# Linking nitrogen load to the structure and function of wetland soil and rhizosphere microbial communities

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## Abstract

Wetland ecosystems are important reservoirs of biodiversity and significantly contribute to emissions of the greenhouse gases $CO_2$, $N_2O$ and $CH_4$. High anthropogenic nitrogen (N) inputs from agriculture and fossil fuel combustion have been recognized as a severe threat to biodiversity and ecosystem functioning such as control of greenhouse gas emissions. Therefore it is important to understand how increased N input into pristine wetlands affects the composition and activity of micro-organisms, especially in interaction with dominant wetland plants. In a series of incubations analyzed over 90 days, we disentangle the effects of N fertilization on the microbial community in bulk soil and the rhizosphere of *Juncus acutiflorus*, a common and abundant graminoid wetland plant. We observed an increase in greenhouse gas emissions when N is increased in incubations with *J. acutiflorus*, changing the system from a greenhouse gas sink to a source. Using 16S rRNA amplicon sequencing and metagenomics, we determined that the bacterial orders Opitutales, Subgroup-6 Acidobacteria and Sphingobacteriales significantly responded to high N availability and we hypothesize that these groups are contributing to the increased greenhouse gas emissions. These results indicated that increased N input leads to shifts in microbial activity within the rhizosphere, severely altering N cycling dynamics. Our study provides a framework for connecting environmental conditions of wetland bulk and rhizosphere soil to the structure and metabolic output of microbial communities.

## Introduction

Wetlands are globally impacted by agricultural industry through the leaching of various nitrogen (N) forms such as nitrate ($NO_3^-$), and by increased N deposition as a result of high N emissions from fossil fuel burning and agriculture (Galloway *et al.*, 2008). Furthermore, due to reduced oxidation under stagnant, waterlogged conditions, these systems show increased availability of ammonium ($NH_4^+$) (Britto and Kronzucker, 2002). The strongly increased anthropogenic N input influences ecosystem degradation by contributing to biodiversity loss and altering (mostly increasing) greenhouse gas fluxes such as nitrous oxide ($N_2O$), methane ($CH_4$) and carbon dioxide ($CO_2$) (Bobbink *et al.*, 1998; Liu and Greaver, 2009; Van den Heuvel *et al.*, 2011; Soons *et al.*, 2016).

The abundance, composition and activity of micro-organisms strongly influence the biogeochemical cycling of wetland nutrients, particularly those resulting in emissions of greenhouse gases (Lamers *et al.*, 2012; Philippot *et al.*, 2009). Specifically, $N_2O$ emission may increase due to lowering of pH affecting the activity of incomplete denitrifiers (Brenzinger *et al.*, 2015; Van den Heuvel *et al.*, 2011; Liu and Greaver, 2009). $CH_4$ emissions can increase due to competitive inhibition of the key enzyme of aerobic methanotrophs, methane monooxygenase (MMO), by elevated $NH_4^+$, osmotic stress of methanotrophs, or through the stimulation of methanogenic archaea (King and Schnell, 1998; Bodelier and Laanbroek, 2004; Dunfield and Knowles, 1995). Finally, the rate of soil C loss can increase as a result of N addition through the stimulation of heterotrophic respiration (Bragazza *et al.*, 2006). Although it is well established that microbial processes are important drivers of ecosystem functions, such as controls on greenhouse gas emissions and nutrient cycling, there is a lack of understanding of how these functions are linked, both to the environmental conditions and to the composition of the microbial community (Philippot *et al.*, 2009).

Wetland plant roots influence the soil region surrounding the root, known as the rhizosphere, by altering the availability of oxygen, organic matter, and organic plant exudates (Smith and Delaune, 1984; Abou Seada and Ottow, 1985; Bardgett and van der Putten, 2014). The total area of soil influenced by roots can be considerable, meaning that this definition of the rhizosphere may extend to the vast majority of the upper soil layer (Robinson *et al.*, 2003). The rhizosphere is an active, complex ecosystem where viruses, bacteria, archaea, fungi and protozoa interact with plant roots (Fierer *et al.*, 2007b). These microorganisms significantly contribute to nutrient cycling and ecosystem structure by channeling energy into higher trophic levels (reviewed in Curl & Harper 1990; Hinsinger et al. 2009).

While the rhizosphere has been studied for decades, the effects of eutrophication on the plant-microbe interactions are of more recent interest. Specifically, it is of interest how N availability influence plant physiology and ultimately C and N cycling in the rhizosphere. On the global scale, soil microbial communities differ depending on the regional and local N regime; although, the diversity of these communities does not seem to vary much (Fierer *et al.*, 2012). Interestingly, variation in microbial community composition seems to be predictable based on local nutrient regimes (Leff *et al.*, 2015; Ramirez *et al.*, 2012). Even though these studies demonstrate the link between nutrient loading and community structure, they do not demonstrate how changes in the microbial community are functionally relevant to the ecosystem..

To build dynamic models of plant-microbe interactions, it is necessary to gain a robust understanding of the connection between environmental conditions (i.e., N availability) and microbial community structure and function (i.e., the bulk biological processes resulting in greenhouse gas emissions). In this study, we aimed at assessing the impact of increased N input into wetland systems on the

rhizosphere microbial community and its functions related to greenhouse gas production. To achieve this, we used *Juncus acutiflorus* (Sharp-flowered Rush), a very common graminoid plant in European wetlands that forms a dense vegetation and is known for radial oxygen loss from roots (ROL; Lamers et al. 2012). Furthermore, it has a high tolerance for increased N inputs (van Diggelen *et al.*, 2016). In a longitudinal study we determined greenhouse gas emissions increase as a result of N addition in incubations with *J. acutiflorus*, but not in incubations with only bulk wetland soil, under controlled stable experimental conditions. Additionally, functional responses were linked to shifts in the dominant members of the microbial community. We hypothesize that certain key microbial groups contribute to greenhouse gas emissions, either directly or indirectly through the food web. Our study takes the first steps toward a predictive understanding of microbial dynamics within the rhizosphere, linking nutrient load, microbial community structure and function.

