A Microbial Link between Elevated CO2 and Methane Emissions that is Plant Species-Specific

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Abstract Rising atmospheric CO2 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 CO2 (700 ppm). We hypothesized that elevated atmospheric CO2 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 CO2, while no changes in methane levels were detected from P. arundineca. 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 CO2 levels shifted the composition of the microbial community under S. cyperinus, while no changes were detected under P. arundineca. More detailed analysis of microbial abundance showed no impact of elevated CO2 on a fatty acid indicative of methanotrophic bacteria (18:2ω6c), 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 13C 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 18:1ω9t). The δ13C isotopic values indicate that the contribution of plant-derived carbon to microbial lipids could differ in rhizospheres of CO2-responsive plant species, such as S. cyperinus in this study. The results from this study show that the CO2–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 CO2.

Introduction

Concerns over the impacts of climate change on greenhouse gas emissions have peaked interest in examining microbial responses to elevated atmospheric levels of CO2 [1–3]. In wetlands, methane (CH4) 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 CO2 levels. The CO2 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 CO2 levels. Differential growth responses of plant species to elevated atmospheric CO2 have been found [4, 5], yet few studies examine how methane dynamics are altered with rising CO2 levels. Given the importance of natural wetlands in comprising 20–39 % of total global methane emissions [6], it is important to understand how changing CO2 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...
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 CO₂ 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 paddies showed enhanced emissions of methane with CO₂ fumigation [8–13]. However, no linkages of elevated CO₂ to soil methanogen or methanotroph population changes have been published from these field-based CO₂ 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 CO₂ fumigation [14]. Several controlled environment studies have reported a change in microbial community structure under elevated CO₂ levels linked to methane dynamics [9, 15, 16]. The lack of field-based evidence for a microbially mediated CO₂–methane link suggests that methanogen and methanotroph communities become acclimated to high CO₂ 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 CO₂ 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 CO₂–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 13C isotopic shift of the phospholipid fatty acids from the plant–soil systems grown under depleted 13CO₂ versus ambient 13CO₂. The δ13C isotopic signal of depleted CO₂ 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 CO₂ enrichment was replicated in three greenhouse rooms, and three additional rooms provided ambient CO₂ levels. Individual seedlings 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 CO₂ fumigation with increased biomass accumulation, as was found in many C₃ 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 CO₂ 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 CO₂ 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 CO₂ concentrations in the greenhouses averaged 380 ppm in the ambient rooms and 700 ppm in the elevated rooms. The
gas used to augment the CO2 concentrations in the greenhouses was derived from commercial sources as C from petroleum byproducts depleted in 13C. The mean δ13C of commercially sourced CO2 averages between −27 and −45‰ and ambient atmospheric CO2 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

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 et al. [17]. 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:B:ωC’ 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-isomethyl 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/TGCTCAGTAAACGCT-3′) and Ar912r (5′-CTCCCAGCCATTCCCTTA-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 TaqI (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 CO2 rooms. The relative amount of plant-derived C (Fplant) used as microbial C source in the elevated CO2 treatment was calculated using the \( ^{13} \)C isotope shift of both PLFA and plant input materials between the ambient CO2 treatment and the elevated CO2 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 CO2 treatments (Fig. 2).

\[
F_{\text{plant}} = \frac{\delta^{13} \text{C}_{\text{PLFA-elevated}} - \delta^{13} \text{C}_{\text{PLFA-ambient}}}{\delta^{13} \text{C}_{\text{plant-elevated}} - \delta^{13} \text{C}_{\text{plant-ambient}}}
\]  

where \( F_{\text{plant}} \) is the relative contribution of plant C to each individual PLFA, \( \delta^{13} \text{C}_{\text{PLFA-elevated}} \) and \( \delta^{13} \text{C}_{\text{PLFA-ambient}} \) are the \( \delta^{13} \)C values of PLFA in the elevated and ambient CO2 treatments, respectively, and \( \delta^{13} \text{C}_{\text{plant-elevated}} \) and \( \delta^{13} \text{C}_{\text{plant-ambient}} \) are the \( \delta^{13} \)C values of shoots in the elevated and ambient CO2 treatments, respectively.

