the differences in $\delta^{13}$C values merely provide a contrasting label (more negative $\delta^{13}$C values) that is useful in distinguishing the contribution of plant C from soil organic C in microbial PLFAs from the two different plant species rhizospheres. The method does not distinguish if there is a CO$_2$ effect on microbial PLFAs. Using contrasting $\delta^{13}$C isotopic values as a label, researchers found that Gram-negative bacteria prefer recent plant material as a C source, while Gram-positive bacteria use substantial amounts of soil organic C in two agricultural soils that underwent vegetation change from C$_3$ to C$_4$ plants in the past few decades [34]. In contrast, we found that both Gram-negative and Gram-positive bacteria showed similar patterns in $\delta^{13}$C values in our study of wetland plant rhizospheres.

Many studies have used $^{13}$C-enriched CO$_2$ to pulse label the plants and trace the recently derived plant C into microbial lipids over a short period of time [46–49]. Only a few studies have used steady-state continuous labeling with $^{13}$C-depleted CO$_2$ under ambient CO$_2$ concentration [50] or elevated CO$_2$ concentration [51] to investigate the microbial group-specific utilization of rhizodeposition. The species-specific differences in the contribution of plant C to microbial lipid C could be indicating additional microorganisms that contribute to methane emissions from wetlands. Most of our present knowledge on methane dynamics is attributed largely to bacterial methanotrophs and archaeal methanogens, while the role of more complex or unexpected community associations are yet to be explored [7]. Many more patterns

establishing a link in plant–microbial physiology can be elucidated using additional microbial community characterization methods [16].

It is important to reemphasize that the T-RFLP data in this study are specific to archaea, whereas the PLFA data indicate bacterial and fungal composition and abundance. The PLFA results indicate a compositional shift in the rhizosphere bacterial and fungal community specific to *S. cyperinus*. As no changes in methanogen relative abundance occurred in *S. cyperinus* rhizospheres under CO$_2$ enrichment, the rise in methane levels could be related to shifts in community composition (total PLFAs) specific to microorganisms utilizing rhizodeposits or exudates. As PLFAs are degraded rapidly in soils, they provide a snapshot of active microorganisms in the soil, as opposed to most nucleic acid-based profiling techniques that amplify residual DNA present in soils leading to confounding soil legacy effects [21, 22, 52]. The data all together show that PLFA and T-RFLP differ in characterizing the CO$_2$-induced changes in methanotroph and methanogen community profiles across plant species. The results from this study indicate that multiple techniques are needed to fully characterize the changes in microbial community structure associated with CO$_2$-induced alterations in methane dynamics across plant species. Understanding the complexity of the methane–CO$_2$ link requires broader analysis of the suite of microbial community shifts resulting from plant stimulation under elevated CO$_2$ [53, 54]. Although plant biomass is easy to measure as a CO$_2$ fertilization response, other plant physiological measurements are needed to characterize potential impacts of CO$_2$ stimulation on the belowground community [55, 56]. Net methane emissions in wetland systems ultimately depend on the sediment–plant–atmosphere pathway, and more accurate measurements across the gas production, consumption, and transport pathways would improve modeling methane dynamics under climate change.

## References

1. Janus LR, Angeloni NL, McCormack J, Rier ST, Tuchman NC, Kelly JJ (2005) Elevated atmospheric CO$_2$ alters soil microbial communities associated with trembling aspen (*Populus tremuloides*) roots. Microb Ecol 50:102–109. doi:10.1007/s00248-004-0120-9

2. van Groenigen KJ, Osenberg CW, Hungate BA (2011) Increased soil emissions of potent greenhouse gases under increased atmospheric $CO_2$. Nature 475:214–U121. doi:10.1038/nature10176

3. Dijkstra FA, Prior SA, Runion GB, Torbert HA, Tian HQ, Lu CQ, Venterea RT (2012) Effects of elevated carbon dioxide and increased temperature on methane and nitrous oxide fluxes: evidence from field experiments. Front Ecol Environ 10:520–527. doi:10.1890/120059

4. Poorter H (1993) Interspecific variation in the growth response of plants to an elevated ambient $CO_2$ concentration. Vegetation 104-105:77–97

5. Poorter H, Navas ML (2003) Plant growth and competition at elevated $CO_2$: on winners, losers and functional groups. New Phytol 157:175–198. doi:10.1046/j.1469-8137.2003.00680.x