## Materials and Methods

### Sample Collection and experimental set up.

Plants and sandy soil were sampled from the Ravenvennen (51.4399 N, 6.1961 E) in Limburg, The Netherlands (August, 2015) and returned to the Radboud University greenhouse facilities for conditioning. The Ravenvennen is a protected marshy area consisting of sandy soil, rich in vegetation with a high prevalence of *Juncus spp.* Plants were removed from soil, rhizomes were cut into eight 2 cm fragments and reconditioned on hydroculture in a nutrient rich medium (as described in Hoagland & Arnon 1950). After sufficient root development (to approximately 25 cm after 2 weeks), eight plants and eight bulk soil incubations were randomly assigned to high or low nitrogen experimental groups (Supplementary table 1; Supplementary figure 1). Soil collected from the field, was homogenized and sieved to remove any contaminating roots and potted. The reconditioned plants were transferred to pots with a diameter of 19 cm at the base, 26 cm at the top and a height of 19 cm containing the prepared soil, moved to an indoor water bath set to 15°C (cryostat, NESLAB, Thermoflex 1400, Breda, The Netherlands) and cultivated with a day/night cycle of 16 hours light and 8 hours dark (Master Son-T PiaPlus, Philips, Eindhoven, The Netherlands). Pots were kept waterlogged with a 2 cm water layer on top. A drip-percolation based system ensured a constant supply of nutrients. The low N input nutrient solution contained 12.5 µM $NH_4NO_3$, corresponding to an N loading rate of 40 kg N $ha^{-1}$ $yr^{-1}$. The high N input solution contained 250 µM $NH_4NO_3$, corresponding to 800 kg N $ha^{-1}$ $yr^{-1}$. These rates fall within N loading of wetlands in agricultural catchments, thus represent contrasting extremes (Verhoeven et al., 2006).

### Incubation measurements.

Five representative *J. acutiflorus* specimens were harvested for initial measurements of plant dry weight, C:N ratios. At the final time point ($T_f$ = 90 days), all plants were harvested to measure dry weight and C:N ratios of roots, shoots and rhizomes. Pore water was extracted using 0.15 µm porous soil moisture samplers (SMS rhizons, Rhizosphere Research Products, Wageningen, The Netherlands) and measured over the course of the experiment to determine inorganic nutrients as well as metals using an Autoanalyzer (Autoanalyzer 3, Bran+Luebbe, Germany) and ICP-OES (iCAP6000, Thermo Scientific,Waltham, MA). To reduce the impact of soil heterogeneity, samples were extracted in duplicate and mean values were calculated.

### Greenhouse gas measurements.

To determine greenhouse gas fluxes, a cylindrical transparent collection chamber (7.5 x 30cm) was used to measure accumulation or depletion of $CO_2$, $CH_4$ and $N_2O$ in the headspace. $CO_2$ and $CH_4$

fluxes were measured at $T_m$ (45 days) and $T_f$ and $N_2O$ fluxes were measured at $T_f$. Fluxes were measured using a Picarro G2308 NIRS-CRD greenhouse gas analyzer (Picarro Inc., Santa Clara, CA, USA). Fluxes were determined by fitting a smoothed spline to the time series using the R function *sm.spline* from the pspline package and the average rate of change was calculated (Ramsay *et al.*, 1997).

**Denitrification potential.**

To measure denitrification potential, two soil slurries were made from each experimental pot by mixing 50g soil with 100mL milliQ water, divided into control and experimental bottles and made anoxic by flushing with argon gas. Bottles were pre-incubated overnight at 15°C to allow for residual unlabeled $NO_3^-$ to be consumed. A $^{15}N$-labeled $NaNO_3$ solution was added to the experimental bottles to a final concentration of 500 µM and a KCl solution was added to the control bottles to a final concentration of 500 µM. Production of $N_2O$ and $N_2$ were measured by taking samples 2, 7 and 22 h after adding substrate on a GC-MS (5975C, Agilent Technologies, Santa Clara, USA).

**DNA extraction, 16S rRNA Amplicon and Metagenomic sequencing.**

Soil was collected from three time points, one initial soil sample from the site, and $T_m$ and $T_f$ samples from each of the 16 incubations. A single core per pot was taken using a 1x7cm corer. DNA was extracted using the PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA, U.S.A.). 16S rRNA genes were amplified in triplicate reactions using IonTorrent sequencing adapter-barcoded primers 341F (CCATCTCATCCCTGCGTGTCTCCGACTCAGxxxxxxxxxxGATCCTACGGGNGGCWGCAG) and 785R (CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGACTACHVGGGTATCTAATCC) and pooled. The pooled amplicons were cleaned with Ampure beads (Beckman Coulter Inc., Fullerton, USA) and subsequently prepared for sequencing on the IonTorrent PGM using the manufacturer's instructions (Life Technologies, Inc., Carlsbad, CA, USA).

From the same DNA samples, total DNA was sheared into approximately 400 bp fragments via sonication. Resulting fragments were prepared for sequencing following the manufacturer's instructions with the Ion Plus Fragment Library Kit (Life technologies, Carlsbad, CA). Raw reads were submitted to NCBI and archived under the SRA accession number SRP099838.

**Data analysis.**

16S rRNA gene amplicons were quality filtered using QIIME v1.9 (Caporaso *et al.*, 2010). Quality controlled reads were then clustered into OTUs at a 97% identify and phylogenetically classified by utilizing the NINJA-OPS v1.3 pipeline (Al-Ghalith *et al.*, 2016). The reference database used for taxonomic assignment was the SILVA database version 123 (Quast *et al.*, 2013). The resulting OTU table was used for downstream analysis in R (R Core Team, 2016). Count data was normalized to relative abundances to account for differing sequence depth between samples and a square root transformation was applied. The *vegan* R package was used to calculate Shannon diversity with the *diversity* function, Bray-Curtis dissimilarity matrices with the *vegdist* function, and to estimate compositional variance with the *betadisper* function (Oksanen *et al.*, 2015). Principal component analysis (PCA) was performed using the *princomp* function in R. The *RandomForest* R package was used for classification and regression (Liaw and Wiener, 2002). Linear models were fit with the *glm* function in the *stats* package. Metagenomic reads were quality filtered ($Q > 25$) and small fragments (< 100bp) were removed using PrinSeq (Schmieder and Edwards, 2011).

The metagenomic reads were compared to custom nitrogen and methane cycling protein databases and the NCBI nr databases with Diamond (Buchfink *et al.*, 2014; Lüke *et al.*, 2016). A bit score ratio (BSR) between the hit to the custom databases and to the NCBI nr database was used to identify false positives hits. A strict BSR of 0.85 was used as a cutoff. Gene abundances were normalized and expressed relative to the single copy RNA polymerase *rpoB* gene abundance. These relative values were then scaled for comparison within genes. Reads from all metagenomes were assembled using metaSPAdes (version 3.7; Bankevich et al. 2012) and resulting contigs were compared against all publicly available Bacteroidetes, Acidobacteria and Verrucomicrobia genomes in the NCBI database using Blastn. Furthermore, contigs were assessed for the presence of N or $CH_4$ cycling genes by comparing them with Diamond to the previously mentioned N and $CH_4$ cycling custom databases.

