Intra- and inter-annual variability of nitrification in the rhizosphere of field-grown bioenergy sorghum

Mark B. Burnham[1,2]*, Sandra J. Simon[1,3], D.K. Lee[1,2,4], Angela D. Kent[1,2,5], Evan H. DeLucia[1,2,6,7], Wendy H. Yang[1,2,6,7,8]

[1]Center for Advanced Bioenergy and Bioproducts Innovation, Urbana, IL, USA 61801

[2]University of Illinois at Urbana-Champaign Institute for Genomic Biology, Urbana, IL, USA 61801

[3]Department of Biology, West Virginia University Institute of Technology, Beckley, WV, USA 25801

[4]Department of Crop Sciences, University of Illinois at Urbana-Champaign, Urbana, IL, USA 61801

[5]Department of Natural Resources and Environmental Sciences, University of Illinois at Urbana-Champaign, Urbana, IL, USA 61801

[6]Department of Plant Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA 61801

[7]Institute for Sustainability, Energy, and Environment, University of Illinois at Urbana-Champaign, Urbana, IL, USA 61801

[8]Department of Geology, University of Illinois at Urbana-Champaign, Urbana, IL, USA 61801

*Corresponding author:

1200 IGB
1206 West Gregory Dr.
Urbana, IL 61801
(413) 834-0056
mburnham@illinois.edu

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Abstract

Biological nitrification inhibition (BNI) and plant-microbe competition for ammonium (NH_4^+) by sorghum (*Sorghum bicolor* (L.) Moench) have the potential to suppress nitrification, reducing nitrate (NO_3^-) and nitrous oxide (N_2O) production for more sustainable bioenergy feedstock production. However, it is unknown how variability in environmental factors, field management, and plant growth affect the suppression of nitrification. We conducted a field trial with four genotypes of energy sorghum and four fertilization rates in central Illinois, USA, and measured soil N pools, potential nitrification and denitrification rates, and microbial community composition in bulk and rhizosphere soils to assess nitrification suppression throughout the 2018 and 2019 growing seasons. Concentrations of NO_3^- and NH_4^+ were very low in rhizosphere soil regardless of fertilization level, suggesting strong N demand by plants and microbes. Potential nitrification was lower in the rhizosphere soil than bulk soil, and this suppression was strongest mid-season ~2 months after planting in both years (20% suppression in 2018 and 58% in 2019). Since precipitation was lower during the mid-growing season of 2019 compared to 2018, we speculate that hydrophilic BNI root exudates accumulated in the rhizosphere and suppressed nitrification more than in 2018 when soil moisture was higher. Unfertilized plots had greater nitrification suppression than fertilized plots during the mid-season in 2018, but otherwise nitrification suppression was insensitive to fertilizer treatment. Potential denitrification was stimulated in the rhizosphere compared to bulk soil in both study years, suggesting that heterotrophic activity was stimulated by plant carbon inputs, possibly further suppressing slower-growing chemoautotrophic nitrifying microbes. Overall, we found inter- and intra-annual variation in nitrification suppression in the rhizosphere of field-grown biomass sorghum,

suggesting that plant phenology and environmental conditions should be considered when devising strategies to improve the nitrogen sustainability of this annual bioenergy crop.

Keywords: nitrification, biological nitrification inhibition, bioenergy, sorghum, denitrification, rhizosphere

1. INTRODUCTION

A nearly four-fold increase in synthetic fertilizer application in the second half of the 20th century (Howarth et al., 2002) has led to elevated nitrate (NO_3^-) leaching into surface and groundwater as well as increased nitrous oxide (N_2O) emissions to the atmosphere (Gelfand et al., 2016; Van Meter et al., 2017). Nitrification is a key biogeochemical process regulating ecosystem nitrogen (N) loss because it transforms ammonium (NH_4^+) to NO_3^-, which is a highly mobile form of N susceptible to leaching and denitrification to N_2O (Farquharson, 2016). Synthetic nitrification inhibitors can effectively reduce N losses from agricultural fields (Di and Cameron, 2002; Gilsanz et al., 2016) but cost is prohibitive for widespread use (Yang et al., 2016). Nitrification inhibition can also occur naturally through direct biological nitrification inhibition (BNI) by plant root exudates and indirect nitrification suppression due to plant N uptake and immobilization of N by heterotrophic microbes in the rhizosphere. Some pasture grasses and cereal crops exude biological nitrification inhibition (BNI) compounds from their roots (Subbarao et al., 2007; Coskun et al., 2017a), so breeding or engineering crops for BNI presents another option for managing agricultural N losses (Subbarao et al., 2013b; Coskun et al., 2017b). However, land managers have less control over suppression of nitrification by plants than application of synthetic nitrification inhibitors, and plant development and climate conditions could affect nitrification suppression in the field. Thus, we must better understand the occurrence and magnitude of suppression of nitrification by plants across intra- and inter-annual variability in field conditions to control field N losses and increase crop sustainability.

Sorghum (*Sorghum bicolor* (L.) Moench), an important grain crop worldwide and an emerging candidate bioenergy feedstock, can suppress nitrification through both direct and indirect mechanisms. The production and release of secondary metabolites from sorghum roots

directly inhibits nitrification in laboratory and greenhouse settings (Zakir et al., 2008; Subbarao et al., 2013a; Weston et al., 2013; Tesfamariam et al., 2014; Sarr et al., 2019; Nardi et al., 2020). Notably, inhibited activity of the model nitrifying bacterial isolate *Nitrosomonas europaea* reflect direct BNI by sorgoleones, sakuranetin and methyl 3-(4-hydroxyphenyl) propionate (MHPP) (Subbarao et al., 2013a). Sorgoleones, a class of hydrophobic *p*-benzoquinone compounds (Czarnota et al., 2001), also suppress the relative abundance of ammonia-oxidizing archaea (AOA) within the nitrifying microbial community in the rhizosphere of greenhouse-grown sorghum (Sarr et al., 2019). However, estimation of sorghum BNI in the field is nearly absent, with only one study measuring end-of-season BNI in the field to complement a set of laboratory experiments (Tesfamariam et al., 2014). In addition to direct BNI by root exudates, other rhizosphere processes can also indirectly suppress nitrification. Root exudates can serve as sources of labile carbon (C) to fuel the activity of faster-growing heterotrophic microbes (Kuzyakov, 2002), which are better competitors for NH_4^+ than relatively slow-growing autotrophic nitrifying bacteria and archaea (Verhagen et al., 1995). Increased heterotrophic activity along with root uptake of NH_4^+ (Herman et al., 2006) can indirectly suppress nitrification rates since NH_4^+ supply is considered a primary control over nitrification rates (Stienstra et al., 1994; Booth et al., 2005). Given the strong seasonal patterns in root production by annual crops (Black et al., 2017) and changes in root exudate composition and amount throughout plant development (Gransee and Wittenmayer, 2000), temporal dynamics of BNI compound production, root N uptake, and rhizosphere heterotrophic activity could exert substantial control over rhizosphere nitrification.

Given that farmers typically apply N fertilizer early in the growing season and that the highest NO_3^- leaching flux occurs prior to the establishment of annual crops (Stenjem et al.,

2019), seasonal variation in plant-induced nitrification suppression could affect its impact on ecosystem N losses. Root exudate amount and chemical profile vary with plant age and nutrient demand (Chaparro et al., 2013; Oburger and Jones, 2018). In sorghum, sorgoleone production and direct BNI increase later in the plant phenology with the strongest BNI effects after a month or more of growth (Zakir et al., 2008; Sarr et al., 2019). Sorghum only exerts significant influence over its rhizobacterial community after approximately a month of growth (Schlemper et al., 2017), with this pattern potentially associated with changes in root exudate profile or simply with the time needed for community structure to change in response to root exudates. Additionally, annual crop root biomass and N uptake increase as the plants develop (van Oosterom et al., 2010a; Black et al., 2017), making indirect pathways of nitrification suppression more important later in the growing season. Therefore, rhizosphere nitrification suppression may be temporally decoupled from peaks in ecosystem N losses that occur earlier in the growing season.

Soil moisture dynamics can also mediate direct and indirect rhizosphere nitrification suppression pathways. Hydrophobic BNI compounds, such as sorgoleones, may remain concentrated near roots whereas hydrophilic BNI compounds, such as sakuranetin and MHPP, may more readily diffuse away from roots under moist soil conditions. When low soil moisture limits solute diffusion through soil pore water (Raynaud, 2010), even hydrophilic compounds may have restricted movement, leading to greater direct BNI in the rhizosphere. Lower soil moisture could also further reduce NH_4^+ diffusion into the rhizosphere and thus increase the contribution of indirect competitive pathways to rhizosphere nitrification suppression. The potential for seasonal variability in BNI compound production combined with seasonal and inter-

annual soil moisture variability could lead to temporally variable nitrification suppression in the rhizosphere of field-grown crops.

