Response of total (DNA) and metabolically active (RNA) microbial communities in *Miscanthus* × *giganteus* cultivated soil to different nitrogen fertilization rates

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Running Title: Comparing DNA and RNA approaches in soil microbiomes

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Abstract

Miscanthus x *giganteus* is a promising high-yielding perennial plant to meet growing bioenergy demands but the degree to which the soil microbiome affects its nitrogen cycling and subsequently, biomass yield remains unclear. In this study, we hypothesize that contributions of metabolically active soil microbial membership may be underestimated with DNA-based approaches. We assessed the response of the soil microbiome to nitrogen availability in terms of both DNA and RNA soil microbial communities from the Long-term Assessment of Miscanthus Productivity and Sustainability (LAMPS) field trial. DNA and RNA were extracted from 271 samples, and 16S SSU rRNA amplicon sequencing was performed to characterize microbial community structure. Significant differences were observed in the resulting soil microbiomes and were best explained by the sequencing library of origin, either DNA and RNA. Similar numbers of taxa were detected in DNA and RNA microbial communities, with more than 90% of taxa shared. However, the profile of dominant taxa within DNA and RNA differed, with varying proportions of *Actinobacteria* and *Proteobacteria* and *Firmicutes* and *Proteobacteria*. Only RNA microbial communities showed seasonal responses to nitrogen fertilization, and these differences were associated with nitrogen-cycling bacteria. The relative abundance of bacteria associated with nitrogen cycling was 7-folds higher in RNA than in DNA, and genes associated with denitrifying bacteria were significantly enriched in RNA, suggesting that these bacteria may be underestimated with DNA-only approaches. Our findings indicate that RNA-based SSU characterization can be a significant and complementing resource for understanding the role of soil microbiomes in bioenergy crop production.

Importance

Miscanthus x *giganteus* is becoming a cornerstone of bioeconomy cropping systems, but it remains unclear how the soil microbiome supplies nitrogen to this low-input crop. DNA-based techniques are used to provide community characterization but may miss important metabolically active taxa. By analyzing both DNA- and actively transcribed RNA-based microbial communities, we found that nitrogen cycling taxa in the soil microbiome may be underestimated using only DNA-based approaches. Accurately understanding the role of microbes and how they cycle nutrients is important for the development of sustainable bioenergy crops, and RNA-based approaches are recommended as a complement to DNA approaches to better understand the microbial, plant, and management interactions.

Introduction

The sterile allopolyploid (2n=3x=57) *Miscanthus* × *giganteus* (Greef et Deu.) is a promising perennial grass bioenergy crop because of its ability to produce large amounts of biomass with little fertilizer compared to hay or grain crops (1–4). The peak biomass production of *M.* x *giganteus* has been observed to be up to three times higher than switchgrass (*Panicum virgatum* L. cv. Cave-in-Rock), similar to willow (*Salix schwerinii* E. Wolf × viminalis L.), three times higher than reed canary grass (*Phalaris arundinacea* L.), and two times higher than triticale (*Triticosecale* Wittmack) (5–7). Additionally, *M.* x *giganteus* production has been shown to have decreased environmental impact, with decreased requirements of nitrogen and pesticides (8, 9) and reduced nitrate leaching relative to other bioenergy crops (10, 11). These advantages of *M.* x *giganteus* and its ability to maintain high productivity for up to 20 years compared to other energy crops have contributed to its increased cultivation (9, 12–14).

To support its growth, environmental and management factors that can affect the productivity of *M.* x *giganteus* have been evaluated. Previously, *M.* x *giganteus* has been observed to decrease in productivity at low temperatures (15, 16). It has also been observed to have relatively high water demand (17, 18) and to require cultivation for at least three years to obtain adequate yield (16, 19–25). Recommendations for nitrogen fertilization of *M.* x *giganteus* are inconsistent, with previous studies showing that fertilization can have little to no effect (26–31) or contribute to its productivity (32–35).

Previously, it has been estimated that *M.* x *giganteus* can obtain 16% of its nitrogen demand from the atmosphere during the growing season (36). Nitrogen can also be provided by the activity of nitrogen-fixing bacteria in the rhizobiome of *M.* x *giganteus* (37), which are enriched early after *M.* x *giganteus* planting (36). Nitrogen fixation genes have been observed to be more abundant in

M. x *giganteus* relative to other energy crops planted in similar soils (38, 39). Specific phyla which have been identified in *M.* x *giganteus* rhizobiomes include *Actinobacteria* and *Proteobacteria*, which include known nitrogen-fixing families such as *Hyphomicrobiaceae*, *Bradyrhizobiaceae*, *Rhodospirillaceae*, and *Geobacteraceae* (40).