## Results

### Plant physiology

*J. acutiflorus* and bulk soil were incubated over a course of 90 days. The soil collected from the sampling site and used in the incubations was a sandy soil with low organic matter content. Soil samples were taken at an initial time point ($T_0$), a mid-point ($T_m$; t = 45 days) and final time point ($T_f$; t = 90 days) (Supplementary Table 1). By $T_m$, *J. acutiflorus* incubations had significant root development throughout the incubated soil, and as a result the rhizosphere was sufficiently sampled such that the soil sampled was clearly dominated by root biomass. To determine the N utilization of the plants and to identify growth responses to N inputs, the total dry weight biomass of roots, rhizomes and shoots and total N and C content of *J. acutiflorus* tissue were measured from plants at $T_f$. Although there was no significant difference in total biomass and root:shoot ratio of *J. acutiflorus* between incubations , the average total N content of plant tissue was approximately twice as high (65 mg $g^{-1}$) in incubations with a high N input (t = 2.66; p = 0.037; Supplementary Table 2). Correspondingly, total C:N (averaged across the whole plant) was significantly higher in *J. acutiflorus* incubations with a low N input (t = -2.964; p = 0.009; Supplementary Table 2). Interestingly, this elevated C:N ratio was observed only for rhizome and shoot tissue, while the root C:N did not significantly differ between incubations (Supplementary Table 2).

### Greenhouse gas fluxes

To link greenhouse gas fluxes with microbial community structure, gas flux measurements were performed at the same time points as soil sampling. Greenhouse gases were measured in both light and dark conditions, at $T_m$ and $T_f$ for $CO_2$ and $CH_4$, and at $T_f$ for $N_2O$ (Figure 1). Bulk soils generally did not have significant greenhouse gas fluxes (fluxes were not significantly different from 0) and will not further be discussed here. In the *J. acutiflorus* incubations, $CO_2$ fluxes followed a day-night rhythm. Daytime $CO_2$ fluxes were generally negative, indicating net $CO_2$ fixation, with the largest rates significantly higher in high N *J. acutiflorus* incubations at $T_f$ (t = -5.28, p = 0.005; Figure 1A). Under dark conditions, $CO_2$ fluxes were positive only under the high N treatment while other treatments were not significantly different from 0(t = 3.52, p = 0.01; Figure 1B). $CH_4$ and $N_2O$ emissions did not vary between dark and light conditions and therefore these conditions will not be compared. $CH_4$ fluxes increased from $T_m$ to $T_f$ and emissions tended to be highest in the *J. acutiflorus* incubations with a high N input, however there was large variability in this group (t = 2.165; p = 0.064; Figure 1C). $N_2O$ emissions were highest in the high N treatment (t = 2.56, p = 0.04; Figure 1D), while a negative $N_2O$ flux was observed in *J. acutiflorus* incubations receiving a low N input (Figure 1D).

### Denitrification potential

To understand how increased N input influenced N cycling within bulk and *J. acutiflorus* rhizosphere soils, soil slurries were taken at $T_f$ and their denitrification potential was measured. There was significantly higher $N_2O$ production from slurries originating from high N treatment soils ($t = 2.41$; $p = 0.045$; Supplementary Figure 2). There was no significant difference in the $N_2$ production between high or low N treatments ($t = 0.32$; $p = 0.75$; Supplementary Figure 2). Additionally, the average $N_2$:$N_2O$ ratio was approximately 10 times higher in low N input slurries (5.36 +/- 7.39; $N_2$:$N_2O$ production) as compared to high N slurries (0.58 +/- 0.61), though not significantly different at $p < 0.05$ ($t = -1.84$; $p = 0.11$; Supplementary Figure 2).

## Microbial community structure

The v3-v4 fragment of the 16S rRNA gene was amplified and sequenced resulting in, on average, over 1100 post-quality control (QC) sequences per sample. Each sample contained on average 264 +/- 136 Operational Taxonomic Units (OTUs +/- s.d.). Over the course of the incubation, the dominant microbial group changed (Figure 2A). Solibacteriales were most abundant at $T_0$ and at $T_m$, but by $T_f$ Rhizobiales became the prominent group (Figure 2B). On average, microbial diversity increased between $T_m$ and $T_f$, ($t = 2.516$; $p = 0.0176$; Supplementary Figure 3A). Within each time point, diversity did not differ significantly between *J. acutiflorus* and bulk soil incubations, nor did N input have an impact (Supplementary Figure 3B+C). To assess how community composition varied across the different incubations, the Bray-Curtis dissimilarity index was used to calculate compositional differences between microbial communities. The compositional variation did not significantly vary between $T_m$ and $T_f$, indicating that community variability did not change within the different experimental groups across time (Supplementary Figure 4A-C). The most variable communities were observed for low N *J. acutiflorus* incubations at $T_m$, which furthermore were significantly different from the low N input bulk soil incubations (Tukey's HSD; $p = 0.0184$; Supplementary Figure 4B). At $T_f$ there were no significant differences in community variation among bulk soil or *J. acutiflorus* incubations, or between low and high N loading.

## Linking microbial community members to function

In order to understand how the microbial community members were linked to environmental conditions and greenhouse gas emissions, a random forest classifier was used to identify microbial taxa whose abundance was affected by N input, time of sampling or presence of *J. acutiflorus*. Additionally, random forest was also used for regression to determine connections between abundance of these groups and environmental conditions or greenhouse gas fluxes, and these associations were further analyzed by fitting linear models.

The top three microbial groups that significantly responded to N input were the Opitutales (Verrucomicrobia) and Sphingobacteriales (Bacteroidetes), which were more abundant in the high N treatment group, and G6 Acidobacteria, which were more abundant in in the low N treatment (Figure 2B; Table 1). More specifically, the relative abundances of these three orders could be linked to $N_2O$ emissions (Table 1). Opitutales and Sphingobacteriales were positively associated with $N_2O$ fluxes, while a negative association was observed for the G6-Acidobacteria. In addition, Sphingobacteriales were correlated to $CO_2$ fixation (Table 1).

The top bacterial order distinguishing microbial communities from rhizosphere and bulk soil were the Alphaproteobacterial Caulobacterales, which were more abundant in the rhizosphere than in bulk soil and had a negative association with elevated $NO_3^-$ concentrations (Table 1). The Rhizobiales and Solibacterales orders of the Alphaproteobacteria class and Acidobacteria phylum, respectively, were most distinctive for the microbial communities sampled at $T_m$ versus $T_f$ (Figure 2; Table 1).

Rhizobiales abundance was negatively associated with $CO_2$ fluxes in dark conditions while the Solibacterales were correlated to pore water alkalinity, which is a proxy for anaerobic decomposition (Figure 2; Table 1).