6. Solomon S, Qin D, Manning M (eds) (2007) IPCC Fourth Assessment Report–Climate Change 2007: The Physical Science Basis. Cambridge University Press, New York

7. Le Mer J, Roger P (2001) Production, oxidation, emission and consumption of methane by soils: a review. Eur J Soil Biol 37:25–50. doi:10.1016/s1164-5563(01)01067-6

8. Dacey JWH, Drake BG, Klug MJ (1994) Stimulation of methane emission by carbon dioxide enrichment of marsh vegetation. Nature 370:47–49. doi:10.1038/370047a0

9. Inubushi K, Cheng WG, Aonuma S, Hoque MM, Kobayashi K, Miura S, Kim HY, Okada M (2003) Effects of free-air $CO_2$ enrichment (FACE) on $CH_4$ emission from a rice paddy field. Glob Chang Biol 9:1458–1464. doi:10.1046/j.1365-2486.2003.00665.x

10. Xu ZJ, Zheng XH, Wang YS, Han SH, Huang Y, Zhu JG, Butterbach-Bahl K (2004) Effects of elevated $CO_2$ and N fertilization on $CH_4$ emissions from paddy rice fields. Global Biogeochem Cycles 18. doi: 10.1029/2004gb002233

11. Marsh AS, Rasse DP, Drake BG, Megonigal JP (2005) Effect of elevated $CO_2$ on carbon pools and fluxes in a brackish marsh. Estuaries 28:694–704. doi:10.1007/bf02732908

12. Zheng XH, Zhou ZX, Wang YS, Zhu JG, Wang YL, Yue J, Shi Y, Kobayashi K, Inubushi K, Huang Y, Han SH, Xu ZJ, Xie BH, Butterbach-Bahl K, Yang LX (2006) Nitrogen-regulated effects of free-air $CO_2$ enrichment on methane emissions from paddy rice fields. Glob Chang Biol 12:1717–1732. doi:10.1111/j.1365-2486.2006.01199.x

13. Yun SI, Kang BM, Lim SS, Choi WJ, Ko J, Yoon S, Ro HM, Kim HY (2012) Further understanding $CH_4$ emissions from a flooded rice field exposed to experimental warming with elevated $CO_2$. Agric For Meteorol 154:75–83. doi:10.1016/j.agrformet.2011.10.011

14. Angel R, Kammann C, Claus P, Conrad R (2012) Effect of long-term free-air $CO_2$ enrichment on the diversity and activity of soil methanogens in a periodically waterlogged grassland. Soil Biol Biochem 51:96–103. doi:10.1016/j.soilbio.2012.04.010

15. Kao-Kniffin J, Freyre DS, Balser TC (2011) Increased methane emissions from an invasive wetland plant under elevated carbon dioxide levels. Appl Soil Ecol 48:309–312. doi:10.1016/j.apsoil.2011.04.008

16. Lee SH, Kim SY, Kang H (2012) Effects of elevated $CO_2$ on communities of denitrifying bacteria and methanogens in a temperate marsh microcosm. Microb Ecol 64:485–498. doi:10.1007/s00248-012-0036-8

17. Kao-Kniffin J, Freyre DS, Balser TC (2010) Methane dynamics across wetland plant species. Aquat Bot 93:107–113. doi:10.1016/j.aquabot.2010.03.009

18. Galatowitsch SM, Anderson NO, Ascher PD (1999) Invasiveness in wetland plants in temperate North America. Wetlands 19:733–755

19. Schooler SS, McEvoy PB, Coombs EM (2006) Negative per capita effects of purple loosestrife and reed canary grass on plant diversity of wetland communities. Divers Distrib 12:351–363. doi:10.1111/j.1366-9516.2006.00227.x

20. Pataki DE, Ellsworth DS, Evans RD, Gonzalez-Meler M, King J, Leavitt SW, Lin GH, Matamala R, Pendall E, Siegwolf R, Van Kessel C, Ehleringer JR (2003) Tracing changes in ecosystem function

under elevated carbon dioxide conditions. Bioscience 53:805–818. doi:10.1641/0006-3568(2003)053[0805:tciefu]2.0.co;2

21. White DC, Davis WM, Nickels JS, King JD, Bobbie RJ (1979) Determination of the sedimentary microbial biomass by extractable lipid phosphate. Oecologia 40:51–62. doi:10.1007/bf00388810

