Title: The impact of stand age and fertilization on the soil microbiome of *Miscanthus* × *giganteus*

List of authors: Lanying Ma,[1,2] Fernando Igne Rocha,[1,3] Jaejin Lee,[1,2] Jinlyung Choi,[1,2] Mauricio Tejera[4], Thanwalee Sooksa-Nguan,[1,2] Nicholas Boersma,[2,4] Andy VanLoocke,[2,4] Emily Heaton,[2,4] Adina Howe[1,2]§

Institute:

[1]Department of Agricultural and Biosystems Engineering, Iowa State University, Ames IA, United States

[2]Center for Advanced Bioenergy and Bioproducts Innovation, Urbana IL, United States

[3]Department of Soil, Universidade Federal Rural do Rio de Janeiro, Seropédica, Brazil

[4]Department of Agronomy, Iowa State University, Ames IA, United States

§Corresponding author

Name: Adina Howe

Postal address: 1201 Sukup Hall, Ames IA. 50011 United States

Telephone: +1-515-294-0176

E-mail: adina@iastate.edu

Keywords: Soil bacterial community structure, *Miscanthus* × *giganteus*, staggered start experiment, nitrogen response

Paper type: Original Research

Abstract

Yield of the perennial grass *Miscanthus* × *giganteus* has shown an inconsistent and unpredictable response to nitrogen (N) fertilizer, yet fertilization underpins the crop's environmental and economic sustainability. The interactions among soil microbial communities, N availability, and *M.* × *giganteus* and management may explain changes in plant productivity. In this study, soil samples from different stand ages of *M.* × *giganteus* in a replicated chronosequence field trial were used to investigate the effects of stand age and N fertilizer rates on microbial community structure. We hypothesized that there is a definable *M.* × *giganteus* soil microbiome and that this community varies significantly with stand age and fertilization. Our results showed that the main phyla in soil microbial communities, regardless of plant age, are similar but microbial community structures are significantly different. The variation in observed microbial communities generally decreases in older stand ages. The amount of N fertilizer applied also affected the microbial community structure associated with different aged *M.* × *giganteus*. Specifically, the relative abundance of *Proteobacteria* (Alphaproteobacteria and Gammaproteobacteria) and *Acidobacteria* (Subgroup Gp1) increased shortly after fertilization and were more associated with younger *M.* × *giganteus*. Further, our results show a significant relationship between bacterial alpha diversity and fertilization rates and that this response is also impacted by stand age. Overall, our results emphasize linkages between microbial community structure, plant age, and fertilization in *M.* × *giganteus*.

Introduction

Perennial biomass crops promise to provide both renewable energy and ecosystem services, but their sustainability hinges critically on crop management (Davis et al. 2013). Harnessing the phytobiome may increase the sustainability of these crops by increasing nutrient use efficiency and thus reducing the need for economically and environmentally expensive synthetic nitrogen (N) fertilizer (Robertson et al. 2017). In temperate rainfed regions, the grass *Miscanthus* × *giganteus* (Greef et Deu.) stands out as a potential bioenergy crop because of its high yields and low-input requirements (Heaton et al. 2004, Heaton et al. 2008, Heaton et al. 2010). Further, it has relatively high cold tolerance while still producing high yields of lignocellulosic biomass (LeBauer et al. 2017; Wang et al. 2008). Estimates of the maximum rainfed simulated end-of-growth-season biomass are ca. 40 Mg ha^{-1} for *M.* × *giganteus*, twice as much as switchgrass estimates (LeBauer et al. 2017; Miguez et al. 2012).

Despite high potential as a bioenergy feedstock, the most appropriate management strategies for the growth of *M.* × *giganteus* are still being explored, especially with regard to fertilization needs (Tejera et al. 2019). Generally, the long term growth of *M.* × *giganteus* would result in the eventual depletion of soil N and thus fertilization is recommended (Cadoux et al., 2012). However, previous studies have reported inconsistent responses of *M.* × *giganteus* to N fertilization (LeBauer et al. 2017). For instance, *M.* × *giganteus* yields have been shown to increase as a result of increased N supply (0, 60, and 120 kg ha^{-1}) in a mediterranean climate (Cosentino et al. 2007). Similarly, N fertilization was found to have a positive effect on yield in Europe (Ercoli et al. 1999; Iqbal et al. 2015; Stépień et al. 2014). In contrast, no significant fertilizer effects on yield of *M.* × *giganteus* were observed at rates 0, 60, and 120 kg ha^{-1} in England (Christian et al. 2008) or with ammonium nitrate at rates of 0 and 100 kg ha^{-1} in North

Carolina (Christian et al. 2008; Rinta-Kanto et al. 2005; Teat et al. 2015). These inconsistent observations of *M.* × *giganteus* response likely represent variations in environmental conditions and soil types as well as sources of N. Another potential reason for the observed differences may be the stand ages of the *M.* × *giganteus* in the various studies, where annual harvesting may cause N depletion in the soils (Arundale et al. 2014). For example, specific *M.* × *giganteus* plants were found to respond to N fertilizer only after the third growing season (Miguez et al. 2008).

One of the factors impacting the availability of nitrogen to plants is the soil's underlying microbial communities (Moreau et al. 2019; Tao et al. 2019). In *M.* × *giganteus*, this microbial community has been identified to provide benefits associated with biological nitrogen fixation (Christian et al. 1997; Davis et al. 2010; Keymer and Kent 2014). Our understanding of soil, plant, and microbial community interactions helps to guide sustainable agroecosystem management (e.g., N additions). Similar to observations of biomass yield, soil microbial communities of *M.* × *giganteus* have been observed to have inconsistent responses to nitrogen fertilization. In a long-term field experiment, nitrogen fertilization had little effect on soil microbial communities (Liu and Ludewig 2019). In a study of *M.* × *giganteus* from four sites in Illinois, Kentucky, Nebraska, and New Jersey, no significant differences in nitrogen-fixing microbial communities were observed between N application rates (Li et al. 2016). In contrast, the microbial composition and biomass of five year old stands of *M.* × *giganteus* were observed to change significantly as a result of fertilization (Oates et al. 2016). It is possible that these observed mixed responses of *M.* × *giganteus* to fertilization could be associated with its perennial lifestyle and studying plants at different stages of establishment. The high N-use efficiency and low N leaching of *M.* × *giganteus* has previously been attributed to the plant's

ability to translocate nutrients to rhizomes at the end of the growing season (Beale and Long 1997). Potentially, as a stand ages and the associated rhizomes mature, the plant's capacity to store nutrients and associated microbial communities may change.

In this study, we explore the relationships of N fertilization on *M.* × *giganteus* of varying stand ages and its associated soil bacterial communities. Unlike previous studies that assessed stand age effects by following the same stands over multiple growing seasons, we used a staggered start experimental design (Loughin 2006), in which we planted replicated plots of *M.* × *giganteus* over three years within a randomized plot layout (Supplementary Fig. S1). This allowed us to compare replicated stands of different ages of *M.* × *giganteus* within the 2018 growing season (Tejera et al. 2019) and to separate the effect of stand age from that of the seasonal environment. We compared three stand ages of *M.* × *giganteus* and three N fertilization rates (0, 224, and 448 kg ha^{-1}) during one growing season. We hypothesized that diversity and membership of microbial communities within the soils of *M.* × *giganteus* are influenced by stand age and N fertilization rate. Further, we expect that there is a relationship between observed diversity and above ground biomass production of *M.* × *giganteus.* Identification of the relationships between diversity, microbial composition, stand age, fertilization and productivity will help to better predict their impacts on *M.* × *giganteus* sustainable growth.