**Soil metagenomics**

In addition to the 16S rRNA gene, total DNA was sequenced from 5 soils representing $T_0$, and rhizosphere and bulk soil samples at $T_m$ and $T_f$ from the high N treatment. These libraries resulted in on average 1 million post-QC reads per library (Supplementary Table 3). In addition to statistically linking the abundance of microbial groups to environmental conditions and greenhouse gas fluxes via the 16S rRNA amplicon analyses, we wanted to identify the genetic potential for N and C cycling processes within the N-amended samples. Furthermore, we aimed at linking these genetic functions to the groups identified as being most strongly influenced by increased N loading. To do this, custom databases of genes involved in N and C cycling processes (Lüke et al., 2016) were used to identify metagenomic reads of major N (*amoA* and *hao*, involved in $NH_4^+$ oxidation; *narG*, *nirK*, *nirS*, *norB* and *nosZ*, involved in denitrification; *nrfA*, involved in dissimilatory nitrite reduction to ammonia; and *nifH*, involved in N fixation) and $CH_4$ cycling genes (*pmoA and mmoX*, inovled in $CH_4$ oxidation; *phnGHI* and *mcrA*, involved in methanogenesis), and their abundance in the high N incubations (abbreviations found in Supplementary Table 5). There were no *nirS* detected in the dataset and only two reads annotated as *mcrA* were detected in the metagenomes from *J. acutiflorus*. All other N and $CH_4$ cycling genes were present. $CH_4$ cycling genes *pmoA*, *hao* and *phn* were most abundant in the initial soil and the *J. acutiflorus* rhizosphere (Supplemental Figure 5). Interestingly, the *nrfA* gene, involved in dissimilatory nitrite reduction to ammonia (DNRA) which competes with denitrification for nitrite, was least abundant in the initial soil and in the rhizosphere soil at the final time point. *amoA* was mostly abundant in bulk soil metagenomes and the initial soil.

In addition, over 4.8 million soil metagenome reads were assembled into 129,476 contigs with a maximum length of 23kbp and a mean length of 597bp (+/- 368bp). Assembled contigs were compared to publicly available bacterial genomes from the Bacteroidetes, Verrucomicrobia and Acidobacterial phyla to identify genome fragments derived from the species identified in our previous analysis. Across all metagenomes, 5454 reads mapped to 145 contigs which had high identity to a Subgroup 6 Acidobacterial genome (CP015136.1; 84.5 +/- 7.1% identity), 6831 and 22 reads mapped to 352 and 5 contigs which aligned to an Opitutales (CP016094.1; 85.5 +/- 7.9% identity) and Sphingobacteriales (CP003349.1; 86.3 +/- 7.7% identity) genomes respectively (Supplementary Table 4).

## 1 Discussion

Greenhouse gas emissions remain a global challenge. A mechanistic understanding of the factors that alter microbial community structure and function, such as increased N input, is important in developing management strategies for greenhouse gas emissions. This is particularly important in ecosystems as extensive as wetlands. With an estimated area of up to 12.8 million $km^2$ worldwide, wetlands considerably contribute to the total terrestrial carbon storage (Zedler and Kercher, 2005; Nahlik and Fennessy, 2016). Here we studied the impact of increased N input on the microbial community and greenhouse gas fluxes from the rhizosphere of *Juncus acutiflorus*, a very common plant in European wetland ecosystems, and a model for other *Juncus* species globally. We found characteristic shifts in the microbial community structure and a stimulation of greenhouse gas fluxes in *J. acutiflorus* incubations in response to N input.

**Plant physiological shifts as a response to high N inputs.**

The plant plays a prominent role in the maintenance of the rhizosphere microbial community (Reinhold-Hurek *et al.*, 2015). Roots release oxygen through radial oxygen loss providing an oxic niche in otherwise anoxic wetland soils (Armstrong, 1971). Plants also release labile organic matter in the form of organic acids, neutral sugars and amino acids (Kamilova *et al.*, 2006; Jones, 1998). The composition of this organic matter structures the microbial community within the rhizosphere by providing different substrates for heterotrophic micro-organisms (Haichar *et al.*, 2008). The exuded organic acids also acidify the surrounding soil, preventing many microbial species from thriving within the rhizosphere, but also modifying nutrient availability (Marschner *et al.*, 1987; Petersen and Böttger, 1991). The quantity of organic matter released is closely associated with photosynthesis rates. As plants are often N limited in natural systems, relieving this limitation promotes plant growth (Reich *et al.*, 2006). In this study we observed that when incubated under high N input *J. acutiflorus* showed increased C fixation rates (Figure 1A) and plant tissue becomes saturated with N (Supplementary Table 2). This also suggests that *J. acutiflorus* without N limitation excretes larger amounts of labile carbon into the surrounding soil, which is also evident from the observed decreases in pore water pH in the high N incubations. Additionally, due to root derived oxygen, increased nitrification rates could contribute to this observed drop in pH (Lamers et al. 2012; Supplementary Figure 5). Together, higher N input could result in higher photosynthetic rates in *J. acutiflorus* specimens, likely depositing larger amounts of organic matter into surrounding soil, stimulating the heterotrophic microbial community in return (Figure 2; Figure 3).

**Greenhouse gas fluxes as a result of high N input.**

N availability has been shown to alter greenhouse gas emission dynamics in previous studies (Philippot *et al.*, 2009). Here we observed that greenhouse gas fluxes in *J. acutiflorus* incubations were stimulated as a response to increased N input (Figure 1). $CO_2$ fixation rates were highest in *J. acutiflorous* incubations with high N input in the light conditions, likely due to increased photosynthetic activity of the plant and photosynthetic microorganisms. In the dark, the same *J. acutiflorus* incubations showed elevated $CO_2$ emissions, likely due to increased plant and microbial respiration (Figure 1).

In this study, the highest $CH_4$ emissions were observed in *J. acutiflorus* incubations with high N input, although with high variability (Figure 1C). Still, the elevated emission rates suggest that the *J. acutiflorus* rhizosphere could become a net source of $CH_4$ under high N input. The total amount of $CH_4$ released reflects the sum of $CH_4$ production (methanogenesis) and consumption (methanotrophy). Methanogenesis has been linked to plant productivity, thought to be due to increased availability of labile organic carbon from photosynthate exudates (Whiting and Chanton, 1993; Aulakh *et al.*, 2001). Furthermore, methanogens can be stimulated through an indirect priming mechanism. Labile organic matter from plant photosynthate can stimulate microbial activity responsible for degrading recalcitrant organic matter, which in turn makes this carbon source available to methanogens (Jenkinson *et al.*, 1985; Kotsyurbenko, 2005; Kotsyurbenko *et al.*, 1993; Tveit *et al.*, 2015). Alternatively, net $CH_4$ emissions can be increased by inhibiting $CH_4$ consumption, for instance through the competitive inhibition of the key enzyme methane monooxygenase by $NH_4^+$ (Bosse *et al.*, 1993; Conrad and Rothfuss, 1991).