Statistical Analysis

The data were analyzed using plant species and CO2 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 CO2 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 CO2 enrichment

We found that the increase in CO2 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 CO2 atmosphere.

Despite the lack of a significant CO2 fertilization effect for both plant species, the higher CO2 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 CO2 levels (Fig. 1). There was no corresponding shift detected for P. arundinacea PLFA profiles, which suggests that the CO2-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 CO2 levels. Unexpectedly, the lipid indicative of methanotrophic bacteria (18:1ω7c) showed no changes with CO2 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
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Table 1  Plant biomass (grams per pot), methane emission (milligrams CH4-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 δ13C values (%) of plant (shoots) and soil organic carbon in the four treatments at the end of the experiment

| Treatment          | Shoot biomass | Root biomass | CH4 emission | PLFA | T-RFLP | Fungi/bacteria ratio | δ13Cplant | δ13Csoil |
|--------------------|---------------|--------------|--------------|------|--------|----------------------|------------|----------|
| P. arundinacea     | 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 |
| (ambient CO2)      |               |              |              |      |        |                      |            |          |
| P. arundinacea     | 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 |          |
| (elevated CO2)     |               |              |              |      |        |                      |            |          |
| S. cyperinus       | 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 |
| (ambient CO2)      |               |              |              |      |        |                      |            |          |
| S. cyperinus       | 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 |          |
| (elevated CO2)     |               |              |              |      |        |                      |            |          |

ANOVA P value

| Plant           | <0.001 | 0.002 | <0.001 | 0.650 | 0.042 | 0.523 | 0.012 | 0.296 |
|-----------------|--------|-------|--------|-------|-------|-------|-------|-------|
| CO2             | 0.707  | 0.871 | 0.007  | 0.957 | 0.007 | 0.673 | <0.001 |       |
| Plant X CO2     | 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 CO2 levels for either plant species (Table 1).

13C Depletion in Plant tissues and Microbial PLFAs

Continuous labeling of plant tissues with depleted 13CO2 resulted in plant tissues highly depleted in 13C for both species. The concentration of CO2 in the greenhouses averaged 380 ppm in the ambient rooms and 700 ppm in the elevated rooms, ensuring a strong 13C label in the CO2 source. The mean δ13C values (%) in plant tissues of P. arundinacea were lower in the elevated CO2 rooms compared with the ambient CO2 rooms (−41.4% versus −28.2%) (Table 1). A similar difference in tissue isotopic signatures was found in S. cyperinus, where lower 13C values were obtained in elevated CO2 rooms (−42.7‰ versus −31.2‰).

Plant C depleted in 13C was traced into the PLFAs of rhizosphere microorganisms as a measure of plant C assimilation. The 13C values of 16 common PLFAs indicative of four main groups of soil microorganisms are listed in Fig. 2. Seven PLFAs (11:4, 11:5, 11:6, 11:7, 11:8, and 11:9) 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 saprophotrophic fungi, and 16:1ω5c indicates arbuscular mycorrhizal fungi (AMF) [30]. In general, most of the lipids included in Fig. 2 are 13C-depleted compared with the C sources (plant C or soil organic C). It is consistent with the general pattern that 13C fractionation occurs during the biosynthesis of lipids. The two-way ANOVA (species and CO2) results indicated that rhizosphere microorganisms from the elevated CO2 rooms showed significant reductions in the δ13C values of most PLFAs (except 11:6:0, 18:1ω7c, and cy19:0) compared with the ambient CO2 rooms. The lower δ13C values of the PLFAs in the elevated CO2 rooms are likely due to greater depletion in the δ13C values of the microorganism’s C source.