Materials and methods

Site description: This study used the Long-term Assessment of Miscanthus Productivity and Sustainability (LAMPS) site located in Central IA (42.013° N, 93.743 ° W). The site has poorly drained soil (fine-loamy, mixed, superactive, mesic Typic Endoaquoll) and four replications of each growing treatment as previously described (Tejera et al. 2019). In summary, treatment

levels include three planting years (2015, 2016, and 2017) in 24 m × 120 m main plots with split-plot treatments of five N fertilization rates (0, 112, 224, 336, and 448 kg ha^{-1}) in 24 m × 12 m plots (Supplementary Fig. S1). *M.* × *giganteus* clone "Freedom" (AgGrow Tech, Greensboro, NC, USA) was planted in 0.3-m rows at a density of around 11 rhizome m^{-2} by a specialized plot planter. This clone has no discernible genetic differences from the "Illinois" or "Hornum" clone used elsewhere in the literature (Głowacka et al. 2015). With the exception of plots with no fertilization, N fertilization was annually applied as an aqueous solution of urea-ammonium nitrate in a single application. Fertilizer was side-dressed into the soil at 0.1 m depth, following coulter wheels spaced 1.5 m apart that cut field residue and opened the soil to avoid surface plant residue. For the year of this study, N fertilizer was applied on May 9, 2018. Historically, herbicide was applied to all stand ages during their first two years of growth to control weeds, and standing biomass was annually removed mid-winter after full crop senescence and substantial leaf drop (see Tejera et al. (2019) for full management details). Standing crop biomass was measured on November 13, 2018 in two 1-m quadrants per plot following the methods of Tejera et al. (2019) (Supplementary Table S1).

Sampling and DNA extraction: Soil samples were taken in 2018 from plots planted with *M.* × *giganteus* in 2015, 2016, and 2017 representing four-year, three-year, and two-year stand ages, respectively. Fertilizer was applied on May 9. Soils from blocks treated with 0, 224, and 448 kg ha^{-1} N fertilizer were collected on April 25, April 30, May 14, May 30, and July 3 or -14, -10, 5, 21, 55 days relative to fertilization, respectively. Bulk soils were obtained from within a 10 cm radius of the plant using a sampling core with a 30.5 cm wet sample tube and 1.75 cm in diameter (Clements Associates Inc, Newton, IA, USA). Roots collected within the sample were not separated. The first 10 cm depth of soil was collected and stored immediately in sampling

bags on dry ice; samples were stored at -80°C upon return to the laboratory. For each plot, two to three samples were obtained and analyzed independently (e.g., not composited). In total, 432 samples were collected (4 blocks x 3 plots x 3 fertilization rates x 5 sampling days x 2-3 replicates). For DNA extraction, each soil sample was homogenized and aliquoted into a 0.25 g subsample. DNA extraction was performed using MagAttract PowerSoil DNA EP kit for DNA (Qiagen, Germantown, MD, USA) following the standard protocol of this kit and the liquid handling of the Eppendorf epMotion 5075 (Eppendorf, Enfield, CT, USA).

16S rRNA gene amplicon sequencing: Deoxyribonucleic acid samples with concentration above 10 ng μl^{-1} were diluted to 10 ng μl^{-1} prior to sequencing. Samples with concentration lower than 10 ng μl^{-1} were submitted directly for amplicon sequencing. In summary, the V4 region of the bacterial 16S rRNA gene was amplified. Amplification was performed using 10 µM each of 16S rRNA v4 region primers. The forward primer, 515F, used was GTGYCAGCMGCCGCGGTAA, and the reverse primer, 806R, used was GGACTACNVGGGTWTCTAAT. The target amplicon size was 390 bp. The PCR amplification parameters were as follows for a 384-well plate: 94 ^{o}C for 3 min and then 94 ^{o}C for 60 s, 50 ^{o}C for 60 s, and 72 ^{o}C for 105 s repeated for 35 cycles with a final extension of 72 ^{o}C for 10 min. The specific protocol is described at https://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s. Sequencing of bacterial amplicons was performed on Illumina Miseq with Miseq Reagent Kit V2 (Illumina, San Diego, CA, USA) at Argonne National Laboratory (Argonne, IL, USA), and sequencing libraries were comprised of 150 bp paired-end reads. Sequencing data is deposited in the NCBI Short Read Archive (SRA) as project PRJNA601860 (sample specific data is shown in Supplementary Table S1).

Amplicon bioinformatic analysis: The sequencing data was analyzed by DADA2 package (version 1.13.1) to determine abundances of amplicon sequence variants (ASVs) (Callahan et al. 2016). Truncated sequence read length was set to 145 bp to remove low quality tails based on inspection of quality control profiles. The filtering parameters were set to be truncLen=c(145,145), truncQ=2, rm.phix=TRUE, compress=TRUE. The taxonomic identity of each observed ASV was determined using sequence similarity to representatives in the Ribosomal Database Project (RDP) Classifier (version 11.5). ASVs observed in less than 10 samples were removed. Samples with less than 9,000 reads were removed, resulting in a total of 416 samples (Supplementary Table S1). Each sequencing library was adjusted to 9,000 reads for estimation of ASV abundances unless otherwise indicated.

Statistical analysis: All statistical analysis was performed in R (R Core Team, 2019). We estimated alpha diversity in samples, expressed as Shannon diversity and Chao 1 richness, using the function *estimate_richness()* included in the phyloseq package, version 1.30.0. Alpha diversity was estimated for each stand age and sampling day to identify differences between N fertilization rates. Generalized linear mixed-effect models (GLMM) fit by maximum likelihood were applied to test the effects of plant above-ground biomass, stand age, and N fertilization rates on soil alpha diversity (lme4 package, version 1.1-23 and lmerTest, version 3.1-2). Experimental factors were considered as both main fixed effects, in interactions, and as a nested random effect within blocks. The Wald chi-square test and least-square means for pairwise t-test with false discovery rate correction for multiple comparisons were used to test the significance of relationships between soil microbial richness and diversity and N fertilization and plant biomass. The rank-based coefficient Kendall's (τ) non-parametric test was used to estimate the amount and direction of correlation between evaluated factors and alpha-diversity measures.

Permutational multivariate analysis of variance (PERMANOVA) distance matrices was performed with the *adonis()* function from the *vegan* package, version 2.5 - 5, based on the Bray-Curtis dissimilarity distances between samples with p-values for the test statistic (pseudo-F) based on 999 permutations. As the experimental plot was identified as a major factor to structure the microbial composition, PERMANOVA was also performed using the "strata" argument for plot sites to better identify the impacts of stand age and N fertilization rates. This analysis restricts permutations to samples within each block and was used to quantify variations between and within treatments (e.g. N fertilization rates and stand age). To test differences in the variability of soil bacterial communities between N fertilization, we used PERMDISP analyses for each experimental period. Multivariate dispersions, based on distances of observations to their centroid, were first calculated using the *betadisper()* function of *vegan*. The mean dispersion was next compared between groups via the *permutest()* function (based on 999 permutations). Principal Coordinates Analysis (PCoA) was performed to visualize the dissimilarity among samples using the Bray-Curtis dissimilarity matrix.

We identified representative ASVs within each stand age and required that each ASV be detected with at least ten reads in a single sample. ASVs were next determined as unique to a stand age or shared between stand ages. To represent these results, a venn diagram was created using the VennDiagram package (version 1.6.20). To identify significantly enriched ASVs between stand ages, differential abundance analysis based on the negative binomial distribution with Wald's test was performed on the experimental factors of block and N fertilization rate. This analysis was performed by the function DESeq with non-rarefied data from the R package DESeq2, version 3.8. Pairwise comparisons to unfertilized treatments were performed, including

comparisons of 224 to 0 kg ha^{-1} N and 448 to 0 kg ha^{-1} N under each stand age of *M.* × *giganteus.*

Code for all described analysis is available at https://github.com/germs-lab/LAMPS-miscanthus-microbiome.