The reduction of $NO_x$ to $N_2$ is often incomplete, resulting in the production of the greenhouse gas $N_2O$. Incomplete denitrification occurs when microbial species do not utilize $N_2O$ as an electron acceptor either due to physiological constraints or induced by certain environmental conditions (Philippot, 2002; Wallenstein *et al.*, 2006). It has been observed that N fertilization has the largest impact on $N_2O$ emissions when considering all terrestrial ecosystems, with $NO_3^-$ availability being

the main driver (Liu and Greaver, 2009). As denitrification is largely a microbial process, the composition of the microbial community plays an important role in the total amount of N emitted from soils. Representatives from a diverse set of phyla are known to denitrify (Philippot, 2002; Philippot *et al.*, 2009) and denitrification rates are therefore considered to be robust to changes in the microbial community composition (Enwall *et al.*, 2005). Here we observed elevated $N_2O$ emissions in *J. acutiflorus* incubations under high N input, whereas there were negative $N_2O$ fluxes in the low N incubations. Interestingly, $N_2O$ emissions by bulk soil were not significantly influenced by the tested N regimes, indicating that *J. acutiflorus* plays a substantial role in stimulating N reducing microbial species, probably by supplying labile carbon. In addition there was an almost 10-fold shift in the release of $N_2O$ relative to $N_2$ as a response to N input suggesting a high N input can shift the community towards partial denitrifiers in the rhizosphere, which is important given the strong greenhouse potential of $N_2O$.

**Shifts in microbial community structure as a response to high N input.**

Associating microbial metabolisms (i.e., those resulting in greenhouse gas emission) to the structure of microbial communities and abiotic factors defined by the environment is essential to predict how the structure and function of these microbial ecosystems may adapt to future conditions. Bulk and rhizosphere soils contain diverse microbial communities with equally diverse metabolisms (Philippot *et al.*, 2013; Torsvik and Øvreås, 2002). It remains a challenge to understand the role that key groups play in these systems, and how they affect their environment.

We link the abundance of three bacterial orders to N input and greenhouse gas emissions (Figure 2; Table 1). The verrucomicrobial Optitutales were associated with high N input and elevated $N_2O$ emissions. Members of this order are diversely associated with different rhizospheres, ranging from sugar cane to wetland plants (Dedysh *et al.*, 2006; van Passel *et al.*, 2011). They have been physiologically described as anaerobic polysaccharide utilizing bacteria that are capable of reducing $NO_3^-$ to $NO_2^-$ (Chin *et al.*, 2001). Apart from the $O_2$ derived from the plant roots, which is quickly consumed by aerobic heterotrophs, wetland soils are waterlogged systems resulting in an anoxic environment. Assembled sequences from the metagenomes obtained in this study aligned to an Optitutales genome (CP016094.1), which encodes $NO_3^-$ and $NO_2^-$ reductases. Additionally, two of our assembled contigs contained open reading frames for the copper-containing nitrite reductase (NirK). It is likely that members of this order are utilizing plant derived organic matter as their electron donor and $NO_3^-$ as their electron acceptor (Figure 3).

The Sphingobacteriales from the phylum Bacteroidetes were also overrepresented in the high N input incubations (Figure 2; Table 1). Sphingobacteriales are understood as copiotrophic bacteria, referring to their ability to metabolize a wide array of carbon sources and being present at high abundances in soils with high carbon availability (Fierer *et al.*, 2007b; Padmanabhan *et al.*, 2003). In the current study, the majority of organic matter would originate from the plant as the sandy soil used had low organic matter content. Rhizodeposition in this case would be very important to groups such as Sphingobacteriales, not only as a carbon source but as an $O_2$ source as Sphingobacteriales seem to be particularly sensitive to $O_2$ availability. When tested for cellulolytic activity in oxic or anoxic environments they were exclusively active in the oxic treatment, suggesting that this group may require oxygenated environments for carbon degradation (Schellenberger *et al.*, 2009). Here, Sphingobacteriales were more abundant in high N input incubations and were associated with $N_2O$ fluxes and higher $CO_2$ fixation rates, suggesting that they may benefit from oxygen and carbon derived from roots. In addition, multiple contigs from the soil metagenomes aligned to a Sphingobacteriales genome (CP003349.1), which encodes nitrate, nitrite, nitric oxide and nitrous

oxide reductases. Three of these contigs encoded NirKs homologous to one found in a Sphingobacteriales genome (LGEL01000245.1). Considering findings from this study and the literature, we hypothesize that Sphingobacteriales within the *J. acutiflorus* rhizosphere could be facultative anaerobes benefiting from the elevated carbon input from the roots and utilizing available $NO_x$ as electron acceptors (Figure 3).

G6 Acidobacteria were overrepresented in the low N input incubations and there was no significant difference in their abundance between bulk and rhizosphere soils. Unlike Opitutales and Sphingobacteriales, they were negatively correlated with $N_2O$ emissions (Figure 2; Table 1). While the G6 Acidobacteria group is not well studied, one genome (CP015136.1) was recently published (Huang *et al.*, 2016) and was shown to contain nitric and nitrous oxide reductases. 145 contigs of our metagenome aligned to this genome; however none of the assembled contigs encoded proteins involved in denitrification. Genomic and physiological studies of a closely related group (group 1 Acidobacteria) showed that they were anaerobic organoheterotrophs capable of utilizing $NO_3^-$ for respiration and $NH_4^+$ as an N source (Dedysh *et al.*, 2012), and other Acidobacteria have also been described as important soil carbon and N cyclers. However, many N-cycling reactions are restricted to particular clades indicating that these functions are heterogeneously represented across the Acidobacteria phylum (Kielak *et al.*, 2016; Koch *et al.*, 2008). Alternatively, Acidobacteria can utilze C derived from autotrophic microorganisms in anoxic environments (Meisinger *et al.*, 2007). They have been reported to utilize various plant and microbe-derived polysaccharides, like xylan, cellobiose and gellan (Janssen *et al.*, 2002; Koch *et al.*, 2008) and thrive in various soils and rhizospheres, including anoxic soils with low pH (Fierer *et al.*, 2007a; Pankratov and Dedysh, 2010). The cultured representatives of Acidobacteria have low growth rates and appear to be adapted to oligotrophic environments (Fierer et al. 2007; Jones et al. 2009). Thus, G6 Acidobacteria may not be competitive under high N availability by fast-growing (partial) denitrifiers. Together, the G6-Acidobacteria may be involved in anaerobic degradation of organic carbon from autotrophic bacteria or plant biomass, and increased N availability might reduce this group's abundance (Figure 3).