Plant species had significant influence on the δ13C values of most of the PLFAs, except i16:0, i17:0, a17:0, 17:1ω6c, 18:1ω7c, and cy17:0. The PLFAs of soils planted with S. cyperinus showed greater depletion in δ13C values compared with those with P. arundinacea. This is likely due to the more 13C-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. 1 Principal component analysis of microbial lipids from P. arundinacea v versus S. cyperinus and elevated CO2 versus ambient CO2 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

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between plant species and CO2 concentration on the $\delta^{13}\text{C}$ values of most PLFAs, except for cy19:0 and 16:1ω5c. We also measured the $\delta^{13}\text{C}$ values of 11 saturated PLFAs, from 11:0 to 20:0. Similar to the pattern of the PLFAs shown above, the $\delta^{13}\text{C}$ values of these saturated PLFAs (except 10:0) ranged from −47.0‰ to −28.5‰ and were more depleted in $S. cypriumus$ and under elevated CO2 conditions (data not shown).

We used the $\delta^{13}\text{C}$ values of the PLFAs in the elevated and ambient CO2 rooms to calculate the relative contribution of plant-derived C to microbial PLFAs for the two plant species. Most of the PLFAs showed a shift in the $\delta^{13}\text{C}$ isotopic signal between the two CO2 treatments, suggesting incorporation of plant-derived C into microbial lipids. Overall, the relative contribution of plant-derived C to PLFA C is larger in $S. cypriumus$ than in $P. arundinacea$. Particularly, the relative amount of plant-derived C in the four PLFAs of Gram-positive bacteria (i14:0, i15:0, a15:0, and 18:1ω9t) was higher in $S. cypriumus$ (34–63 %) than in $P. arundinacea$ (24–48 %). Moreover, three PLFAs (a17:0, cy17:0, and cy19:0) showed plant-derived C signals in $S. cypriumus$ 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 $S. cypriumus$.

**Discussion**

Microbial mediation of the CO2–methane link is highly dependent on plant species. Methane emissions increased 1.5-fold from $S. cypriumus$, but no changes were detected in $P. arundinacea$. The enhanced methane levels from $S. cypriumus$ did not show a corresponding increase in plant shoot or root biomass. Other studies indicating a link between elevated CO2 and methane emissions have found increases 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 invasive cattail *Typha angustifolia* [15]. No similar relationships were found with $S. cypriumus$ despite the significant increase in methane emissions. While increased plant biomass could be impacting methane emissions, other CO2-induced plant physiological changes could be driving changes in microbial activity. In an earlier study, we found changes in the relative abundances of rhizosphere microorganisms, despite the lack of a CO2 fertilization effect on $P. arundinacea$ biomass [25]. Elevated CO2 can impact C flux without a corresponding change in plant biomass [35]. Plant physiological changes other than biomass influenced by elevated CO2 include rhizodeposition and root exudation. Rhizodeposits are composed of plant exudates, lysates, mucilage, 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 CO2 [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 differ-
et measure of root C influence on soil microorganisms. The plants grown in elevated CO2 rooms (receiving a CO2 source highly depleted in δ^{13}C) showed more negative δ^{13}C values in microbial lipids corresponding with more negative δ^{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 CO2, indicating no or little plant contribution to microbial PLFAs. Overall, these results indicate the need for ^{13}C labeling in elevated CO2 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 δ^{13}C values merely provide a contrasting label (more negative δ^{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 CO2 effect on microbial PLFAs. Using contrasting δ^{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 C3 to C4 plants in the past few decades [34]. In contrast, we found that both Gram-negative and Gram-positive bacteria showed similar patterns in δ^{13}C values in our study of wetland plant rhizospheres.

Many studies have used ^{13}C-enriched CO2 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 CO2 under ambient CO2 concentration [50] or elevated CO2 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 CO2 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 CO2-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 CO2-induced alterations in methane dynamics across plant species. Understanding the complexity of the methane–CO2 link requires broader analysis of the suite of microbial community shifts resulting from plant stimulation under elevated CO2 [53, 54]. Although plant biomass is easy to measure as a CO2 fertilization response, other plant physiological measurements are needed to characterize potential impacts of CO2 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.

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