Results

In this study, the *M.* × *giganteus* plots performed typically for Iowa, yielding between 12 and 30 Mg ha^{-1} depending on treatment and stand age (Supplementary Table S1). Stand age had a large effect on biomass yield (P_{Age} = 0.0068). Three- and four-year old stands yielded ~20% more biomass than two-year old stands. Nitrogen fertilization also had a significant effect on biomass yield, with significant correlations with biomass in three- and four-year old stands but not in two-year old stands (P_{Nrate} < 0.0001, Supplementary Fig. S2). Fertilized plots yielded up to 38% more biomass than unfertilized plots, but this response changed with stand age ($P_{Nrate*Age}$ < 0.0001).

In our *M.* × *giganteus* soil samples, we observed a total of 31 bacterial phyla and 39,810 ASVs. ASVs associated with *Proteobacteria, Actinobacteria, Acidobacteria, Verrucomicrobia,* and *Bacteroidetes* were found to be the most abundant and comprising over 80% by relative abundance (Fig. 1A). *Proteobacteria* were dominant in the microbiome, with Alphaproteobacteria comprising the largest proportion followed by Betaproteobacteria, Deltaproteobacteria, and Gammaproteobacteria (Fig. 1B). A total of 39,810 ASVs were observed in sampled soils (Fig. 2). ASVs uniquely associated within each stand age were identified, with 1,550, 812, and 701 ASVs identified in 2, 3, and 4 year-old stands, respectively. Shared between samples from all three stand ages, a total of 27,315 ASVs (68% of total) were identified, and these shared ASVs represent highly abundant membership, comprising over 80%

of the total observed abundance (Fig. 3A). In contrast, unique ASVs identified from each stand age comprised only a small percentage (<1%) of total observed abundance (Fig. 3B). These unique ASVs were represented by diverse phyla but were dominated by Proteobacteria (Alphaproteobacteria, Deltaproteobacteria, and Gammaproteobacteria), Actinobacteria, and Acidobacteria.

Overall, we observed no statistically significant differences in alpha diversity between soils originating from the three stand ages of *M.* × *giganteus* (Supplementary Table S2). Fertilization rates and stand age interactions were observed to effect alpha diversity. With increased fertilization rates, we observed that alpha diversity was negatively affected, especially in two-year old stands (Fig. 4). This trend was also found in response to days since fertilization, where alpha diversity decreased in samples directly after fertilization, particularly in younger stands (Supplementary Table 3, Fig. 4). No significant ($P > 0.05$) correlations between alpha diversity and days relative to fertilization were observed in four-year old stand or samples that received no N fertilizer.

Next, we identified taxa and their associated phyla that were observed to be significantly different between fertilization rates within each stand age (Supplementary Fig. S3). In two- and four-year old plants, a total of 194 and 47 ASVs, respectively, were identified as significantly differentially abundant ASVs under varying treatments of N rates. The most dominant phyla represented by these ASVs in soils from two-year plants were Proteobacteria (classes Alphaproteobacteria and Gammaproteobacteria) and Acidobacteria. Dominant phyla associated with these ASVs in soils from four-year plants also included Proteobacteria (classes Alphaproteobacteria and Gammaproteobacteria), followed by Actinobacteria. Overall, the majority of these ASVs were unique to each stand age, with only 11 of these ASVs were

252 identified as present in both two- and four-year old stands. From three-year old *M.* × *giganteus*,
253 only three ASVs were observed to change significantly due to the N fertilization rates.
254 Generally, we observed differences in the microbial community structure of the youngest
255 stand age (two-year) compared to the older stand ages. First, the ASVs identified as significantly
256 enriched in fertilized treatments of two-year old *M.* × *giganteus* samples represented a greater
257 relative abundance compared to those identified in older stand age samples (12 vs 3% average
258 relative abundance, Supplementary Fig. S3). Second, many of these ASVs were also found to
259 have a seasonal response to fertilization, and these trends were also more pronounced in two-
260 year old stands. Particularly, *Proteobacteria* (especially *Alphaproteobacteria* and
261 *Gammaproteobacteria*) and *Acidobacteria* (especially subdivision 1) increased in relative
262 abundance five days after the 448 kg ha^{-1} fertilization in both two-year and four-year old stands
263 (Supplementary Fig. S4).
264 Beta diversity was calculated based on Bray-Curtis dissimilarity for sample-to-sample
265 comparison. PERMANOVA analysis demonstrated that the largest observed variation to affect
266 this diversity was the plot of sample origination (R^2 = 0.244, F = 4.520, *P* = 0.001), followed by
267 stand age (R^2 = 0.075, F = 21.652, *P* = 0.001) and fertilization rates (R^2 = 0.012, F = 3.532, *P* =
268 0.001). The days relative to fertilization (i.e., sampling day) also affected the microbial
269 community structure significantly (PERMANOVA, R^2 = 0.09, F = 1.424, *P* = 0.04). To further
270 analyze the effects of stand ages and N fertilization rates, we evaluated differences within a
271 block. All PERMANOVA analyses were also performed without 'strata as plot' with similar
272 results observed. Comparison of beta diversity estimates observed between stand ages showed
273 that microbial communities between two- and three- year old *M.* × *giganteus* (PERMANOVA, F
274 = 22.7, *P* = 0.001, strata with plot) and two- and four- year old *M.* × *giganteus* (PERMANOVA,

F = 5.37, P = 0.028, strata with plot) were significantly different (Supplementary Fig. S5). No significant difference was observed in communities from soils collected from the three- and four-year old stands. While the most abundant phyla observed in soils from all three plant ages was consistent, the contribution of specific phylum to the total abundance varied, with *Verrucomicrobia*, *Gemmatimonadetes*, *Planctomycetes,* and *Thaumarcheota* observed as significantly different between all three stand ages (Supplementary Fig. S6).

We evaluated the impact of N fertilization rates on the heterogeneity of microbial composition within each stand age of *M.* × *giganteus*. Generally, we observed the largest variation between samples in two-year old stand age (PERMDISP, F = 11.81; P = 0.001), where significant differences were observed under all N fertilization rates (Supplementary Fig. S7). In three-year old stands, this variation was found to be decreased (PERMDISP, F = 1.49; P = 0.238). In four-year old stands, significant dissimilarity between soil communities was detected (PERMDISP, F = 9.15; P = 0.001), however, only significant differences were observed between unfertilized and fertilized groups (PERMDISP, 0 x 224 kg ha^{-1}: P = 0.003; 0 x 448 kg ha^{-1}: P = 0.001; 224 x 448 kg ha^{-1}: P = 0.244).

Finally, we evaluated our ability to predict the soil microbiome diversity from above-ground biomass. Overall, patterns of increasing biomass with increased fertilization (Supplementary Fig. S2). We developed a predictive model for the effects of the interactions between above-ground biomass and N fertilization rate on alpha-diversity (Fig. 5). This model showed a positive correlation of biomass and alpha diversity from fertilized samples which was not present in non-fertilized samples. While we observed that there is not a direct effect of stand age on alpha diversity (Supplementary Table 2), we identified a relationship between biomass

297 and alpha diversity that varies depending on stand age and fertilization rates (Supplementary Fig.
298 S8).