Plant-induced nitrification suppression may also respond to soil N availability. While it is not known exactly why plants direct resources to BNI compound production, reducing nitrification and leaching of NO_3^- could be a mechanism employed by plants to retain soil N under N-limited conditions (Subbarao et al., 2015). Plants adapted to low-N environments exhibit the greatest levels of BNI (Subbarao et al., 2007), but BNI compound production requires a low level of ammonium (NH_4^+) in solution (~ 1 mM) (Subbarao et al., 2007; Zakir et al., 2008; Subbarao et al., 2013a; Subbarao et al., 2015). This suggests that plants actively regulate BNI compound production in response to availability of NH_4^+, the nitrification substrate pool. Also, since substrate availability strongly controls nitrification levels (Stienstra et al., 1994; Booth et al., 2005; Herman et al., 2006; Wendeborn, 2020), it is likely that reduced competition for NH_4^+ between nitrifiers and roots or heterotrophic microbes in heavily fertilized fields would minimize the effects of these indirect nitrification suppression pathways. Indeed, despite nitrification inhibition by plant root exudates, typical agronomic levels of fertilizer addition still stimulate nitrification in the sorghum rhizosphere (Sarr et al., 2019). The dependence of nitrification in the rhizosphere on plant N status and soil NH_4^+ concentration suggests that management of fertilizer N inputs could serve as an important control on suppression of nitrification in the rhizosphere.

Here, we assessed rhizosphere nitrification suppression by field-grown energy sorghum under different fertilizer application rates and across two growing seasons varying in precipitation amounts. In the first year of the study, we included four energy sorghum genotypes grown for bioenergy production to explore the possibility of genotypic variation in BNI. Biological nitrification inhibition cannot be measured *in situ* in the field, so we inferred

144 nitrification suppression by testing for differences between potential nitrification in rhizosphere
145 soil, the soil that clung to excavated root systems, and in bulk soil collected between planted
146 sorghum rows. The potential nitrification assay allowed us to test for differences in the
147 nitrification capacity of the soil microbial community in different soil environments throughout
148 the growing season, encompassing both direct and indirect suppression of nitrifiers. We expected
149 that plant phenology, variable environmental conditions between seasons, and field N
150 management would interact to control the magnitude of nitrification suppression in rhizosphere
151 soil. Specifically, we hypothesized that 1) the suppression of nitrification would be greatest mid-
152 season during the period of highest sorghum growth, N demand, and exudation of BNI
153 compounds, 2) varying environmental conditions, especially soil moisture, between growing
154 seasons lead to inter-annual variation in the magnitude of the suppression of nitrification in the
155 rhizosphere, 3) increasing available N through fertilizer addition would reduce the suppression of
156 nitrification in the rhizosphere, and 4) denitrification rates would be stimulated in the rhizosphere
157 and rhizosphere microbial communities would be distinct from bulk soil, indicating higher
158 heterotrophic microbial activity and indirect suppression of nitrification via competition for
159 NH_4^+.
160

161 ## 2. MATERIALS AND METHODS

162 ### 2.1 Site description

163 We conducted a two-year field study (2018-2019) of biomass sorghum grown for
164 bioenergy feedstock production at the University of Illinois Urbana-Champaign (UIUC) Energy
165 Farm (N 40.063607, W 88.206926). The soil within this site is predominantly Drummer silty
166 clay loam (fine-silty, mixed, superactive, mesic Typic Endoaquolls), which is very deep and

poorly drained. The 2010-2019 mean annual temperature and mean annual precipitation for this site were 9.0° C and 1023 mm, respectively, and growing season (May 1 – October 31) mean temperature and precipitation were 17.0° C and 598 mm, respectively (NOAA National Climatic Data Center station ID USC00118740, 3.6 km from the field trial).

2.2 Experimental design

We leveraged a sorghum agronomy trial that included four sorghum genotypes grown in factorial combination with four fertilizer application levels in a randomized block design (n = 4) (Schetter et al., 2021). Each of the 64 treatment plots consisted of eight rows, 12 m in length and spaced 76 cm apart, resulting in an overall plot size of ~ 72 m^2. The plots were planted at a population rate of 185,325 seed ha^{-1}. The germplasm was obtained from the Texas A&M University sorghum breeding program. The four genotypes used (TAM08001, TAM17800, TAM17600, and TAM17500) varied in photoperiod sensitivity (Schetter et al., 2021), with TAM17600 and TAM17800 typically flowering earlier than TAM17500 and TAM08001. This potential difference in plant phenology with respect to flowering could influence plant N dynamics by changing the timing of N demand and uptake from the soil (van Oosterom et al., 2010a; van Oosterom et al., 2010b). The four fertilizer treatments included 0, 56, 112, and 168 kg N ha^{-1}, knifed into soil in the center of the alley between planted rows at a depth of 6-7 cm as 28% liquid 1:1:1 urea-ammonium-nitrate, hereafter referred to as N-0, N-56, N-112, and N-168. In 2018, the plots were planted on May 18 and fertilized on May 22. In 2019, the plots were planted on June 1 and fertilized on June 10. The timing of planting and subsequent fertilization depends on field conditions and anticipated rainfall to distribute the fertilizer into the soil.

In the annual crop rotation at this site, sorghum is planted following a soybean crop the season prior. To maintain this rotation, the 2018 and 2019 trials were planted in different fields

located approximately 200 m apart and on the same soil type. Both fields were planted in corn two years prior and soybean one year prior to sorghum. Pre-trial surface (0-10 cm) soil N concentrations were similar between years with slightly higher NO_3^- concentrations in 2018 (Table 1), and soil N concentrations did not differ between blocks within either year. We only measured mineral N concentrations in the top 10 cm of soil in 2018, but in 2019 we also measured soil N from 10-30 cm soil depth (0.5 mg NH_4^+-N kg^{-1} soil, 5.7 mg NO_3^--N kg^{-1} soil). Using bulk density measurements from adjacent fields, we estimate that the pre-fertilization mineral N stocks in the 2019 trial field were ~ 28 kg N ha^{-1} in the top 30 cm of soil. Although we did not quantify soil texture, we observed no soil texture differences within trial or between the two nearby trial fields, which were on the same soil type and ~ 200 m apart. A 4 ha plot adjacent to both trial fields had surface soil textures ranging from 5-13% sand, 51-64% silt, and 30-36% clay, and so it is unlikely that our two trial fields differ significantly in soil texture. Pre-trial soil pH did not differ between fields (Table 1), and more soil properties are reported by Schetter et al. (2021).

In each year of the study, we selected a subset of treatments from the sorghum field trial. In 2018, we sampled soils from all four genotypes across two fertilization levels (N-0 and N-168) to evaluate genotypic variation in nitrification suppression and its potential interaction with management practices. In 2019, we selected only TAM08001, the genotype with nitrification suppression most sensitive to fertilization based on 2018 results. We sampled this genotype across all four fertilization levels in 2019 to better characterize the effect of soil N availability on nitrification suppression.

2.3 Field sampling

We collected bulk and rhizosphere soils from 0-10 cm depth to assess sorghum effects on N cycling potential rates, soil properties, and microbial community composition. To collect rhizosphere soil from each plot, two plants were uprooted from different rows in the plot using a shovel. After knocking off loose soil, soil clinging to the root ball of each plant was hand-collected and pooled as a single rhizosphere soil sample for each plot. On the first sampling date of each growing season, when the plants were still small, we sampled more than two plants as needed to obtain sufficient soil for all analyses. Bulk soil was collected between planted rows within each plot to minimize belowground plant influence while controlling for similar plot conditions experienced by the rhizosphere soil. Since N fertilizer was knifed in directly between planted rows, we sampled bulk soils approximately 25-30 cm into the alley from the row where rhizosphere soils were collected. After collection, soils were split in the field, with one portion stored at 4° C within 2 hours of collection for up to 48 hours prior to analysis of potential N cycling rates and soil properties. The other portion was initially stored at 4° C within 2 hours of collection and then freeze-dried and stored at -20 °C within 48 hours for later soil microbial analyses. To evaluate the response of BNI to changes in plant phenology and environmental conditions, we sampled in the early-, mid-, and late-growing season. This corresponded to 40, 68, and 96 days since sowing in 2018, and 37, 65, and 94 days since sowing in 2019, or V6, V12, and R growth stages of sorghum.

2.4 Soil analyses

We inferred the suppression of nitrification as the difference in potential nitrification between bulk and rhizosphere soils. Although measuring potential nitrification in field-collected soils does not yield *in situ* field nitrification rates, it does allow us to compare the microbial capacity for nitrification between different soil environments and within and across growing seasons to infer variability in the suppression of nitrification in field rhizosphere soil. To estimate potential nitrification rates, we measured nitrification rates in aerobic soil slurries under excess NH_4^+ substrate availability using a method adapted from Belser and Mays (1980). Sodium chlorate was also added to the soil slurries to inhibit nitrite (NO_2^-) oxidation such that nitrification rates could be measured as the accumulation of NO_2^- over the incubation period. Briefly, for each soil sample, three 5 g subsamples were each added to 20 mL of 1 mM NH_4-N as ammonium sulfate along with 0.1 mL of 1.5M sodium chlorate. Two subsamples were treated as technical replicates for which nitrification occurred over 5 h; these subsamples were incubated on an orbital shaker at room temperature to promote the distribution of substrate throughout the slurries. The third subsample was frozen at -20 °C as a control to account for background soil NO_2^- under minimal microbial activity. After incubation, 5 mL of 2 M potassium chloride (KCl) was added, and NO_2^- concentration of the filtered supernatant was quantified using a colorimetric reaction with 40 g L^{-1} sulfanilamide and 2 g L^{-1} N-(1-napthyl) ethylenediamine dihydrochloride in a buffer of 6.8 g L^{-1} imidazole and 0.02 g L^{-1} copper (II) sulfate adjusted to pH 7.8 (Griess-Ilosvay method) (Shinn, 1941; Nydahl, 1976) read with a Genesys 30 visible spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) at 520 nm. Production of NO_2^- was thus the difference in NO_2^- concentrations between the room temperature test samples and chilled control samples after the 5 h soil incubation and was calculated as a daily rate of NO_2^- production. Thus,

potential nitrification rates are the accumulation rate of NO_2^- and are expressed as mg N kg^{-1} dry soil d^{-1}.