**A model microbial food web within bulk soil and the *J. acutiflorus* rhizosphere.**

Increased N input poses a distinct threat to wetland ecosystems, contributing to the degradation of biodiversity and altering greenhouse gas emissions (Bobbink *et al.*, 1998; Philippot *et al.*, 2009). Plants, such as *J. acutiflorus*, influence the abundance and composition of micro-organisms living in the rhizosphere by exuding organic matter and releasing oxygen from their roots (Reinhold-Hurek *et al.*, 2015). In the current study, N addition resulted in increased productivity of *J. acutiflorus*, stimulating the effect of the plant on the microbial community but also directly affecting microbial metabolism. Based on our observations and published knowledge, we built a model of the *J. acutiflorus* microbial food web indicating how N input impacts the soil microbial community (Figure 3).

N fertilization can directly influence the soil microbial community by providing excess $NH_4^+$ and $NO_3^-$. Previous studies have shown that *J. acutiflorus* prefers $NH_4^+$ over $NO_3^-$ as N source, leading to a surplus of $NO_3^-$ in the rhizosphere (Supplementary Figure 5; van Diggelen et al. 2016). This alters N cycling dynamics, favoring microbial species capable of rapidly reducing $NO_3^-$ to $N_2O$ rather than to $N_2$. While complete denitrification supports higher growth yields, it also is energetically more costly and thus unfavorable under lower nutrient availability (i.e., K strategy life style). The combined effect of enhanced plant derived carbon input and higher N availability stimulates heterotrophic activity, resulting in increased $N_2O$ and $CO_2$ emissions (Figure 3). While excess $NO_3^-$ spurs anaerobic respiration, increased $NH_4^+$ concentrations can lead to an inhibition of methane

oxidation, possibly contributing to the heterogeneity observed in $CH_4$ emissions (Figure 1C). High N availability can also have an indirect effect by influencing plant physiology. The observed increased rates of carbon fixation by *J. acutiflorus* under high N input may result in augmented release of organic matter (including organic acids) and oxygen from the roots. This acidifies the rhizosphere soil, which can alter the activity of *nosZ* containing microbes (Liu *et al.*, 2014). Additionally, elevated oxygen availability stimulates heterotrophic activity in an otherwise anoxic environment, leading to higher $CO_2$ emissions. Thus, altered N input in the *J. acutiflorus* rhizosphere leads to increased greenhouse gas fluxes directly by altering the abundance of N-cycling species and indirectly through the stimulation of plant primary productivity (Figure 3).

## CONCLUSIONS

With continued anthropogenic inputs of nitrogen into wetlands, it is critical to mechanistically understand how this activity may affect globally relevant carbon and nitrogen cycling within wetlands. The results here support that under high N input, greenhouse gas emissions from the *J. acutiflorus* rhizosphere increase, shifting the system from a greenhouse gas sink to a source. Three bacterial orders, the Opitutales, G6-Acidobacteria and Sphingobacteriales, respond to increased N availability and genomic evidence supports their involvement in processes leading to changes in greenhouse gas fluxes. Our view is that understanding interactions within the rhizosphere, that result in increased greenhouse gas emissions, is essential for creating management solutions aimed to address greenhouse gas emission goals, efficient agricultural practices, and conservation efforts. To move forward in our understanding of the complex dynamics within ecosystems such as the rhizosphere, future effort needs to be made in building extensive datasets that can be used to build predictive models of how these microbial ecosystems might respond under altered environmental conditions. We propose that mechanistic models, such as our *J. acutiflorus* rhizosphere plant-microbial food web model, should be used to set the framework for building such datasets.

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## Author Contributions

EH, SFH, JvD, LL, MJ, CL, SL, CW designed research; EH, SFH, JvD performed research; EH, SFH, JvD analyzed data; EH, SL, CW wrote the paper; All authors reviewed and agreed with the final version of the manuscript.

## Conflict of Interest

The authors declare no conflicts of interest.

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## Figures

**Figure 1. $CO_2$, $CH_4$ and $N_2O$ fluxes.**

Greenhouse gas fluxes were measured at a midpoint ($T_m$) and final time point ($T_f$) during the 90 day incubation experiment. (A) $CO_2$ light conditions, (B) $CO_2$ dark conditions, (C) $CH_4$ and (D) $N_2O$. Asterisks denote significant differences ($p < 0.05$).

**Figure 2. Microbial community structure and diversity.**

(A) Overview of microbial community structure of the initial soil sample (I), *J. acutiflorus* rhizosphere and bulk soil incubations receiving high or low N input at midpoint ($T_m$) and final time point ($T_f$). (B). Principal component analysis of the microbial community members distinguishing high and low N treatments and midpoint and final sampling time points. Points indicate individual samples taken. Red dashed arrows indicate environmental and gas fluxes that corresponded variation in microbial community member's abundance along the respective axis.

**Figure 3. A *Juncus acutiflorus* rhizosphere microbial food web model.**

In the model of the *J. acutiflorus* rhizosphere, microbial processes are directly (red lines) or indirectly (black lines) influences by N deposition. *J. acutiflorus* preferentially takes up $NH_4^+$ which stimulates plant productivity and rhizodeposition of organic matter and oxygen (Van Diggelen et al., 2015). Released oxygen and labile organic matter contribute to soil acidification in addition to stimulating complex polymer degradation (Sphingobacteriales) and heterotrophic denitrifiers (Opitutales). The production of $N_2$ can be affected by a drop in pH which influences the activity of complete denitrifiers. The Group-6 Acidobacteria are outcompeted at higher N availability. Recalcitrant organic matter degraded by Sphingobacteriales can enter the microbial food web and be fermented by fermenters that in turn provide substrates for methanogens (*mcr*). The activity of phosphonate lyases (*phn*) might also stimulate the production of methane while anaerobic methane oxidation also contributes to methane consumption. Additionally, methane consumption by aerobic methanotrophs

674 through methane monoxgenases (*pmo*) could be inhibited by excess $NH_4^+$ (Dunfield and Knowles
675 1995).

676 **Supplemental Material**

677 **Supplemental Table 1.**

678 Sample overview containing the time of sampling, N load treatment and whether or the sample was
679 bulk or rhizosphere soil. Additionally, the number of post quality filtered reads that were produced
680 and the number of OTUs found in each sample. Finally, greenhouse gas fluxes are reported in (µmol
681 $m^{-2}$ $d^{-1}$).

682 **Supplemental Table 2.**

683 Plant average dry weight and C:N were determined in different sections of the plant including the
684 roots, shoots and rhizomes. Biomass weight was determined as dry weight. The mean values from
685 plants receiving high N and low N are reported (Mean_High and Mean_Low). The p-value is
686 reported as a result of a t-test comparing mean values from high and low N treatments.