299 ## Discussion

300 In this study, we explored the relationships between the soil microbial communities of *M.*
301 × *giganteus*, its stand age, and nitrogen fertilization rates. This community is dominated by
302 Proteobacteria (mainly Alphaproteobacteria), Acidobacteria, and Actinobacteria, which is
303 consistent with previous studies of *M.* × *giganteus* (Yan et al. 2017; Zhalnina et al. 2018). The
304 large majority of membership of this community is present across plants from all stand ages
305 within our study (Fig. 3B), though unique taxa were identified as specific to soils from two-,
306 three-, and four-year old plants.

307 To determine the impacts of plant stand age and fertilization rates on the overall *M.* ×
308 *giganteus* microbiome, we evaluated several characteristics of the soil microbiomes, including
309 estimations of alpha diversity and significantly enriched members between treatments. While
310 there is evidence of a high proportion of the microbial community shared between the samples,
311 impacts of plant stand age and fertilization to alter these communities are illustrated consistently
312 in our results. Between microbiomes originating from plants of varying stand ages, differences
313 between microbial communities were observed to be mainly due to shifts in the proportions of
314 present bacteria rather than different community membership. In response to varying
315 fertilization rates, microbial taxa in each stand age were significantly enriched, with more
316 pronounced shifts in response to fertilization in communities associated with younger stand ages.
317 Additionally, we observe that the microbial communities associated with younger, two-year old
318 stands are more variable in their biodiversity. In comparison, the microbiomes of the four-year
319 old stands were observed to have decreased overall alpha-diversity and appeared more similar in

composition to three-year old than two-year old stands. Overall, these results suggest that stand age shifts the underlying soil microbial communities and is consistent with a previous study in *Pinus elliottii* that showed a significant impact of plant age on microbial communities (Wu et al. 2015).

In the youngest stand age, notable enrichments of *Proteobacteria*, especially Alphaproteobacteria and Gammaproteobacteria, and *Acidobacteria* were observed in response to fertilization. *Acidobacteria* and *Proteobacteria* have previously been associated with N cycling and have been observed to be enriched in response to long-term elevated nitrogen in diverse agricultural soils (Dai et al. 2018; Pan et al. 2014). *Acidobacteria* have previously been found to be sensitive to inorganic and organic nutrients inputs and have been previously associated with nitrate reduction based on both the conservation of nitrate reduction genes and characterization of this activity in isolates (Kielak et al. 2016; Ward et al. 2009). Our results are also consistent with other studies which have observed the enrichment of Alphaproteobacteria and Gammaproteobacteria in *M.* × *giganteus* in rhizomes after fertilization (Liu and Ludewig 2019). Both Alphaproteobacteria and Gammaproteobacteria have also been observed as significant membership in the phyllosphere of *M.* × *giganteus*, with both classes having compensatory patterns over 10-week growing season (Grady et al. 2019). The consistent observations of the enrichment of these bacteria in *M.* × *giganteus* suggests that these bacteria may play a role in the microbial response to fertilization. A future research direction is to use functional studies to understand whether these differences in microbial communities are attributed to functional changes or benefits to the plant or soils.

In association with our observations that specific microbial membership in older Miscanthus stands have a less pronounced response to fertilization, we also observed that the

alpha diversity of older stand ages were generally less variable in response to fertilization compared to alpha diversity estimated in younger stand ages. Our results consistently suggest that interactions between stand age and fertilization are important to the diversity observed in *M.* × *giganteus.* N fertilization has been previously shown to cause shifts of microbial communities in other plant studies (Dai et al. 2018; Fierer et al. 2012; Yu et al. 2016), but within *M.* × *giganteus,* previous studies have shown little effect of N fertilization on soil communities (Li et al. 2016; Liu and Ludewig 2019). Our results indicate that stand age can influence the *M.* × *giganteus* microbial community response to fertilization and thus N availability to the plants. Over multiple growing seasons, plants within aged stands can accumulate increased dead plant organs (litter and roots) both above and below the soil surface and also will develop more mature rhizomes. Potentially, the decreased heterogeneity and variability in the response of the microbiome of older *M.* × *giganteus* is related to its perennial growth and more consistency in the availability of nutrients.

Our observations of role of stand ages in response to fertilization are also consistent with the yield variability at this site (Tejera et al. 2019). We compared observations of alpha diversity within soil microbiomes with above-ground biomass production observed during this sampling year. We identified significant relationships between alpha diversity, stand ages, fertilization and above-ground biomass production, with general patterns of increased above-ground biomass with increased alpha diversity under fertilized conditions. Overall, this result highlights a relationship between microbiome, plant productivity, and fertilization practices. As stands of different ages were planted in different years, a possible confounding factor to these observations are the conditions during the establishment of stands, e.g., climate conditions during the year of planting. Soil properties, such as pH, climate, and organic carbon availability have previously

366 been shown to affect associated microbiomes (Fierer 2017; Lauber et al. 2009). The year of
367 planting has been previously reported to significantly affect biomass yield along with stand age,
368 with planting condition associated with larger effects on one-year old stands but minimal
369 compared to age effects after the second year of growth (Tejera et al. 2019). Conditions
370 associated with planting, especially rhizome condition and weather conditions have been shown
371 to influence stand performance in subsequent years at climatically similar locations in North
372 America and Europe (Lewandowski et al. 2016; Maughan et al. 2012).
373 Overall, this study supports the use of microbial characterization to understand *M.* ×
374 *giganteus* productivity and highlights the need to consider plant age in developing management
375 strategies. Our observations that there are consistent bacterial communities associated with *M.* ×
376 *giganteus* that shifts in response to plant and soil traits justifies future research to better link this
377 microbiome with plant productivity and sustainability. This study specifically focuses on the
378 bacterial community within the *M.* × *giganteus* soil microbiome. Understanding the role and
379 interactions of other living organisms in the soil (e.g., fungi, nematodes, etc.) and their
380 interactions with nutrients represent another opportunity for future research. Additionally, there
381 is a need to obtain better functional information to understand the response of the *M.* × *giganteus*
382 bacterial communities to fertilization and how this helps to meet the N needs of *M.* × *giganteus*
383 over varying plant ages.

Acknowledgements

This work was funded by the DOE Center for Advanced Bioenergy and Bioproducts Innovation (U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research under Award Number DE-SC0018420). The DNA sequencing in this study was performed at Environmental Sample Preparation and Sequencing Facility at Argonne National Laboratory. The LAMPS experiment was funded by USDA National Institute of Food and Agriculture Hatch grant 1008969, the Leopold Center for Sustainable Agriculture, and the Iowa State University Department of Agronomy. FIR was also supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (CAPES; PDSE call nº 41/2018). Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the U.S. Department of Energy, USDA, Leopold Center for Sustainable Agriculture or Iowa State University. Partial support was also provided by USDA Hatch Project IOW04414.