To estimate potential denitrification rates, we measured denitrification rates in anaerobic soil slurries under excess NO_3^- substrate availability using a method adapted from Groffman et al (1999). Acetylene was added to the slurry incubation headspace to inhibit N_2O reduction such that denitrification rates could be estimated from the accumulation of N_2O over the incubation period. Briefly, for each soil sample, a 25 g subsample was added to 25 mL of 10 mM NO_3^--N as potassium nitrate and 60 mM dextrose-C in a 125 mL airtight media bottle. After flushing the bottle headspace with ultra-high purity helium, 15 mL of pure acetylene gas was added and the solution was shaken vigorously for 30 sec to mix in the acetylene. Headspace gas samples were collected every 10 min for 30 min, and subsequently analyzed for N_2O concentration via gas chromatography equipped with an electron capture detector (Shimadzu GC-2014, Shimadzu Scientific Instruments, Inc., Columbia, Maryland, USA). Potential denitrification rates were calculated from the linear rate of increase of N_2O after accounting for aqueous-phase N_2O using Bunsen's constant at the assay temperature of 20 °C. All potential denitrification rates are expressed as mg N kg^{-1} dry soil d^{-1}.

To assess how soil properties and N pools differed among genotypes or treatments, we analyzed mineral N pools (NO_3^- and NH_4^+), soil moisture, and soil pH for all soil samples. Mineral N pools were quantified via colorimetric analysis of 2M KCl soil extracts on a SmartChem 200 discrete analyzer (Unity Scientific, Milford, MA, USA). Concentrations of NH_4^+ were quantified using a method adapted from Weatherburn (1967) for the SmartChem 200 instrument in which NH_4^+ reacts with hypochlorite and sodium salicylate in the presence of sodium nitroprusside catalyst. Concentrations of NO_3^- were quantified using a SmartChem 200

adaptation of the Griess-Ilosvay method. Gravimetric soil moisture was determined by drying soils for 48 hr at 105°C. Finally, pH was measured in a 1:1 mass ratio of field moist soil and deionized water.

2.5 Molecular analyses

To characterize soil microbial community structure and quantify the relative abundance of nitrifiers, microbial DNA was extracted from freeze-dried soil samples using the DNeasy 96 PowerSoil Pro QIAcube HT Kit. Samples were disrupted for DNA extraction by weighing out 0.05 grams of soil into PowerBead Pro Tubes, lysed using a TissueLyser II, and extracted using the QIAcube DNA extraction robot (QIAGEN, Hilden, Germany).

Amplicon sequencing of the V4 region of the 16S rRNA gene was carried out using a Fluidigm Access Array IFC chip (Fluidigm, San Francisco, CA) with single-index barcoded primers 515F (5'- GTGYCAGCMGCCGCGGTAA - 3') and 806R (5'- GGACTACNVGGGTWTCTAAT – 3') (Apprill et al., 2015; Parada et al., 2016), along with barcodes for assigning individual reads to samples. Sequencing was completed using 2 x 250bp paired-end chemistry on an Illumina NovaSeq 6000 Sequencing System (Illumina, San Diego, CA) at the Roy J. Carver Biotechnology Center (Urbana, IL, USA). Paired-end sequences were merged using Fast Length Adjustment of Short reads (FLASH) software (Magoc and Salzberg, 2011), and the FASTX-Toolkit (Hannon, 2014) was used to filter merged sequences using a minimum quality score of 30 across 90% of the bases. USEARCH version 8.1 (Edgar, 2010) was used to remove singletons, chimeras, and cluster OTU sequences based on 97% similarity. Taxonomic classification was assigned with Quantitative Insights into Microbial Ecology (QIIME) (Caporaso et al., 2010) using the UCLUST algorithm and GreenGenes database

(DeSantis et al., 2006). QIIME was also used to assemble OTU tables. All sequence data is archived in the National Center for Biotechnology Information's (NCBI) Sequence Read Archive (accession number SRP326979, project number PRJNA741261).

2.6 Statistical analysis

We constructed linear mixed-effects models using the *lme4* package in R (Bates et al. 2015) to determine (1) if rhizosphere nitrification suppression or rhizosphere effect on denitrification depended upon sorghum growth stage, (2) genotype and fertilizer effects on nitrification suppression or rhizosphere effect on potential denitrification rate, and (3) genotype and fertilizer effects on the relative abundance of nitrifiers in the soil microbial community. In addition, we used linear mixed-effects models to evaluate growth stage, genotype, and fertilization effects on soil NO_3^- and NH_4^+ concentrations. The R package *phyloseq* (McMurdie and Holmes, 2013) was used to subset nitrifying archaeal and bacterial taxa (Nitrosomonadales, Nitrososphaerales, and Nitrospirae) from the 16S OTU table for calculation of relative abundance. Although the order Nitrosomonadales also contains non-nitrifying organisms, nearly all of this group's OTUs in our dataset (98%) were identified as *Nitrosovibrio tenuis*, an ammonia oxidizing bacterium (Harms et al., 1976). Additionally, Nitrospirae are nitrite oxidizing bacteria, and although our potential nitrification assay measures only ammonia oxidation, the indirect suppression of nitrification in rhizosphere soil could also impact nitrite oxidizers. Therefore, we included Nitrospirae when analyzing differences in nitrifier relative abundance between bulk and rhizosphere soils. We also used linear mixed-effects models to determine if the ammonia oxidizer relative abundance affected potential nitrification assay rates. For this analysis, we excluded Nitrospirae because our potential nitrification assay only measured

ammonia oxidation to nitrite. A log of the odds ratio (logit) transformation was applied to relative abundance values to adhere to the model assumption of a boundless continuous response variable for statistical testing. Significance was determined as $P < 0.05$ and means are reported ± 1 standard error. Models included dependent variables of potential nitrification, potential denitrification, or relative abundance of nitrifier operational taxonomic units (OTUs); fixed effects of bulk vs. rhizosphere soil, fertilizer treatment, genotype (for 2018 models only), growth stage, and all interactions between fixed effects; and a random effect of plot nested within block to account for spatial variability across the field. Post-hoc pairwise comparisons of estimated marginal means were computed using the *emmeans* package in the R statistical environment (R Core Team, 2020). To assess the effects of bulk vs. rhizosphere soil, fertilizer addition, genotype (2018 only), and growth stage on the full microbial community composition (non-subsetted community dataset) and nitrifier community composition (subsetted nitrifier dataset), permutational analysis of variance models (PERMANOVA) for each year were used with the *adonis* function from the community ecology R package *vegan* (Oksanen et al., 2008). Differences in the PERMANOVA models were visualized by sample date using non-metric multidimensional scaling (NMDS) ordinations and *ggplot2* (Wickham, 2016).

3. RESULTS

3.1 Edaphic and climate factors

Growing season precipitation and soil moisture dynamics were distinct between the two years of this study, with 2018 being a wetter year than average and 2019 being drier. From May 1 through October 31, rainfall totaled 666 mm in 2018 and only 580 mm in 2019, compared to a

10-year average of 598 mm from 2010-2019 (Figure 1). Much of this difference occurred during the middle of the growing season, as rainfall totaled 122 mm from July 8 to August 20, 2018 but only 36 mm over the same period in 2019. As a result, soil moisture differed significantly between years ($P < 0.01$, Figure 2), with the inter-annual soil moisture difference especially pronounced mid-season (17% gravimetric water content in 2018 vs. 10% in 2019) and late-season (24% in 2018 vs. 15% in 2019).

Soil moisture dynamics showed similar growing season patterns over both years of the study, but differences between bulk and rhizosphere soil moisture occurred only in 2019 (Figure 2). In both years, soil moisture dropped significantly from the V6 to the V12 growth stage and then recovered by the R growth stage (2018, $P < 0.001$; 2019, $P < 0.001$). Under the drier conditions in 2019, rhizosphere soil exhibited higher moisture than bulk soil in the V12 and R growth stages (growth stage * bulk vs. rhizosphere effect: $P < 0.001$; bulk vs. rhizosphere post-hoc Tukey's test, $P < 0.001$).

Soil NH_4^+ concentrations exhibited different growing season patterns, fertilization effects, and bulk vs. rhizosphere soil effects in 2018 versus 2019 (Figure 3, Table 2). In 2018, soil NH_4^+ concentration varied with plant phenology and the effect of sorghum genotype depended upon growth stage, with TAM17600 having higher soil NH_4^- than TAM08001 and TAM17500 in the V6 stage (growth stage, $P = 0.03$; genotype x growth stage, $P = 0.009$; Supporting Information: Fig. S1). There were no differences between bulk and rhizosphere soils nor fertilizer effects on soil NH_4^+ in 2018, possibly due to a lack of rainfall immediately following fertilization favoring ammonia (NH_3) volatilization. In contrast, 15.7 mm of rain fell after fertilization in 2019, which typically decreases NH_3 loss (Fillery and Khimashia, 2016). These effects and the resulting soil N concentrations could also have contributed to lower

sorghum tissue N in 2018 (Schetter et al., 2021). In 2019, NH_4^+ concentration increased with fertilizer level in the bulk soil but not in the rhizosphere ($P = 0.001$). There was no difference between bulk and rhizosphere soils by the R growth stage when NH_4^+ was depleted throughout the soil in all plots.