22. Amelung W, Brodowski S, Sandhage-Hofmann A, Bol R (2008) Combining biomarker with stable isotope analyses for assessing the transformation and turnover of soil organic matter. Adv Agron 100(100):155–250. doi:10.1016/s0065-2113(08)00606-8

23. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J of Biochem and Physiol 37:911–917

24. White DC, Ringelberg DB (1998) Signature lipid biomarker analysis. In: Burlage RS, Atlas R, Stahl D, Geesey G, Sayler G (eds) Techniques in Microbial Ecology. Oxford University Press, Inc., New York, pp 255–272

25. Kao-Kniffin J, Balser TC (2007) Elevated $CO_2$ differentially alters belowground plant and soil microbial community structure in reed canary grass-invaded experimental wetlands. Soil Biol Biochem 39:517–525

26. Arao T (1999) In situ detection of changes in soil bacterial and fungal activities by measuring C-13 incorporation into soil phospholipid fatty acids from C-13 acetate. Soil Biol Biochem 31:1015–1020

27. Baath E, Anderson TH (2003) Comparison of soil fungal/bacterial ratios in a pH gradient using physiological and PLFA-based techniques. Soil Biol Biochem 35:955–963. doi:10.1016/s0038-0717(03)00154-8

28. Steenwerth KL, Jackson LE, Calderon FJ, Stromberg MR, Scow KM (2003) Soil community composition and land use history in cultivated and grassland ecosystems of coastal California. Soil Biol Biochem 35:489–450. doi:10.1016/S0038-0717(03)00028-2

29. Haack SK, Garchow H, Odelson DA, Forney LJ, Klug MJ (1994) Accuracy, reproducibility, and interpretation of fatty acid methyl ester profiles of model bacterial communities. Appl Environ Microbiol 60:2483–2493

30. Zelles L (1999) Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review. Biol Fertil Soils 29:111–129. doi:10.1007/s003740050533

31. Gattinger A, Schloter M, Munch JC (2002) Phospholipid etherlipid and phospholipid fatty acid fingerprints in selected euryarchaeotal monocultures for taxonomic profiling. FEMS Microbiol Lett 213:133–139. doi:10.1111/j.1574-6968.2002.tb11297.x

32. Lueders T, Friedrich M (2000) Archaeal population dynamics during sequential reduction processes in rice field soil. Appl Environ Microbiol 66:2732–2742. doi:10.1128/aem.66.7.2732-2742.2000

33. Boschker HTS, Middelburg JJ (2002) Stable isotopes and biomarkers in microbial ecology. FEMS Microbiol Ecol 40:85–95. doi:10.1111/j.1574-6941.2002.tb00940.x

34. Kramer C, Gleixner G (2006) Variable use of plant- and soil-derived carbon by microorganisms in agricultural soils. Soil Biol Biochem 38:3267–3278. doi:10.1016/j.soilbio.2006.04.006

35. Korner C, Asshoff R, Bignucolo O, Hattenschwiler S, Keel SG, Pelaez-Riedl S, Pepin S, Siegwolf RTW, Zotz G (2005) Carbon flux and growth in mature deciduous forest trees exposed to elevated CO2. Science 309:1360–1362. doi:10.1126/science.1113977

36. Lynch JM, Whipps JM (1990) Substrate flow in the rhizosphere. Plant Soil 129:1–10. doi:10.1007/bf00011685

37. Jones DL, Hodge A, Kuzyakov Y (2004) Plant and mycorrhizal regulation of rhizodeposition. New Phytol 163:459–480. doi:10.1111/j.1469-8137.2004.01130.x

38. Uren NC (2007) Types, amounts, and possible functions of compounds released into the rhizosphere by soil-growing plants. In: Pinton R, Varannini Z, Nannipieri P (eds) The Rhizosphere: Biochemical and Organic Substances at the Soil–Plant Interface, 2nd edn. CRC Press, Boca Raton, pp 1–21

39. Mitchell EAD, Gilbert D, Buttler A, Amblard C, Grosvernier P, Gobat JM (2003) Structure of microbial communities in Sphagnum

peatlands and effect of atmospheric carbon dioxide enrichment. Microb Ecol 46:187–199. doi:10.1007/s002480-002-0008-5