References

Arundale, R. A., Dohleman, F. G., Heaton, E. A., Mcgrath, J. M., Voigt, T. B., and Long, S. P. 2014. Yields of *Miscanthus* × *giganteus* and *Panicum virgatum* decline with stand age in the Midwestern USA. GCB Bioenergy 6:1–13. https://doi.org/10.1111/gcbb.12077

Beale, C. V, and Long, S. P. 1997. Seasonal dynamics of nutrient accumulation and partitioning in the perennial C4-grasses *Miscanthus* × *giganteus* and *Spartina cynosuroides*. Biomass and Bioenergy 12:419–428. https://doi.org/https://doi.org/10.1016/S0961-9534(97)00016-0

Cadoux, S., Riche, A. B., Yates, N. E., and Machet, J. M. 2012. Nutrient requirements of

406 *Miscanthus* × *giganteus*: Conclusions from a review of published studies. Biomass and
407 Bioenergy 38:14–22. https://doi.org/10.1016/j.biombioe.2011.01.015

408 Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P.
409 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat.
410 Methods 13:581–583. https://doi.org/10.1038/nmeth.3869

411 Christian, D. G., Poulton, P. R., Riche, A. B., and Yates, N. E. 1997. The recovery of 15N-
412 labelled fertilizer applied to *Miscanthus* × *giganteus*. Biomass and Bioenergy 12:21–24.
413 https://doi.org/https://doi.org/10.1016/S0961-9534(96)00060-8

414 Christian, D. G., Riche, A. B., and Yates, N. E. 2008. Growth, yield and mineral content of
415 *Miscanthus* × *giganteus* grown as a biofuel for 14 successive harvests. Ind. Crops Prod.
416 28:320–327. https://doi.org/10.1016/j.indcrop.2008.02.009

417 Cosentino, S. L., Patanè, C., Sanzone, E., Copani, V., and Foti, S. 2007. Effects of soil water
418 content and nitrogen supply on the productivity of *Miscanthus* × *giganteus* Greef et Deu. in
419 a Mediterranean environment. Ind. Crops Prod. 25:75–88.
420 https://doi.org/10.1016/j.indcrop.2006.07.006

421 Dai, Z., Su, W., Chen, H., Barberán, A., Zhao, H., Yu, M., Yu, L., Brookes, P. C., Schadt, C. W.,
422 Chang, S. X., and Xu, J. 2018. Long-term nitrogen fertilization decreases bacterial diversity
423 and favors the growth of *Actinobacteria* and *Proteobacteria* in agro-ecosystems across the
424 globe. Glob. Chang. Biol. 24:3452–3461. https://doi.org/10.1111/gcb.14163

425 Davis, S. C., Boddey, R. M., Alves, B. J. R., Cowie, A. L., George, B. H., Ogle, S. M., Smith, P.,
426 van Noordwijk, M., and van Wijk, M. T. 2013. Management swing potential for bioenergy
427 crops. GCB Bioenergy 5:623–638. https://doi.org/10.1111/gcbb.12042

Davis, S. C., Parton, W. J., Dohleman, F. G., Smith, C. M., Grosso, S. Del, Kent, A. D., and DeLucia, E. H. 2010. Comparative Biogeochemical Cycles of Bioenergy Crops Reveal Nitrogen-Fixation and Low Greenhouse Gas Emissions in a *Miscanthus* × *giganteus* Agro-Ecosystem. Ecosystems 13:144–156. https://doi.org/10.1007/s10021-009-9306-9

Ercoli, L., Mariotti, M., Masoni, A., and Bonari, E. 1999. Effect of irrigation and nitrogen fertilization on biomass yield and efficiency of energy use in crop production of Miscanthus. F. Crop. Res. 63:3–11. https://doi.org/10.1016/S0378-4290(99)00022-2

Fierer, N. 2017. Embracing the unknown: disentangling the complexities of the soil microbiome. Nat. Rev. Microbiol. 15:579–590. https://doi.org/10.1038/nrmicro.2017.87

Fierer, N., Lauber, C. L., Ramirez, K. S., Zaneveld, J., Bradford, M. A., and Knight, R. 2012. Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. ISME J. 6:1007–1017. https://doi.org/10.1038/ismej.2011.159

Głowacka, K., Clark, L. V., Adhikari, S., Peng, J., Stewart, J. R., Nishiwaki, A., Yamada, T., Jørgensen, U., Hodkinson, T. R., Gifford, J., Juvik, J. A., and Sacks, E. J. 2015. Genetic variation in *Miscanthus* × *giganteus* and the importance of estimating genetic distance thresholds for differentiating clones. GCB Bioenergy 7:386–404. https://doi.org/10.1111/gcbb.12166

Grady, K. L., Sorensen, J. W., Stopnisek, N., Guittar, J., and Shade, A. 2019. Assembly and seasonality of core phyllosphere microbiota on perennial biofuel crops. Nat. Commun. 10:4135. https://doi.org/10.1038/s41467-019-11974-4

Heaton, E. A., Clifton-Brown, J., Voigt, T. B., Jones, M. B., and Long, S. P. 2004. Miscanthus

for renewable energy generation: European union experience and projections for Illinois. Mitig. Adapt. Strateg. Glob. Chang. 9:433–451. https://link.springer.com/content/pdf/10.1023%2FB%3AMITI.0000038848.94134.be.pdf

Heaton, E. A., Dohleman, F. G., and Long, S. P. 2008. Meeting US biofuel goals with less land: The potential of Miscanthus. Glob. Chang. Biol. 14:2000–2014. https://doi.org/10.1111/j.1365-2486.2008.01662.x

Heaton, E. A., Dohleman, F. G., Miguez, A. F., Juvik, J. A., Lozovaya, V., Widholm, J., Zabotina, O. A., McIsaac, G. F., David, M. B., Voigt, T. B., Boersma, N. N., and Long, S. P. 2010. Miscanthus: A promising biomass crop. Adv. Bot. Res. 56:75–137. https://doi.org/10.1016/B978-0-12-381518-7.00003-0

Iqbal, Y., Gauder, M., Claupein, W., Graeff-Hönninger, S., and Lewandowski, I. 2015. Yield and quality development comparison between miscanthus and switchgrass over a period of 10 years. Energy 89:268–276. https://doi.org/10.1016/j.energy.2015.05.134

Keymer, D. P., and Kent, A. D. 2014. Contribution of nitrogen fixation to first year *Miscanthus* × *giganteus*. GCB Bioenergy 6:577–586. https://doi.org/10.1111/gcbb.12095

Kielak, A. M., Barreto, C. C., Kowalchuk, G. A., van Veen, J. A., and Kuramae, E. E. 2016. The Ecology of Acidobacteria: Moving beyond Genes and Genomes. In *Frontiers in Microbiology* (Vol. 7, p. 744). https://www.frontiersin.org/article/10.3389/fmicb.2016.00744

Lauber, C. L., Hamady, M., Knight, R., and Fierer, N. 2009. Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. Appl. Environ. Microbiol. 75:5111–5120. https://doi.org/10.1128/AEM.00335-09

LeBauer, D., Kooper, R., Mulrooney, P., Rohde, S., Wang, D., Long, S. P., and Dietze, M. C. 2017. BETYdb: a yield, trait, and ecosystem service database applied to second-generation bioenergy feedstock production. GCB Bioenergy 10:61–71. https://doi.org/10.1111/gcbb.12420

Lewandowski, I., Clifton-Brown, J., Trindade, L. M., Van Der Linden, G. C., Schwarz, K. U., Müller-Sämann, K., Anisimov, A., Chen, C. L., Dolstra, O., Donnison, I. S., Farrar, K., Fonteyne, S., Harding, G., Hastings, A., Huxley, L. M., Iqbal, Y., Khokhlov, N., Kiesel, A., Lootens, P., … Kalinina, O. 2016. Progress on optimizing miscanthus biomass production for the european bioeconomy: Results of the EU FP7 project OPTIMISC. Front. Plant Sci. 7:1–23. https://doi.org/10.3389/fpls.2016.01620

Li, D., Voigt, T. B., and Kent, A. D. 2016. Plant and soil effects on bacterial communities associated with *Miscanthus* × *giganteus* rhizosphere and rhizomes. GCB Bioenergy 8:183–193. https://doi.org/10.1111/gcbb.12252