Temporal and treatment patterns in soil NO_3^- concentrations were similar in 2018 and 2019, although bulk soil NO_3^- concentrations were higher in 2019 (Figure 3, Table 2). In both years, growth stage, fertilizer treatment, and bulk vs. rhizosphere soil affected soil NO_3^- concentrations (all $P < 0.001$, Table 2). In addition, sorghum genotypes differed in soil NO_3^- concentration in 2018 ($P = 0.006$; Supporting information: Fig. S1). Fertilizer addition increased NO_3^- concentrations in the bulk soil but not rhizosphere soil in both years (2018, $P = 0.035$; 2019, $P = 0.008$). However, bulk soil NO_3^- was depleted by the R growth stage in 2018 ($P < 0.001$). In 2019, bulk soil NO_3^- remained elevated through the V12 growth stage when plots fertilized with 168 kg N ha^{-1} had higher soil NO_3^- than unfertilized plots ($P < 0.001$) (Figure 3).

3.2 Potential nitrification rates

In 2018, interactions among bulk and rhizosphere soil differences, sorghum growth stages, and fertilizer treatments suggested complex controls on the suppression of nitrification in the field (Table 3). Across all growth stage sampling dates, fertilizer treatments, and genotypes, potential nitrification averaged 17.5 ± 0.5 mg NO_2^--N kg $soil^{-1}$ d^{-1} in bulk soil and 16.0 ± 0.6 mg NO_2^--N kg $soil^{-1}$ d^{-1} in the rhizosphere, with the higher rates in bulk soil compared to the rhizosphere indicating the suppression of nitrification in the rhizosphere ($P < 0.001$). Sorghum suppressed potential nitrification only during the V12 growth stage mid-season, when

rhizosphere soil exhibited 20% lower potential nitrification rates than bulk soil across all fertilization levels and genotypes ($P < 0.001$) (Figure 4).

In 2019, potential nitrification rates were lower overall compared to 2018 but nitrification suppression in the rhizosphere was more pronounced than in 2018 in terms of both absolute and relative reduction of potential nitrification rates in the rhizosphere compared to bulk soil. Across all growth stages and fertilizer treatments, potential nitrification averaged 12.7 ± 0.7 mg NO_2^--N kg $soil^{-1}$ d^{-1} in bulk soil and 8.4 ± 0.5 mg NO_2^--N kg $soil^{-1}$ d^{-1} in the rhizosphere ($P < 0.001$). Similar to 2018, the suppression of nitrification was greatest during the V12 stage mid-season, when rhizosphere potential nitrification was 58% lower than bulk soil across fertilization levels ($P < 0.001$) (Figure 4, Table 3). In contrast to 2018, significant nitrification suppression was also observed during the R stage, when rhizosphere potential nitrification was 22% lower than bulk soil across all fertilization levels ($P < 0.001$). Fertilizer treatment did not affect the difference between bulk and rhizosphere soil potential nitrification on any sampling date during the 2019 growing season.

3.3 Potential denitrification rates

In 2018, potential denitrification was higher in the rhizosphere than in bulk soil, but was also affected by growth stage, fertilizer, and genotype. The mean potential denitrification was 0.8 ± 0.07 mg N_2O-N kg $soil^{-1}$ d^{-1} in bulk soil and 1.2 ± 0.07 mg N_2O-N kg $soil^{-1}$ d^{-1} in the rhizosphere in 2018 across all growth stages, genotypes, and fertilization treatments ($P < 0.001$, Figure 5, Table 3). Potential denitrification rates changed over the growing season, with V6 stage rates approximately double the later rates ($P = 0.001$). The greatest difference between bulk and rhizosphere soil occurred during the V12 growth stage when potential denitrification was 171%

higher in the rhizosphere than in bulk soil ($P < 0.001$) (Figure 5). Neither genotype nor fertilizer treatment altered the difference between bulk and rhizosphere soils.

Drier conditions in 2019 led to an order of magnitude lower potential denitrification rates compared to 2018, with 2019 rates averaging only 0.047 ± 0.01 mg N_2O-N kg soil^{-1} d^{-1} in bulk soil and 0.075 ± 0.01 mg N_2O-N kg soil^{-1} d^{-1} in the rhizosphere (Figure 5). Despite the dry conditions, potential denitrification still differed significantly between bulk and rhizosphere soils, with 63% higher rates in rhizosphere soil than bulk soil across all treatments ($P = 0.01$, Figure 5). We observed the largest difference between bulk and rhizosphere soil during the R growth stage when rhizosphere potential denitrification was 125% higher than in bulk soil ($P = 0.04$). In 2019, V12 growth stage potential denitrification was 71% and 79% lower than the earlier V6 and later R growth stage rates, respectively ($P < 0.001$). Fertilizer treatment did not affect potential denitrification on any sampling date in 2019.

3.4 Soil microbial community

Soil bacterial and archaeal community composition differed between bulk and rhizosphere soils and by sorghum growth stage and fertilizer treatment. The overall microbial community composition differed between bulk and rhizosphere soils in both 2018 and 2019 ($P < 0.001$, $R^2 = 0.02$, and $P < 0.001$, $R^2 = 0.02$, respectively, Figure 6). Bulk and rhizosphere soil microbial communities diverged more as the season progressed in both years as well (2018, $P < 0.001$, $R^2 = 0.02$; 2019, $P = 0.02$, $R^2 = 0.02$). Soil microbial community composition also varied by fertilizer treatment in both years (2018, $P < 0.001$, $R^2 = 0.01$; 2019, $P < 0.001$, $R^2 = 0.05$; Supporting Information: Fig. S3).

The composition of nitrifiers also differed between bulk and rhizosphere soils and by sorghum growth stage and fertilizer treatment. During both years, the nitrifier community differed between bulk and rhizosphere soils (2018, $P = 0.023$, $R^2 = 0.01$; 2019, $P = 0.01$, $R^2 = 0.02$). The nitrifier community also diverged between bulk and rhizosphere soils throughout both growing seasons (2018, $P = 0.005$, $R^2 = 0.01$; 2019, $P = 0.031$, $R^2 = 0.03$). Soil nitrifier community composition varied by fertilizer treatment as well (2018, $P = 0.001$, $R^2 = 0.02$; 2019, $P = 0.001$, $R^2 = 0.10$).

Total (archaea + bacteria) nitrifier relative abundance was lower in the rhizosphere compared to bulk soil in both 2018 and 2019 ($P < 0.001$ and $P < 0.001$, respectively; Figure 7, Table 3). In 2018, the difference between bulk and rhizosphere soils was marginally significantly greater in the V6 and V12 growth stages than the later R stage ($P = 0.07$). Archaeal nitrifier relative abundance was also lower in the rhizosphere than bulk soil in both study years, but this effect did not change through the growing season (2018, $P < 0.001$; 2019, $P = 0.02$). However, in 2018, the difference between relative abundance of bacterial nitrifiers in bulk versus rhizosphere soil differed among growth stages ($P = 0.002$, Supporting Information: Fig. S4). In the V6 and V12 stages, bacterial nitrifier relative abundance was lower in the rhizosphere, but in the R stage it was higher in the rhizosphere compared to bulk soil. In contrast, in 2019, bacterial nitrifier relative abundance differed among growth stages ($P < 0.001$) but there was no difference between bulk and rhizosphere soils. Sorghum genotype and fertilizer treatment did not affect relative abundances of total nitrifiers, archaeal nitrifiers, or bacterial nitrifiers.

In 2018, the relative abundance of ammonia oxidizers had no relationship with potential nitrification rates across all sample dates. When isolating the mid-season date when rhizosphere effects were the strongest, there was a trend towards lower potential nitrification rate at lower

462 relative abundances of both archaeal (P = 0.09) and bacterial ammonia (P = 0.09) oxidizers.
463 However, in 2019, when rhizosphere effects on nitrification were much stronger, potential
464 nitrification rate significantly declined with lower relative abundance of total ammonia oxidizers
465 (P = 0.006) and both archaeal (P = 0.02) and bacterial ammonia oxidizers (P = 0.03)
466 individually.

467

468 **4. DISCUSSION**

469 The expanding body of literature on BNI suggests that many plant species may be
470 capable of reducing nitrification in the rhizosphere (Subbarao et al., 2007; Chowdhury et al.,
471 2017; Coskun et al., 2017a; Janke et al., 2018). In the agricultural setting, direct and indirect
472 suppression of nitrification could reduce NO_3^- losses from annual cropping systems, with the
473 agronomic benefit of increased fertilizer use efficiency and environmental benefits of reduced
474 NO_3^- flow into downstream waterways and N_2O emissions into the atmosphere. Across two
475 growing seasons, we detected lower potential nitrification in the rhizosphere soil of field-grown
476 sorghum, a species with direct BNI capacity, with nitrification suppressed 20-58% during peak
477 growth in the mid-season. This is comparable to the sorghum direct BNI effect of 20-60%
478 measured in laboratory and greenhouse studies (Subbarao et al., 2013a; Tesfamariam et al.,
479 2014; Sarr et al., 2019). Importantly, we showed that plant phenology exerts substantial control
480 over rhizosphere soil nitrification and that the most suppression occurs when plants were
481 growing fastest mid-season. Intra- and inter-annual variation in soil moisture and N availability
482 also affected soil potential nitrification and its suppression in rhizosphere soils. Thus, these
483 factors influence how we can leverage sorghum rhizosphere dynamics to reduce N losses and
484 improve the sustainability of biomass sorghum as a bioenergy feedstock.