687 **Supplemental Table 3.**

688 Metagenome library overview including the time of sampling (Time), number of post-QC reads
689 (Reads), average length (Avg_len) and the standard deviation in read length (Sd_len).

690 **Supplemental Table 4.**

691 Number of reads and contigs assigned to one of three publicly available soil bacterial genomes.
692 Number of reads each metagenome contained are reported as well as the number of contigs that were
693 assembled that aligned to these genomes.

694 **Supplemental Table 5.**

695 Gene abbreviations.

696 **Supplemental Figures**

**Supplemental Figure 1.**

Experimental design schema depicting sample replicates per treatment in either rhizosphere/bulk soil. Additionally, the sampling points are denoted by colored boxes.

**Supplemental Figure 2.**

The Shannon diversity index (H') was calculated for all microbial communities. The Shannon diversity of all samples was compared from $T_m$ and $T_f$ (A). Diversity of experimental groups (High/Low N + Rhizosphere/Bulk) of all $T_m$ (B) and $T_f$ (C) samples were compared using multiple comparisons

**Supplemental Figure 3.**

Microbial community variation was estimated by calculating the Bray-Curtis dissimilarity for each sample in a pairwise fashion resulting in a square distance matrix. These pairwise distances were then reduced to two dimensions using multidimensional scaling. A centroid was calculated for each group being compared and each sample's Euclidean distance to its respective group centroid was calculated. $T_m$ and $T_f$ were compared in panel A, $T_m$ (B) and $T_f$ (C) samples were compared within respective groups (High/Low N + Rhizosphere/Bulk)

**Supplemental Figure 4.Denitrification potential from soil slurries.**

$N_2$ and $N_2O$ production rates were estimated to determine potential denitrification of the soil and rhizosphere microbial communities.

**Supplemental Figure 5. Heatmap of C and N cycling gene abundance from metagenomes.**

**Metagenome of Initial, N-loaded rhizosphere representative (at Tm/Tf), N-loaded bulk soil representative (at Tm/Tf). Supplemental Figure 6. Pore water inorganic nutrients, pH and alkalinity.**

Concentration of inorganic nutrients, pH and alkalinity in pore water sampled throughout the incubation.

721 **Table 1. Correlations of microbial community members to environmental conditions and greenhouse gas fluxes.**

722 The mean relative abundance of top bacterial families distinguishing high v low N, rhizosphere v bulk soil or $T_m$ v $T_f$ sampling time points

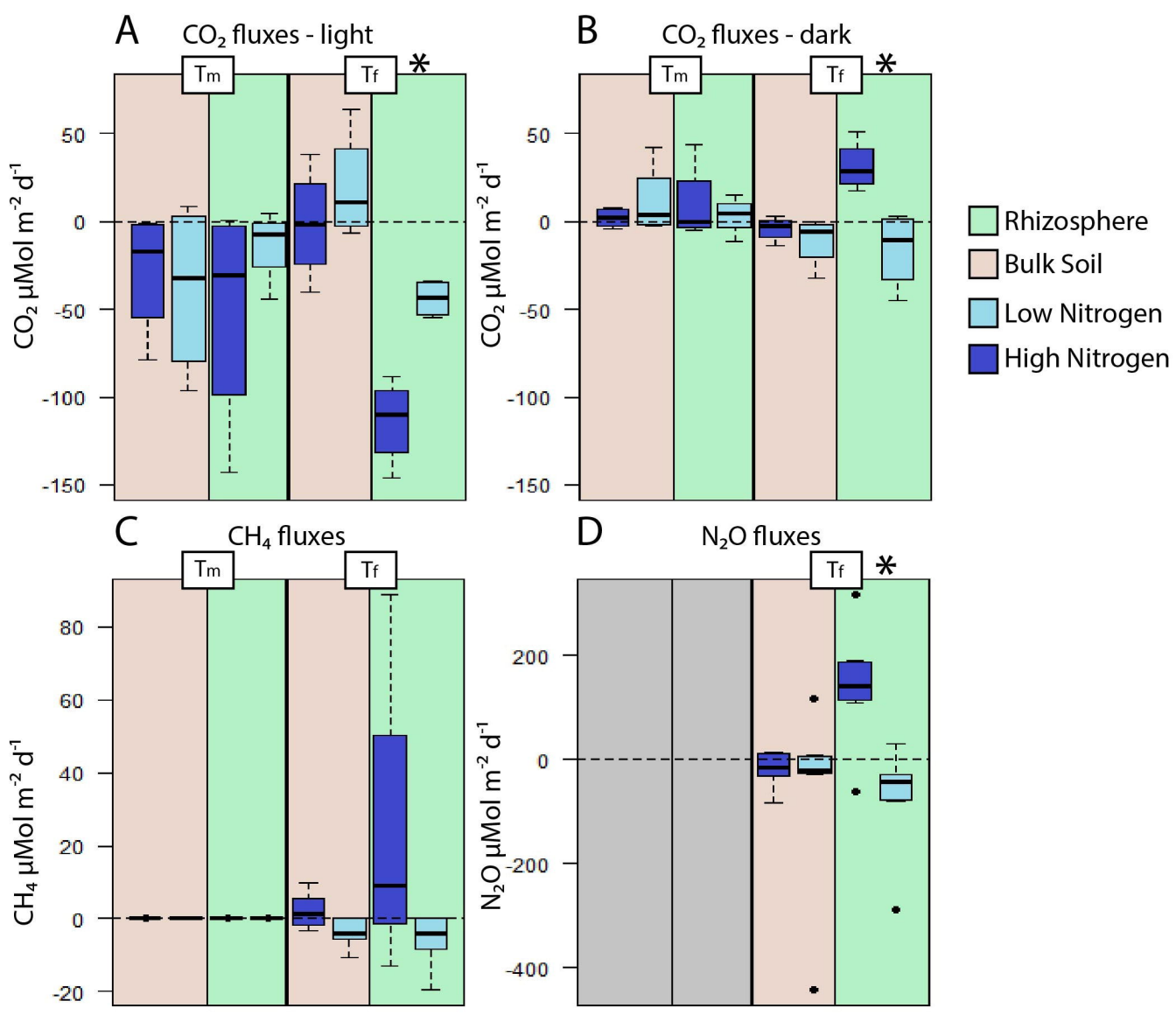
723 are indicated as is the t-test result and statistics. Additionally, the top environmental or functional traits correlated with these groups were