Liu, Y., and Ludewig, U. 2019. Nitrogen-dependent bacterial community shifts in root, rhizome and rhizosphere of nutrient-efficient *Miscanthus* × *giganteus* from long-term field trials. GCB Bioenergy 11:1334–1347. https://doi.org/10.1111/gcbb.12634

Loughin, T. M. 2006. Improved experimental design and analysis for long-term experiments. Crop Sci. 46:2492–2502. https://doi.org/10.2135/cropsci2006.04.0271

Maughan, M., Bollero, G., Lee, D. K., Darmody, R., Bonos, S., Cortese, L., Murphy, J., Gaussoin, R., Sousek, M., Williams, D., Williams, L., Miguez, F., and Voigt, T. 2012. *Miscanthus* × *giganteus* productivity: The effects of management in different environments. GCB Bioenergy 4:253–265. https://doi.org/10.1111/j.1757-1707.2011.01144.x

494 Miguez, F. E., Maughan, M., Bollero, G. A., and Long, S. P. 2012. Modeling spatial and
495 dynamic variation in growth, yield, and yield stability of the bioenergy crops *Miscanthus* ×
496 *giganteus* and Panicum virgatum across the conterminous United States. GCB Bioenergy
497 4:509–520. https://doi.org/10.1111/j.1757-1707.2011.01150.x

498 Miguez, F. E., Villamil, M. B., Long, S. P., and Bollero, G. A. 2008. Meta-analysis of the effects
499 of management factors on *Miscanthus* × *giganteus* growth and biomass production. Agric.
500 For. Meteorol. 148:1280–1292. https://doi.org/10.1016/j.agrformet.2008.03.010

501 Moreau, D., Bardgett, R. D., Finlay, R. D., Jones, D. L., and Philippot, L. 2019. A plant
502 perspective on nitrogen cycling in the rhizosphere. Funct. Ecol. 33:540–552.
503 https://doi.org/10.1111/1365-2435.13303

504 Oates, L. G., Duncan, D. S., Sanford, G. R., Liang, C., and Jackson, R. D. 2016. Bioenergy
505 cropping systems that incorporate native grasses stimulate growth of plant-associated soil
506 microbes in the absence of nitrogen fertilization. Agric. Ecosyst. Environ. 233:396–403.
507 https://doi.org/10.1016/j.agee.2016.09.008

508 Pan, Y., Cassman, N., De Hollander, M., Mendes, L. W., Korevaar, H., Geerts, R. H. E. M., Van
509 Veen, J. A., and Kuramae, E. E. 2014. Impact of long-term N, P, K, and NPK fertilization
510 on the composition and potential functions of the bacterial community in grassland soil.
511 FEMS Microbiol. Ecol. 90:195–205. https://doi.org/10.1111/1574-6941.12384

512 R_Core_Team. 2019. R: A language and environment for statistical computing. R Found. Stat.
513 https://www.r-project.org/

514 Rinta-Kanto, J. M., Ouellette, A. J. A., Boyer, G. L., Twiss, M. R., Bridgeman, T. B., and
515 Wilhelm, S. W. 2005. Quantification of toxic Microcystis spp. during the 2003 and 2004

blooms in western Lake Erie using quantitative real-time PCR. Environ. Sci. Technol. 39:4198–4205. http://www.ncbi.nlm.nih.gov/pubmed/15984800

Robertson, G. P., Hamilton, S. K., Barham, B. L., Dale, B. E., Izaurralde, R. C., Jackson, R. D., Landis, D. A., Swinton, S. M., Thelen, K. D., and Tiedje, J. M. 2017. Cellulosic biofuel contributions to a sustainable energy future: Choices and outcomes. Science (80-.). 356:1–9. https://doi.org/10.1126/science.aal2324

Stépień, W., Górska, E. B., Pietkiewicz, S., and Kalaji, M. H. 2014. Long-term mineral fertilization impact on chemical and microbiological properties of soil and *Miscanthus* × *giganteus* yield. Plant, Soil Environ. 60:117–122.

Tao, K., Kelly, S., and Radutoiu, S. 2019. Microbial associations enabling nitrogen acquisition in plants. Curr. Opin. Microbiol. 49:83–89. https://doi.org/https://doi.org/10.1016/j.mib.2019.10.005

Teat, A. L., Neufeld, H. S., Gehl, R. J., and Gonzales, E. 2015. Growth and Yield of *Miscanthus* × *giganteus* Grown in Fertilized and Biochar-Amended Soils in the Western North Carolina Mountains. Castanea 80:45–58. https://doi.org/10.2179/14-021R1

Tejera, M., Boersma, N., Vanloocke, A., Archontoulis, S., Dixon, P., Miguez, F., and Heaton, E. 2019. Multi-year and Multi-site Establishment of the Perennial Biomass Crop *Miscanthus* × *giganteus* Using a Staggered Start Design to Elucidate N Response. BioEnergy Res. 12:471–483. https://doi.org/10.1007/s12155-019-09985-6

Wang, D., Portis, A. R., Moose, S. P., and Long, S. P. 2008. Cool C4 Photosynthesis: Pyruvate Pi Dikinase Expression and Activity Corresponds to the Exceptional Cold Tolerance of Carbon Assimilation in *Miscanthus* × *giganteus*. Plant Physiol. 148:557–567.

https://doi.org/10.1104/pp.108.120709

Ward, N. L., Challacombe, J. F., Janssen, P. H., Henrissat, B., Coutinho, P. M., Wu, M., Xie, G., Haft, D. H., Sait, M., Badger, J., Barabote, R. D., Bradley, B., Brettin, T. S., Brinkac, L. M., Bruce, D., Creasy, T., Daugherty, S. C., Davidsen, T. M., DeBoy, R. T., … Kuske, C. R. 2009. Three Genomes from the Phylum Acidobacteria Provide Insight into the Lifestyles of These Microorganisms in Soils. Appl. Environ. Microbiol. 75:2046 LP – 2056. https://doi.org/10.1128/AEM.02294-08

Wu, Z., Elizabeth Haack, S., Lin, W., Li, B., Wu, L., Fang, C., and Zhang, Z. 2015. Soil Microbial Community Structure and Metabolic Activity of Pinus elliottii Plantations across Different Stand Ages in a Subtropical Area OPEN ACCESS. PLoS One 10:1–11. https://doi.org/10.1371/journal.pone.0135354

Yan, G., Xing, Y., Xu, L., Wang, J., Dong, X., Shan, W., Guo, L., and Wang, Q. 2017. Effects of different nitrogen additions on soil microbial communities in different seasons in a boreal forest. Ecosphere 8:1–19. https://doi.org/10.1002/ecs2.1879

Yu, C., Hu, X., Deng, W., Li, Y., Han, G., and Xiong, C. 2016. Response of Bacteria Community to Long-Term Inorganic Nitrogen Application in Mulberry Field Soil. PLoS One 11:1–12. https://doi.org/10.1371/journal.pone.0168152

Zhalnina, K., Louie, K. B., Hao, Z., Mansoori, N., da Rocha, U. N., Shi, S., Cho, H., Karaoz, U., Loqué, D., Bowen, B. P., Firestone, M. K., Northen, T. R., and Brodie, E. L. 2018. Dynamic root exudate chemistry and microbial substrate preferences drive patterns in rhizosphere microbial community assembly. Nat. Microbiol. 3:1–11. https://doi.org/10.1038/s41564-018-0129-3

Figures:

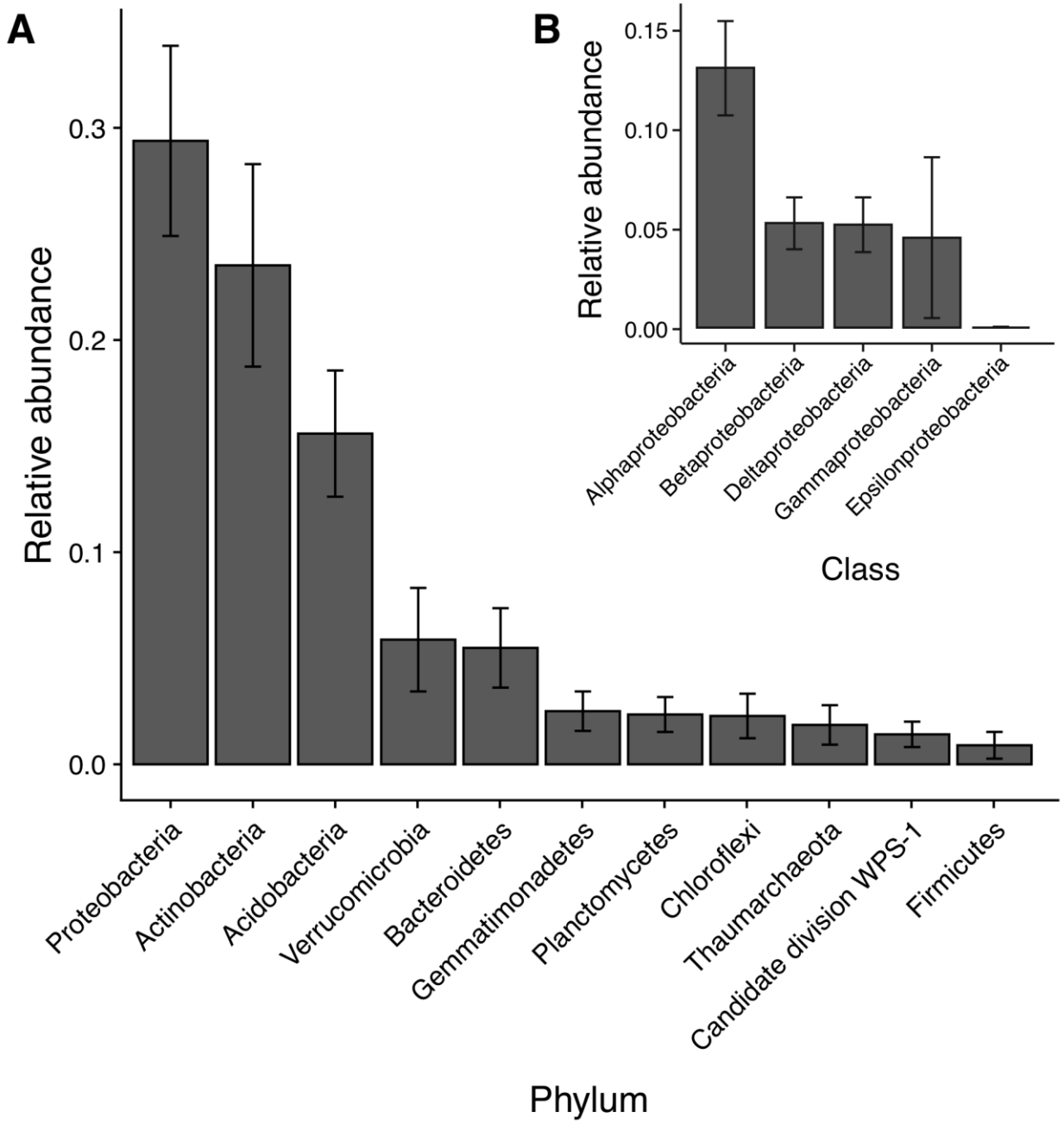

Fig. 1. Phylogenetic distribution of *M.* × *giganteus* microbiome. **A,** The first eleven most abundant phyla across all samples. **B,** Classes associated with Proteobacteria.

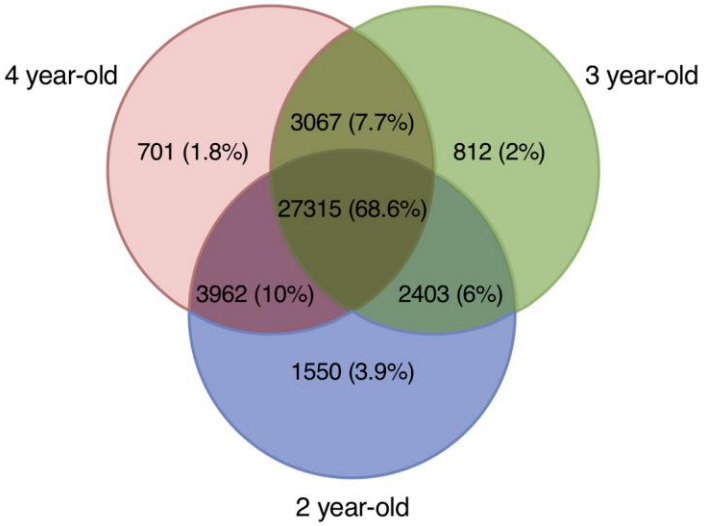

Fig. 2. Venn diagram of the total number (and percentile) of amplicon sequence variations (ASVs) associated with different stand ages of *M.* × *giganteus*.

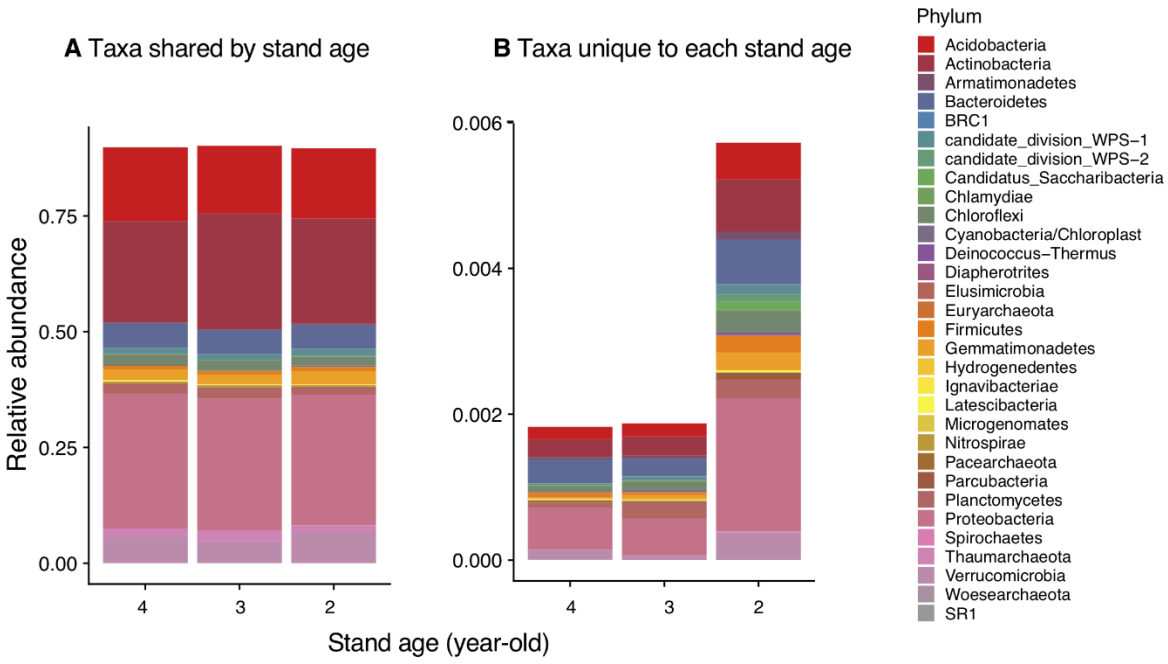

Fig. 3. A, Taxonomic distribution of the ASVs shared by soils from each stand age of *M.* × *giganteus* as measured during the summer growing season in 2018. **B,** Taxonomic distribution of the ASVs unique to each stand age of *M.* × *giganteus*.