Dynamics of sorghum nitrification suppression

Estimating rhizosphere nitrification suppression in an agricultural field over two full growing seasons allowed us to explore a possible role of plant phenology in controlling rhizosphere nitrification dynamics. Direct BNI likely plays a significant role in suppression of nitrification along growing roots, and production of BNI compounds by sorghum increases as plants develop such that we expected to observe temporal changes in BNI throughout the growing season. In both study years, rhizosphere nitrification potential was reduced during the mid-season V12 sorghum growth stage but not in the early-season, consistent with the typical pattern of increasing BNI root exudate production and release as sorghum plants develop (Zakir et al., 2008; Subbarao et al., 2013a; Sarr et al., 2019). The mid-season nitrification suppression effect was also during the period of maximum growth rate and highest N demand for biomass sorghum (van Oosterom et al., 2010a; van Oosterom et al., 2010b; Maughan et al., 2012; Schetter et al., 2021), so the increased nitrification suppression during the V12 growth stage of sorghum likely also resulted from plant N uptake reducing NH_4^+ availability for nitrifiers. Contrary to our expectation, the four genotypes we used had very similar growth rates and flowering times (differing by only ~1 week), resulting in the lack of any effect of genotype on rhizosphere nitrification. However, investigating different sorghum types, such as grain, forage, and biomass sorghum, could reveal stronger temporal changes in production and release of BNI compounds and NH_4^+ competition resulting from plant N uptake. Thus, the temporal dynamics of nitrification suppression within a growing season is likely the result of both indirect suppression of nitrifiers driven by growth rate and N demand as the plant develops, as well as direct suppression of nitrifier activity through increasing release of BNI compounds.

Inter-annual variability in the strength of nitrification suppression suggests that there is significant environmental control over rhizosphere nitrification in the field. The main difference

between the two years in our study was lower precipitation and soil moisture in 2019, particularly mid-season from early-July through mid-August (Figure 3). Soil water content and exudate compound diffusivity are key drivers of the movement of exudates away from roots (Raynaud, 2010), such that hydrophilic compounds are retained and concentrated in closer proximity to the root in dry years. Of the three known BNI compounds exuded by sorghum roots, MHPP and sakuranetin are hydrophilic, while sorgoleones are hydrophobic (Subbarao et al., 2013a). Although sorgoleones have gained more attention for their role in sorghum BNI (Dayan et al., 2010; Sarr et al., 2019), the concentration of hydrophilic BNI compounds in the rhizosphere under the drier soil conditions in 2019 may have led to a greater BNI effect compared to 2018. Additionally, lower soil moisture restricts diffusion of NH_4^+ (Stark and Firestone, 1995; Agehara and Warncke, 2005), which likely interacted with plant NH_4^+ uptake and microbial immobilization in the rhizosphere to further reduce NH_4^+ availability for relatively slow-growing nitrifiers, especially mid-season in 2019. This suggests that inter-annual variability in growing season precipitation and soil moisture should be accounted for when predicting how plants contribute to nitrification suppression and mitigation of ecosystem N losses.

Although we found evidence for phenological and environmental controls over rhizosphere nitrification dynamics, changes in soil N availability across a range of fertilization levels did not affect nitrification suppression as expected. In our study, fertilizer was knifed into the soil between planted rows immediately after planting, so one potential caveat to our design is the possibility that bulk soils were affected more by fertilization than rhizosphere soils, confounding the fertilizer and bulk vs. rhizosphere soil effects. However, suppression of nitrification in the rhizosphere did not differ between fertilization levels in either year (Table 3), and NH_4^+ concentration did not differ between the bulk and rhizosphere soils in 2018 and was

only higher in bulk soil of the highest fertilization treatment in early- and mid-2019 (Figure 3). Thus, plant-induced nitrification suppression rather than fertilizer placement most likely led to our measured nitrification dynamics. Interestingly, our sorghum plants were generally unresponsive to N addition, with minimal effect on biomass yield (Schetter et al., 2021). Since the experimental plots were located on fertile soil in fields historically managed as a fertilized maize-soybean rotation, it is likely that the sorghum stands were not N-limited even in the absence of fertilizer application in the study years. Additionally, direct BNI may be generally unresponsive to plant N status so long as there is at least a low NH_4^+ concentration present in soil solution (~ 1 mM) to stimulate BNI compound production (Zakir et al., 2008; Subbarao et al., 2013a; Subbarao et al., 2017). As a result, we expect that BNI will occur at agronomic levels of N fertilization, potentially playing a role in reducing fertilizer N loss as NO_3^-. Determining whether NO_3^- leaching is reduced by BNI crops across a range of fertilization levels compared to non-BNI crops is important for evaluating the potential for BNI to contribute to sustainable production of high-yielding crops that often require N inputs.

Greater rhizosphere denitrification potential relative to bulk soil during both growing seasons provides support for heterotrophic competition with nitrifiers for NH_4^+ contributing indirectly to the suppression of nitrification. Given that denitrifiers represent a diverse group of facultative anaerobes that use organic carbon for aerobic heterotrophic respiration under oxic soil conditions, the stimulation of rhizosphere denitrification potential starting in mid-2018 and late-2019 suggests that heterotrophic activity may have been broadly stimulated in the rhizosphere by root exudation of labile carbon compounds during the V12 and R growth stages of sorghum. Lower relative abundance of nitrifiers in the rhizosphere compared to bulk soil is also consistent with increased abundance of heterotrophic microbes. Thus, competition for NH_4^+ via the

554 combination of stimulated heterotrophic activity and increased plant uptake of N (van Oosterom
555 et al., 2010a) likely exerted significant control over rhizosphere nitrification and contributed to
556 the strong seasonal changes in the suppression of nitrification in the rhizosphere. Although our
557 design does not allow us to compare the relative importance of indirect and direct pathways of
558 nitrification suppression, we speculate that the indirect mechanisms are at least as important as
559 direct BNI starting mid-season.

560 In conclusion, we demonstrated that nitrification suppression in field-grown energy
561 sorghum exhibits considerable intra- and inter-annual variability likely associated with plant
562 phenology and environmental conditions. Since it is not constant throughout the growing season,
563 the potential mismatch between the timing of greatest NO_3^- production and greatest rhizosphere
564 nitrification suppression could reduce the effectiveness or dependability of engineering BNI to
565 mitigate ecosystem N losses. Additionally, climate variability and resulting differences in soil
566 moisture control the magnitude of nitrification inhibition, with weaker nitrification suppression
567 during moist periods when microbial activity and NO_3^- leaching would be the greatest. However,
568 we found that rhizosphere nitrification can be suppressed in fertile soils even with high rates of
569 fertilizer addition, indicating that it can limit NO_3^- production when NH_4^+ availability is high, as
570 is often the case in intensively managed annual cropping systems. Together our results suggest
571 that carefully considering how interactions between plant development, local climate, and
572 rhizosphere microbes affect N cycling and loss would maximize the role of rhizosphere
573 nitrification suppression in reducing agroecosystem N losses. Strategies that match the timing of
574 sorghum nitrification suppression with NH_4^+ availability and selecting for greater direct BNI
575 expression earlier in plant development will increase the effectiveness of managing plant-

576 rhizosphere interactions for ecosystem N retention and maximizing the sustainability of biomass
577 sorghum as a bioenergy crop.

578

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750

751 **Figure legends**

752

753 Figure 1. Cumulative growing season (May 1 – October 31) rainfall in 2018 (blue) and 2019
754 (orange). The 2010-2019 mean cumulative growing season rainfall is shown in black, with the
755 gray shading indicating one standard error around the mean. Points along each line indicate
756 planting, fertilization, and different sorghum growth stages when we sampled within year, with
757 year indicated by point color.

758 Figure 2. Gravimetric soil moisture in bulk and rhizosphere soils (yellow and green bars,
759 respectively) by sorghum growth stage in 2018 (a) and 2019 (b). Given no statistically
760 significant fertilizer treatment or genotype effects on any sampling date, all fertilizer treatments
761 and genotypes were averaged for each soil type within growth stage. Asterisks denote significant
762 differences between bulk and rhizosphere soil ($P < 0.05$).

763 Figure 3. Soil NH_4^+ (a-f) and NO_3^- (g-l) concentrations in bulk and rhizosphere soils (yellow and
764 green bars, respectively) by fertilization treatment at the V6, V12, and R sorghum growth stages
765 in 2018 (averaged across genotypes) and 2019. 2018 genotype effects are shown in figure S1.
766 Only two fertilizer treatments, 0 and 168 kg N ha^{-1}, were sampled in 2018. Differing letters
767 indicate significant differences in soil N within sorghum growth stage sampling date ($P < 0.05$),
768 and panels with no letters have no significant differences.

769 Figure 4. Potential nitrification rates in bulk and rhizosphere soils (yellow and green bars,
770 respectively) by fertilization treatment in the V6, V12, and R sorghum growth stages in 2018 (a-
771 c) and 2019 (d-f). Given no statistically significant genotype effects, all genotypes were averaged
772 for each soil type within sorghum growth stage sampling date. Asterisks denote significant

773 differences between bulk and rhizosphere soils ($P < 0.05$), with lower rates in rhizosphere soils
774 indicating suppression of nitrification.