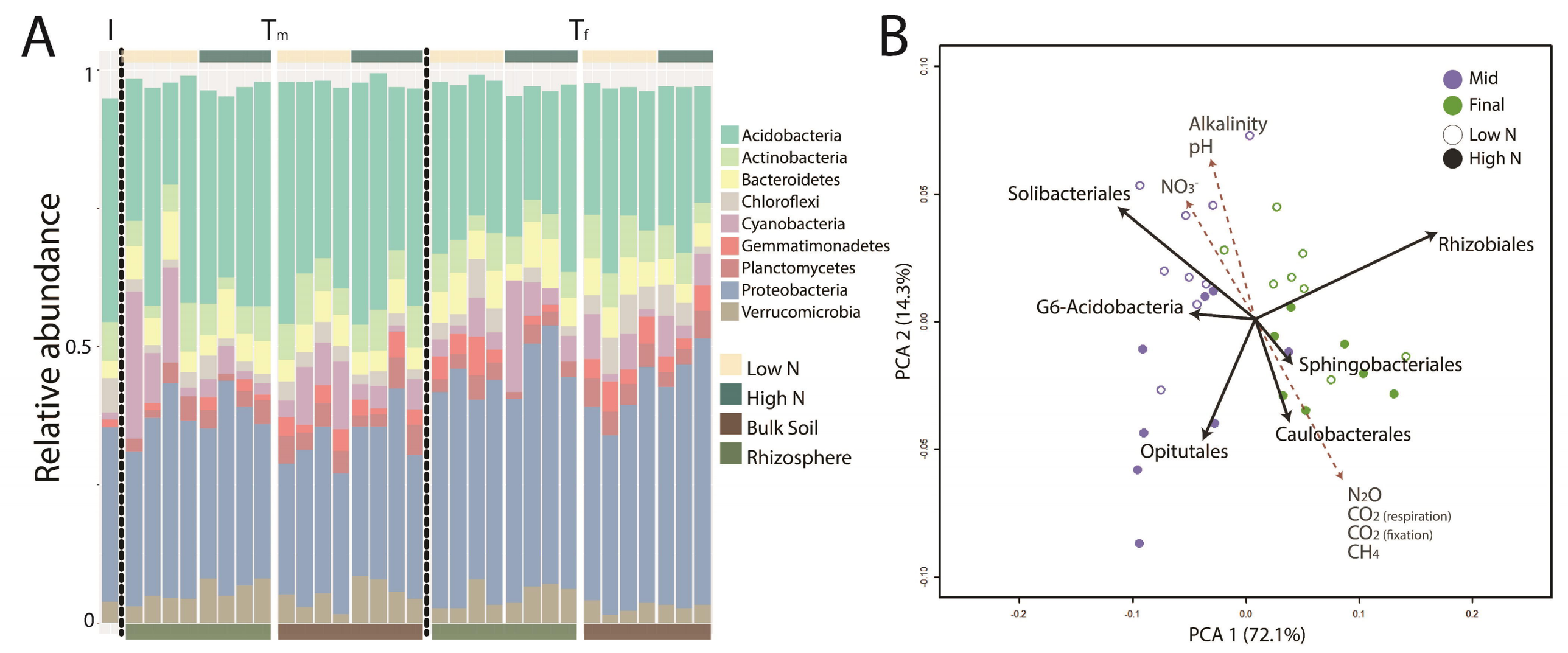
724 reported along with linear model statistics.

| | t | p-value | Mean Relative Abundance | | Correlate | adj R2 | coef | p-value |
|---|---|---|---|---|---|---|---|---|
| ***High versus low N*** | | | *High N* | *Low N* | | | | |
| Opitutales | 4.17 | < 0.001 | 0.040 | 0.010 | $N_2O$ | 0.11 | 3.50E-04 | 0.012 |
| G6-Acidobacteria | -4.22 | < 0.001 | 0.007 | 0.020 | $N_2O$ | 0.19 | -3.18E-05 | 0.058 |
| Sphingobacteriales | 2.88 | 0.008 | 0.010 | 0.005 | $N_2O$ | 0.32 | 3.10E-05 | 0.016 |
| | | | | | $CO_{2\ (fixation)}$ | 0.29 | 7.07E-05 | 0.011 |
| ***Rhizosphere versus bulk*** | | | *Rhizosphere* | *Bulk* | | | | |
| Caulobacterales | -3.46 | 0.002 | 0.052 | 0.032 | $NO_3^-$ | 0.21 | -8.50E-05 | 0.003 |
| ***$T_m$ versus $T_f$*** | | | *$T_m$* | *$T_f$* | | | | |
| Rhizobiales | 6.66 | < 0.001 | 0.099 | 0.184 | $CO_{2\ (respiration)}$ | 0.27 | -6.40E-04 | 0.001 |
| Solibacterales | -4.76 | < 0.001 | 0.179 | 0.116 | *Alkalinity* | 0.26 | -2.00E-02 | 0.002 |

725

726


A
$CO_2$ fluxes - light
Tm
Tf
*
$CO_2$ µMol $m^{-2}$ $d^{-1}$
50
0
-50
-100
-150
B
$CO_2$ fluxes - dark
Tm
Tf
*
$CO_2$ µMol $m^{-2}$ $d^{-1}$
50
0
-50
-100
-150
Rhizosphere
Bulk Soil
Low Nitrogen
High Nitrogen
C
$CH_4$ fluxes
Tm
Tf
$CH_4$ µMol $m^{-2}$ $d^{-1}$
80
60
40
20
0
-20
D
$N_2O$ fluxes
Tf
*
$N_2O$ µMol $m^{-2}$ $d^{-1}$
200
0
-200
-400


A
I
Tm
Tf
1
0.5
0
Relative abundance
Acidobacteria
Actinobacteria
Bacteroidetes
Chloroflexi
Cyanobacteria
Gemmatimonadetes
Planctomycetes
Proteobacteria
Verrucomicrobia
Low N
High N
Bulk Soil
Rhizosphere
B
Mid
Final
Low N
High N
Alkalinity
pH
NO3-
Solibacteriales
Rhizobiales
G6-Acidobacteria
Sphingobacteriales
Caulobacterales
Opitutales
N2O
CO2 (respiration)
CO2 (fixation)
CH4
PCA 2 (14.3%)
PCA 1 (72.1%)
0.10
0.05
0.00
-0.05
-0.10
-0.2
-0.1
0.0
0.1
0.2

Effect of N addition

Direct

Indirect

Inhibition

$NH_4NO_3$

Water layer

Oxic Zone

Anoxic Zone

$NH_4^+$

Roots

Organic Matter → pH↓

VI-Acidobacteria

Sphingobacteriales

$NO_3^-$

$NH_4^+$

$NH_4^+$

$CH_4$

Opitutales

$CO_2$ $CH_4$ $N_2$ $N_2O$