40. Phillips RP, Finzi AC, Bernhardt ES (2011) Enhanced root exudation induces microbial feedbacks to N cycling in a pine forest under long-term CO$_2$ fumigation. Ecol Lett 14:187–194. doi:10.1111/j.1461-0248.2010.01570.x

41. Grayston SJ, Wang SQ, Campbell CD, Edwards AC (1998) Selective influence of plant species on microbial diversity in the rhizosphere. Soil Biol Biochem 30:369–378. doi:10.1016/s0038-0717(97)00124-7

42. Marilley L, Hartwig UA, Aragno M (1999) Influence of an elevated atmospheric CO$_2$ content on soil and rhizosphere bacterial communities beneath *Lolium perenne* and *Trifolium repens* under field conditions. Microb Ecol 38:39–49. doi:10.1007/s002489900155

43. Cadillo-Quiroz H, Yavitt JB, Zinder SH, Thies JE (2010) Diversity and community structure of archaea inhabiting the rhizoplane of two contrasting plants from an acidic bog. Microb Ecol 59:757–767. doi:10.1007/s00248-009-9628-3

44. Marschner P, Crowley D, Yang CH (2004) Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. Plant Soil 261:199–208. doi:10.1023/B:PLSO.0000035569.80747.c5

45. Jones DL, Nguyen C, Finlay RD (2009) Carbon flow in the rhizosphere: carbon trading at the soil–root interface. Plant Soil 321:5–33. doi:10.1007/s11104-009-9925-0

46. Butler JL, Williams MA, Bottomley PJ, Myrold DD (2003) Microbial community dynamics associated with rhizosphere carbon flow. Appl Environ Microbiol 69:6793–6800. doi:10.1128/aem.69.11.6793-6800.2003

47. Lu YH, Murase J, Watanabe A, Sugimoto A, Kimura M (2004) Linking microbial community dynamics to rhizosphere carbon flow in a wetland rice soil. FEMS Microbiol Ecol 48:179–186. doi:10.1016/j.femsec.2004.01.004

48. Treonis AM, Ostle NJ, Stott AW, Primrose R, Grayston SJ, Ineson P (2004) Identification of groups of metabolically-active rhizosphere microorganisms by stable isotope probing of PLFAs. Soil Biol Biochem 36:533–537. doi:10.1016/j.soilbio.2003.10.015

49. Denef K, Bubenheim H, Lenhart K, Vermeulen J, Van Cleemput O, Boeckx P, Muller C (2007) Community shifts and carbon translocation within metabolically-active rhizosphere microorganisms in grasslands under elevated CO$_2$. Biogeosciences 4:769–779

50. Paterson E, Gebbing T, Abel C, Sim A, Telfer G (2007) Rhizodeposition shapes rhizosphere microbial community structure in organic soil. New Phytol 173:600–610. doi:10.1111/j.1469-8137.2006.01931.x

51. Billings SA, Ziegler SE (2005) Linking microbial activity and soil organic matter transformations in forest soils under elevated CO$_2$. Glob Chang Biol 11:203–212. doi:10.1111/j.1365-2486.2005.00909.x

52. Moore-Kucera J, Dick RP (2008) PLFA profiling of microbial community structure and seasonal shifts in soils of a Douglas-fir chronosequence. Microb Ecol 55:500–511. doi:10.1007/s00248-007-9295-1

53. Paterson E, Hall JM, Rattray EAS, Griffiths BS, Ritz K, Killham K (1997) Effect of elevated CO$_2$ on rhizosphere carbon flow and soil microbial processes. Glob Chang Biol 3:363–377. doi:10.1046/j.1365-2486.1997.t01-1-00088.x

54. Zak DR, Pregitzer KS, King JS, Holmes WE (2000) Elevated atmospheric CO$_2$, fine roots and the response of soil microorganisms: a review and hypothesis. New Phytol 147:201–222. doi:10.1046/j.1469-8137.2000.00687.x

55. Joabsson A, Christensen TR (2001) Methane emissions from wetlands and their relationship with vascular plants: an Arctic example. Glob Chang Biol 7:919–932. doi:10.1046/j.1354-1013.2001.00044.x

56. Chin KJ, Lueders T, Friedrich MW, Klose M, Conrad R (2004) Archaeal community structure and pathway of methane formation on rice roots. Microb Ecol 47:59–67. doi:10.1007/s00248-003-2014-7