775 Figure 5. Potential denitrification rates in bulk and rhizosphere soils (yellow and green bars,
776 respectively) by sorghum growth stage in 2018 (a) and 2019 (b). Given no statistically
777 significant fertilizer treatment effects during any growth stage, all fertilizer treatments were
778 averaged for each soil type within growth stage. All genotypes were averaged for each soil type
779 within growth stage, and 2018 genotype and fertilizer effects are shown in figure S2. Asterisks
780 denote significant differences between bulk and rhizosphere soils ($P < 0.05$).

781 Figure 6. Non-metric multi-dimensional scaling (NMDS) plots of microbial communities in bulk
782 and rhizosphere soils (yellow and green points, respectively) in the V6, V12, and R sorghum
783 growth stages in 2018 (a-c) and 2019 (d-f). Greater distances between points indicate more
784 distinct microbial communities. Fertilizer effects are shown in figure S3.

785 Figure 7. Nitrifier relative abundance in 16S rRNA extracted from bulk and rhizosphere soils
786 (yellow and green bars, respectively) in 2018 (a) and 2019 (b). All fertilizer treatments and
787 growth stage measurements were averaged to illustrate significant bulk vs. rhizosphere effects in
788 each year, and all fertilizer treatment means by date are shown in figure S4. Asterisks denote
789 significant differences between bulk and rhizosphere soils ($P < 0.05$).

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791

Table 1. Pre-trial average soil pH and mineral N concentrations (± SE) in the field sites for 2018 and 2019 sorghum trials.

	pH	**NH_4^+ (mg N kg^{-1} soil)**	**NO_3^- (mg N kg^{-1} soil)**
2018	6.05 (± 0.02)	0.67 (± 0.26)	12.45 (± 1.50)
2019	6.00 (± 0.05)	0.80 (± 0.10)	9.71 (± 0.35)

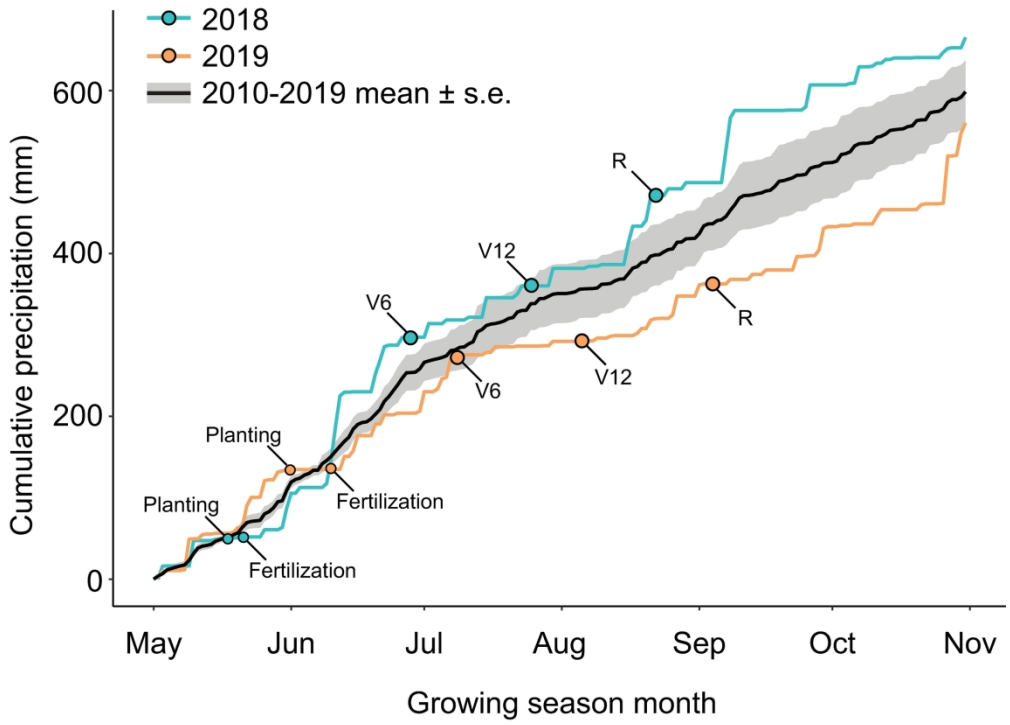
Table 2. Results from linear mixed effects models evaluating effects on soil N pools and soil moisture. All models included a random effect of plot within block to account for the hierarchical experimental layout. Bulk vs. rhizosphere soil effect is abbreviated B-R.

		Soil nitrate concentration		Soil ammonium concentration		Soil moisture	
2018	**df**	**F**	**P**	**F**	**P**	**F**	**P**
Date	2,120	**57.6**	**<0.001**	**3.8**	**0.025**	**568.0**	**<0.001**
Bulk vs. rhizosphere soil (B-R)	1,120	**120.4**	**<0.001**	0.8	0.367	0.2	0.639
Fertilizer	1,21	**28.9**	**<0.001**	0.0	0.940	0.3	0.578
Genotype	3,21	**4.3**	**0.006**	2.2	0.115	1.3	0.307
B-R x Date	2,120	**13.6**	**<0.001**	1.7	0.185	**5.2**	**0.007**
B-R x Fertilizer	1,120	**4.5**	**0.035**	0.6	0.445	**7.3**	**0.008**
B-R x Genotype	3,120	2.4	0.075	0.7	0.584	0.3	0.848
Date x Fertilizer	2,120	0.9	0.390	0.8	0.441	2.1	0.127
Date x Genotype	6,120	0.8	0.609	**3.0**	**0.009**	1.1	0.386
Fertilizer x Genotype	3,21	1.7	0.161	0.7	0.544	0.3	0.834
B-R x Date x Fertilizer	2,120	0.2	0.810	0.4	0.640	**6.5**	**0.002**
B-R x Fertilizer x Genotype	3,120	1.2	0.313	0.9	0.425	0.5	0.675
B-R x Date x Genotype	6,120	0.3	0.939	1.1	0.375	0.1	0.990
Date x Fertilizer x Genotype	6,120	1.2	0.314	0.9	0.500	1.1	0.342
B-R x Date x Fertilizer x Genotype	6,120	0.6	0.748	0.2	0.971	0.8	0.542
2019	**df**	**F**	**P**	**F**	**P**	**F**	**P**
Date	2,60	**7.3**	**0.001**	2.5	0.094	**320.3**	**<0.001**
B-R	1,60	**310.9**	**<0.001**	**40.1**	**<0.001**	**53.9**	**<0.001**
Fertilizer	3,12	**20.1**	**<0.001**	**6.5**	**0.007**	0.3	0.797
B-R x Date	2,60	0.7	0.509	**3.5**	**0.036**	**46.2**	**<0.001**
B-R x Fertilizer	3,60	**8.1**	**<0.001**	**6.8**	**0.001**	0.7	0.577
Date x Fertilizer	6,60	1.2	0.334	2.2	0.056	1.8	0.112
B-R x Date x Fertilizer	6,60	0.1	0.998	1.8	0.115	1.2	0.339

Table 3. Results from linear mixed effects models evaluating effects on potential nitrogen cycling rates and nitrifier relative abundances. All models included a random effect of plot within block to account for the hierarchical experimental layout. Bulk vs. rhizosphere soil effect is abbreviated B-R.

		Potential nitrification rate		**Potential denitrification rate**		**Total nitrifier relative abundance**		**Archaeal nitrifier relative abundance**		**Bacterial nitrifier relative abundance**	
2018	**df**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
Date	2,120	156.2	**<0.001**	44.0	**<0.001**	27.8	**<0.001**	27.7	**<0.001**	3.4	**0.035**
Bulk vs. rhizosphere soil (B-R)	1,120	14.9	**<0.001**	41.4	**<0.001**	11.5	**<0.001**	11.3	**<0.001**	1.7	0.195
Fertilizer	1,21	4.9	**0.038**	0.9	0.355	0.0	0.933	0.0	0.963	0.0	0.960
Genotype	3,21	0.6	0.601	2.2	0.115	2.2	0.114	1.9	0.155	2.0	0.139
B-R x Date	2,120	3.4	**0.035**	7.3	**0.001**	2.7	0.069	1.2	0.291	6.7	**0.002**
B-R x Fertilizer	1,120	0.8	0.371	0.7	0.409	0.0	0.971	0.1	0.756	0.6	0.443
B-R x Genotype	3,120	1.6	0.205	0.2	0.921	1.0	0.388	1.4	0.236	0.4	0.769
Date x Fertilizer	2,120	0.4	0.703	1.8	0.176	3.7	**0.027**	3.6	**0.030**	0.8	0.450
Date x Genotype	6,120	0.5	0.844	1.0	0.422	0.5	0.804	0.5	0.831	0.7	0.676