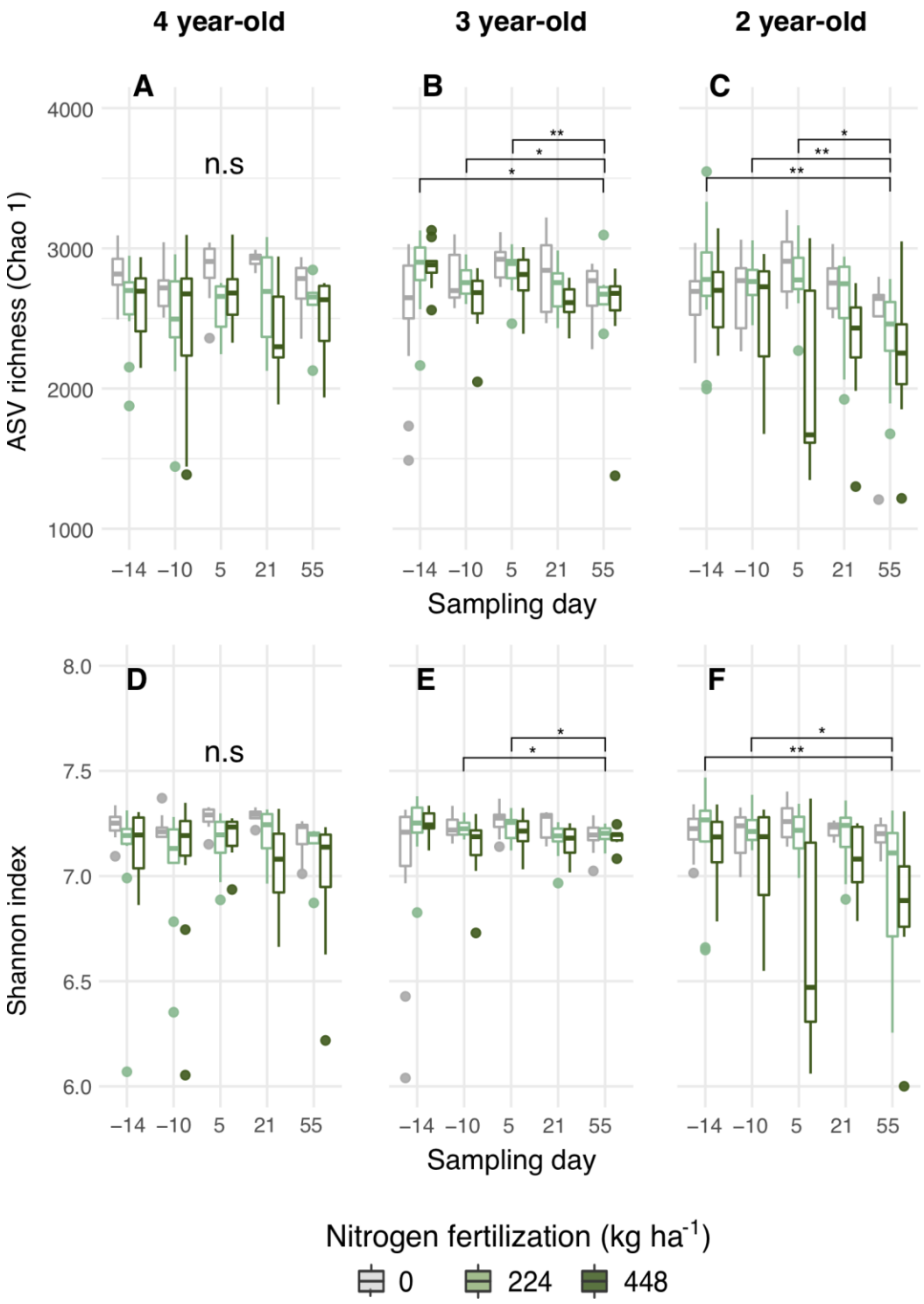

Fig. 4. Short-term effect of N fertilization on the alpha diversity of soil bacterial communities in *M.* × *giganteus* planting areas by stand ages. Chao 1 estimated richness for **A,** four- **B,** three- and **C,** two-year old and Shannon diversity estimates for **D,** four- **E,** three- and **F,** two-year old stand ages shown for varying fertilization rates and over the season. Sampling days are reported relative to the day of fertilizer application. Asterisks indicate significant differences between sampling days, where significance is denoted as * $P < 0.05$, ** $P < 0.01$, and n.s. = not significant.

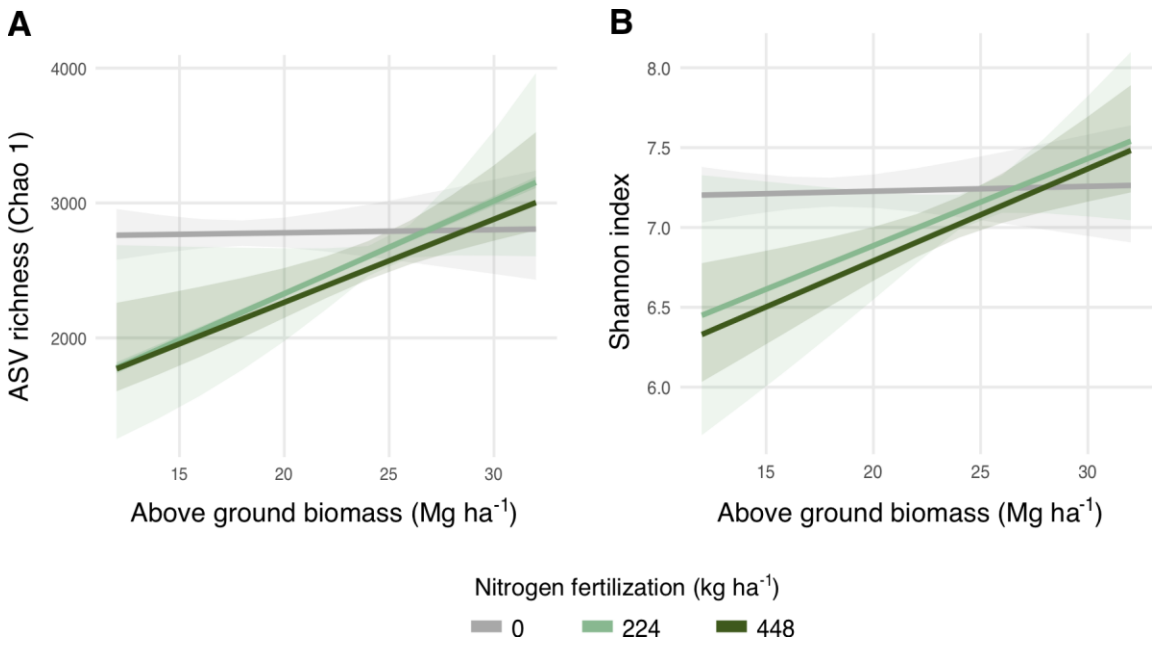

Fig. 5. Alpha-diversity estimates of **A,** richness by estimated Chao 1 and **B,** Shannon diversity of bacterial communities predicted by the interaction between N fertilization and *M.* × *giganteus* above-ground biomass. Estimates are from predicted marginal effects.