Fertilizer x Genotype	3,21	0.7	0.570	0.5	0.663	0.0	0.985	0.1	0.941	0.5	0.657
B-R x Date x Fertilizer	2,120	2.4	0.098	0.6	0.532	1.7	0.195	1.8	0.177	0.2	0.784
B-R x Fertilizer x Genotype	3,120	0.8	0.472	0.3	0.835	0.7	0.583	0.6	0.615	0.9	0.445
B-R x Date x Genotype	6,120	0.6	0.693	0.4	0.888	1.7	0.121	1.7	0.121	1.8	0.106
Date x Fertilizer x Genotype	6,120	1.3	0.251	2.4	**0.029**	1.5	0.178	1.4	0.236	1.2	0.294
B-R x Date x Fertilizer x Genotype	6,120	0.3	0.920	1.6	0.146	1.0	0.440	0.8	0.550	1.2	0.322
2019	**df**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
Date	2,60	4.1	**0.022**	25.2	**<0.001**	2.3	0.112	4.6	**0.014**	5.4	**<0.001**
B-R	1,60	35.4	**<0.001**	7.1	**0.010**	8.7	**<0.001**	5.4	**0.023**	1.6	0.206
Fertilizer	3,12	1.3	0.320	0.5	0.705	0.8	0.543	1.0	0.444	2.1	0.167
B-R x Date	2,60	14.5	**<0.001**	3.6	**0.035**	1.3	0.272	1.7	0.196	1.4	0.252
B-R x Fertilizer	3,60	0.5	0.675	0.5	0.667	2.2	0.095	3.3	**0.027**	0.6	0.648
Date x Fertilizer	6,60	0.7	0.685	1.3	0.287	1.5	0.203	1.6	0.160	0.6	0.702
B-R x Date x Fertilizer	6,60	0.7	0.687	1.2	0.311	0.7	0.680	0.7	0.619	1.6	0.167



Cumulative growing season (May 1 – October 31) rainfall in 2018 (blue) and 2019 (orange). The 2010-2019 mean cumulative growing season rainfall is shown in black, with the gray shading indicating one standard error around the mean. Points along each line indicate planting, fertilization, and different sorghum growth stages when we sampled within year, with year indicated by point color.

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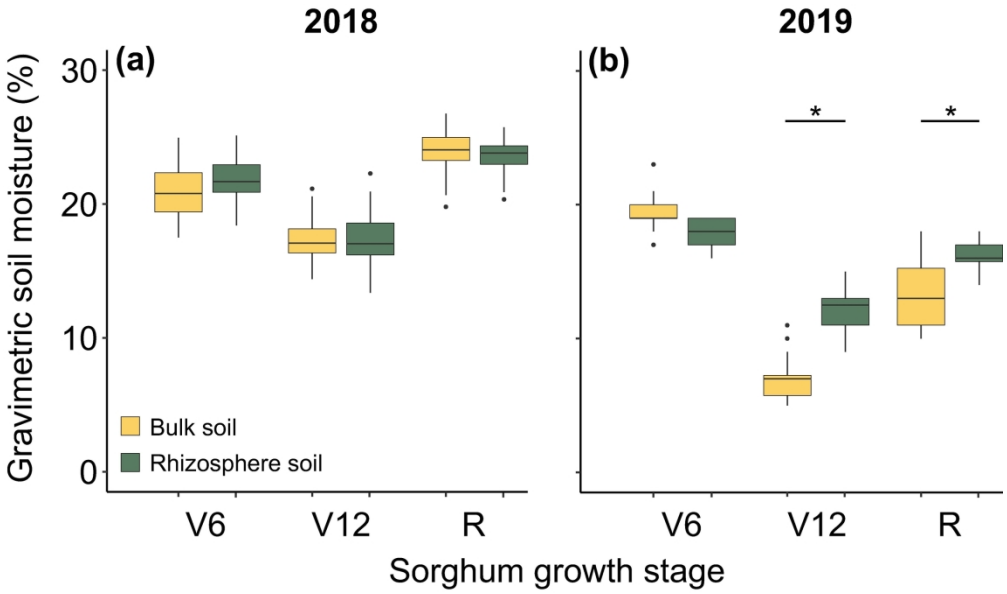

Gravimetric soil moisture in bulk and rhizosphere soils (yellow and green bars, respectively) by sorghum growth stage in 2018 (a) and 2019 (b). Given no statistically significant fertilizer treatment or genotype effects on any sampling date, all fertilizer treatments and genotypes were averaged for each soil type within growth stage. Asterisks denote significant differences between bulk and rhizosphere soil ($P < 0.05$).

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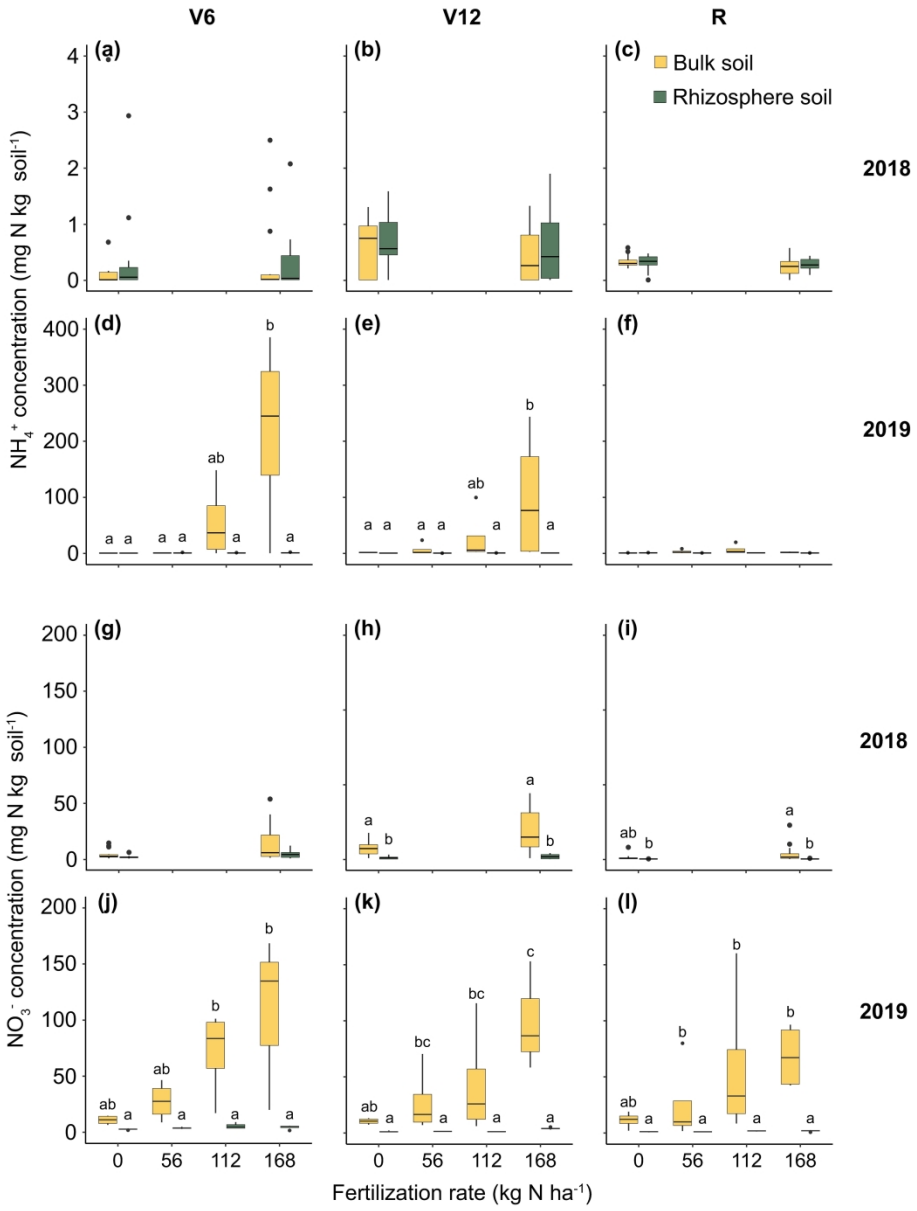

Soil NH4+ (a-f) and NO3- (g-l) concentrations in bulk and rhizosphere soils (yellow and green bars, respectively) by fertilization treatment at the V6, V12, and R sorghum growth stages in 2018 (averaged across genotypes) and 2019. 2018 genotype effects are shown in figure S1. Only two fertilizer treatments, 0 and 168 kg N ha-1, were sampled in 2018. Differing letters indicate significant differences in soil N within sorghum growth stage sampling date ($P < 0.05$), and panels with no letters have no significant differences.

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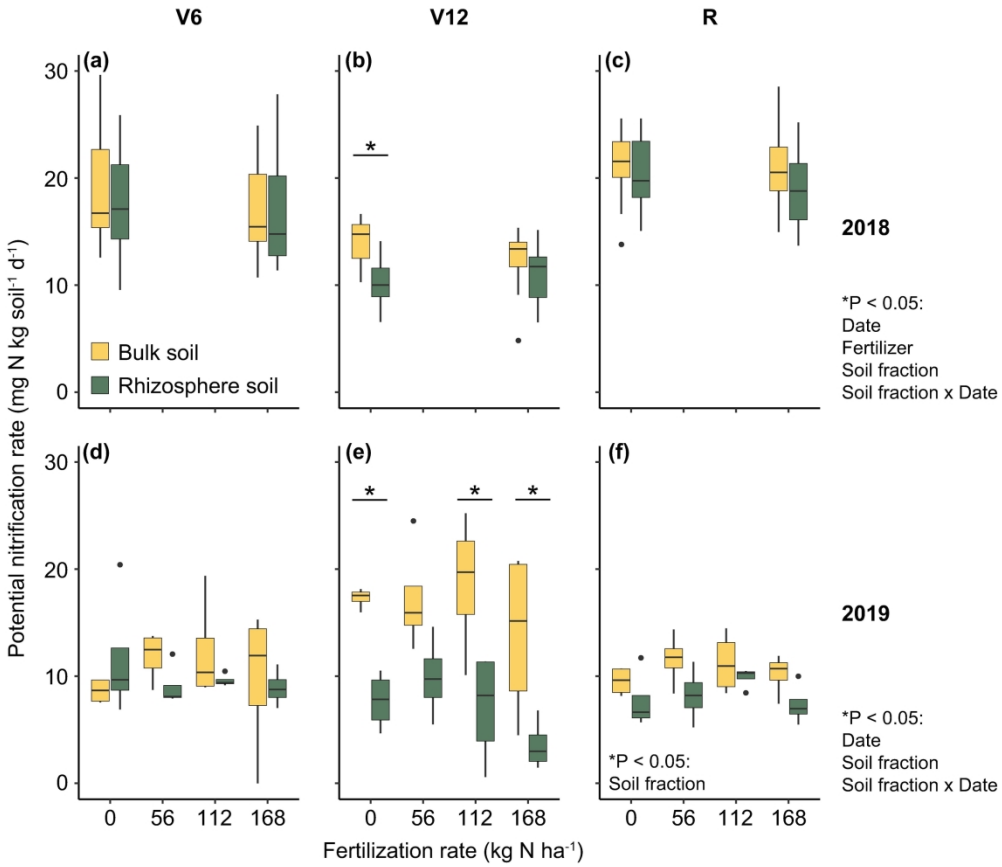

Potential nitrification rates in bulk and rhizosphere soils (yellow and green bars, respectively) by fertilization treatment in the V6, V12, and R sorghum growth stages in 2018 (a-c) and 2019 (d-f). Given no statistically significant genotype effects, all genotypes were averaged for each soil type within sorghum growth stage sampling date. Asterisks denote significant differences between bulk and rhizosphere soils ($P < 0.05$), with lower rates in rhizosphere soils indicating suppression of nitrification.

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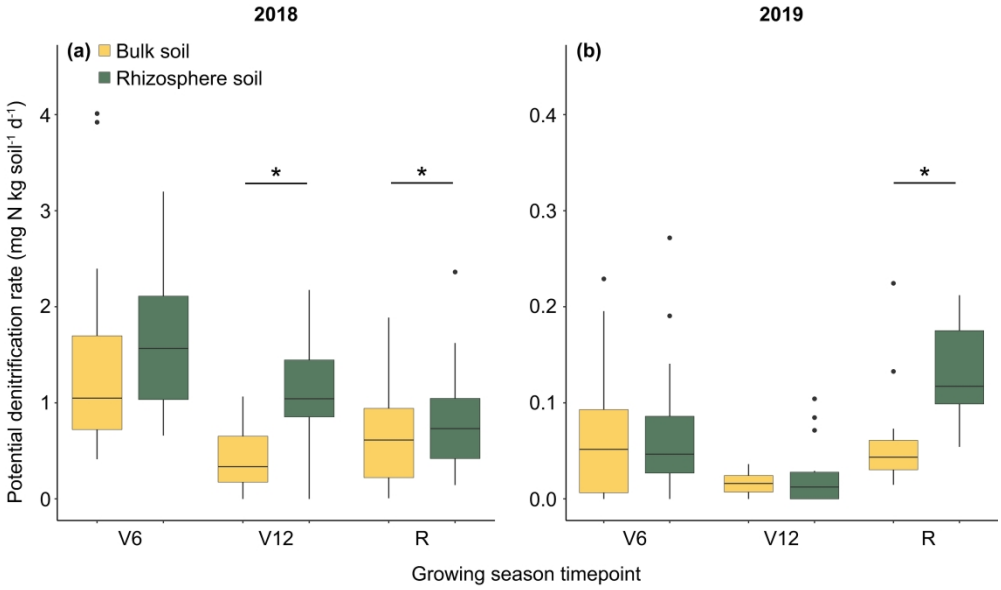

Potential denitrification rates in bulk and rhizosphere soils (yellow and green bars, respectively) by sorghum growth stage in 2018 (a) and 2019 (b). Given no statistically significant fertilizer treatment effects during any growth stage, all fertilizer treatments were averaged for each soil type within growth stage. All genotypes were averaged for each soil type within growth stage, and 2018 genotype and fertilizer effects are shown in figure S2. Asterisks denote significant differences between bulk and rhizosphere soils ($P < 0.05$).

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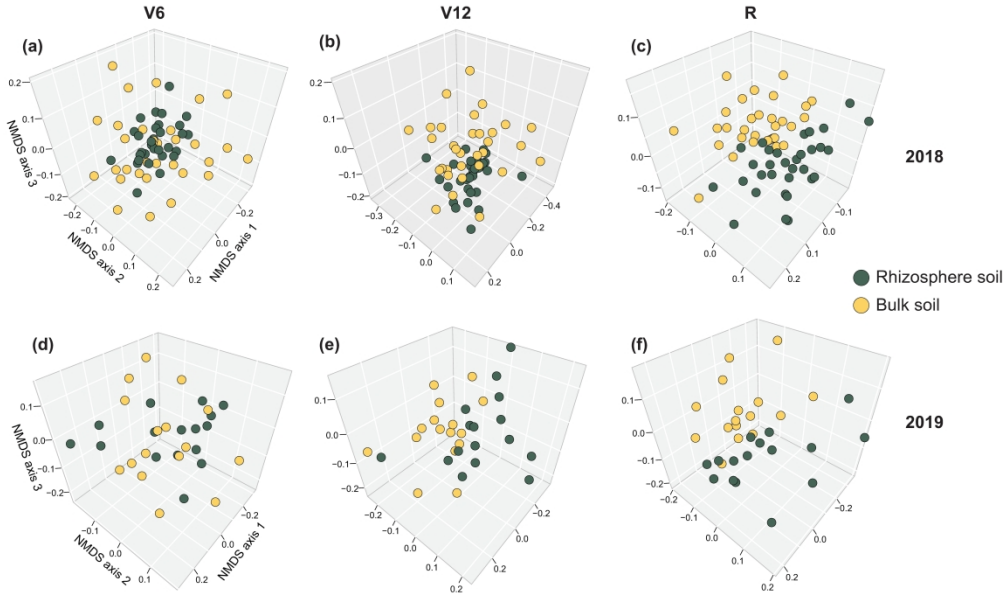

Non-metric multi-dimensional scaling (NMDS) plots of microbial communities in bulk and rhizosphere soils (yellow and green points, respectively) in the V6, V12, and R sorghum growth stages in 2018 (a-c) and 2019 (d-f). Greater distances between points indicate more distinct microbial communities. Fertilizer effects are shown in figure S3.

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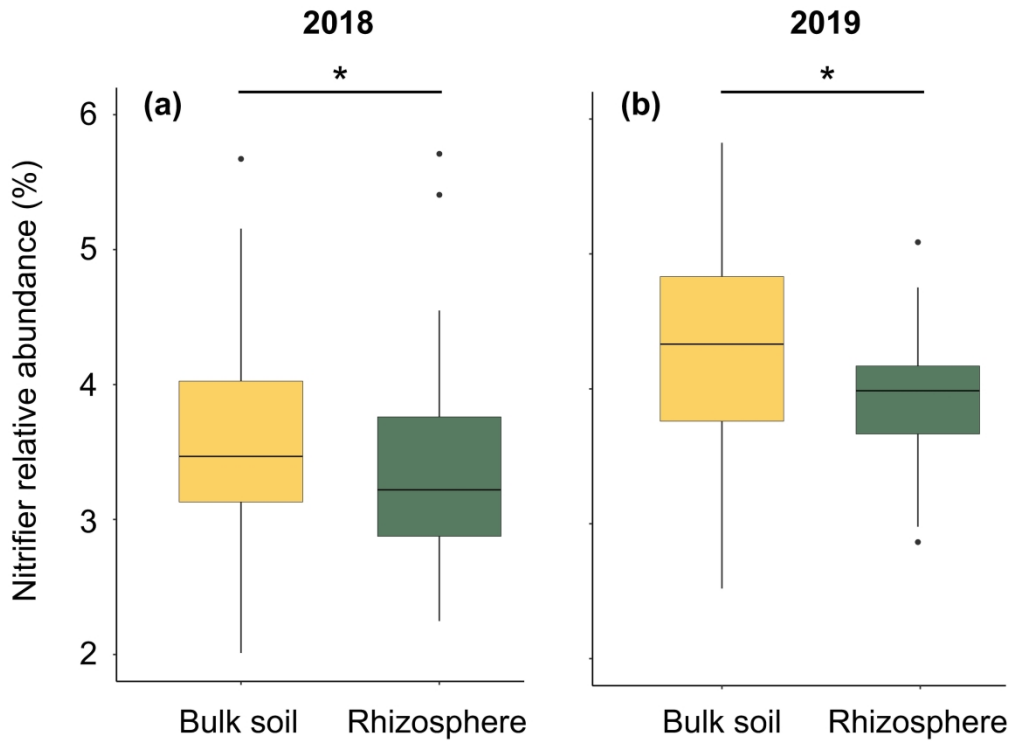

Nitrifier relative abundance in 16S rRNA extracted from bulk and rhizosphere soils (yellow and green bars, respectively) in 2018 (a) and 2019 (b). All fertilizer treatments and growth stage measurements were averaged to illustrate significant bulk vs. rhizosphere effects in each year, and all fertilizer treatment means by date are shown in figure S4. Asterisks denote significant differences between bulk and rhizosphere soils ($P < 0.05$).

285x210mm (300 x 300 DPI)