To date, all studies of *M.* x *giganteus* soil microbial communities and their response to fertilization or biomass production have been limited to the characterization of soil environmental DNA. We previously used sequencing of 16S rRNA genes in DNA to identify significant interactions between microbial diversity, stand age, fertilization, and above-ground biomass in *M.* x *giganteus* (41). However, it is possible that DNA-based analysis may underestimate the number of active taxa, resulting in biased interpretations of how microbial communities respond to the environment (42, 43). By contrast, RNA-based characterization of microbial communities, representing metabolically active or transcribed genes, can better relate community responses to environmental variability (44–48). Additionally, RNA-based studies are more sensitive and have detected underrepresented active bacteria that are below the amplification threshold of DNA-based approaches. Despite the advantages of RNA-based methods, direct comparison of the DNA and RNA methods for microbial community characterization in bioenergy crops soil microbial communities is sparse. One previous study of the bioenergy grass, *Pennisetum purpure*, compared bacterial communities of DNA- and RNA-based denaturing gradient gel electrophoresis (DGGE) profiles and clone libraries and found that RNA-based methods could identify enriched metabolically active membership (49).

In this study, we perform comparison of DNA and RNA approaches to help us better understand how soil microbiome in field-grown *M.* x *giganteus* can inform management and environmental impacts of *M.* x *giganteus* production. We evaluate the effects of stand age

(representing different initial growth environments) and fertilization (representing different N availability) on changes in microbial community membership and structure. We hypothesize that microbiome responses (as indicated by DNA and RNA) to *M.* x *giganteus* management will differ and specifically that metabolically active (RNA) microbial communities will show a more rapid and sensitive response to fertilization than total (DNA) microbial communities. To test these hypotheses, soil samples were collected from the Long-term Assessment of Miscanthus Productivity and Sustainability (LAMPS) site, a replicated chronosequence field previously used to investigate the effects of stand age and nitrogen fertilizer on *M.* x *giganteus* and corn (*Zea mays* L.) (28, 50). DNA and RNA were extracted from these soil samples, and we compared these microbial responses to stand age, N fertilization amount, and time since fertilization.

Material and Methods

Sample description

Soil samples were collected in 2018 from the Long-term Assessment of Miscanthus Productivity and Sustainability (LAMPS) site located in Central IA, USA (42.013° N, 93.743° W). This staggered-start experiment was planted with *M.* x *giganteus* (clone "Freedom", AGgrow Tech, High Point NC, USA) at a density of ~11 plants m^{-2} in replicated blocks (n=4) in each of 2015, 2016 and 2017 as described previously (28). The experimental design is a split-plot replicated block with age (planting year) as the main plot and N fertilization rate as the split plot. Soils at the site are deep loams (>1m) formed over glacial till; the dominant soil type (53%) is a Webster clay loam (fine-loamy, mixed, superactive, mesic Typic Endoaquoll). Fertilizer was applied as banded urea ammonium nitrate (UAN) in aqueous solution and side-dressed into the soil at 0.1 m depth on May 9, 2018, at rates of 0, 224, and 448 kg ha^{-1} N. Soil samples were taken on April 30, May

14, May 30, and July 3. Soils were collected from within 10 cm radius of the *M.* x *giganteus* stems using a sampling core (30.5 cm wet sample tube with 1.75cm diameter, Clements Associates Inc, USA). Soil samples included in this analysis were obtained in triplicate from 60 experimental plots at each time point and analyzed independently. Samples for DNA extraction were stored on dry ice immediately after being taken as described previously (41), and samples for RNA extraction were immediately collected and then frozen in RNAlater (Thermo Fisher Scientific, USA) which offers the advantage of preserving microbial community integrity while preventing RNA degradation (51). All samples were stored in a cooler filled with dry ice during return to the laboratory.

DNA/RNA extraction and 16S rRNA gene amplicon sequencing

DNA and RNA extraction was performed from subsampled 0.25 g soil samples submerged in RNAlater (Thermo Fisher Scientific, USA), using the MagAttract PowerMicrobiome DNA/RNA EP Kit (Qiagen, USA) following the standard protocol in this kit and liquid handling in Eppendorf epMotion 5075 (Eppendorf North America). The extracted RNA was transcribed into cDNA according to a standard protocol using iScript™ cDNA Synthesis Kit (BIO-RAD, USA) for sequencing analysis. The resulting DNA and RNA were analyzed for quantity using an Invitrogen™ Qubit™ 4 Fluorometer (Invitrogen, USA). DNA and RNA sample concentrations above 10 ng ul^{-1} were normalized to 10 ng ul^{-1} prior to sequencing. Samples with concentrations lower than 10 ng ul^{-1} were submitted directly for sequencing. The V4 region of the bacterial 16S rRNA gene was amplified with the conserved primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′) (52, 53). Bacterial amplicon sequencing was performed on Illumina Miseq with Miseq Reagent Kit V2 (Illumina, USA) at Argonne

National Laboratory. The DNA and RNA sequencing data are available at NCBI Short Read Archive PRJNA601860 and PRJNA745191, respectively.

Amplicon bioinformatics and statistical analysis

The *DADA2* package (version 1.13.1) in R (version 4.1.0) was used to perform quality control of sequencing libraries and to determine the abundance of amplicon sequence variants (ASV). The quality filtering parameters for all sequences were the same as previously described for DNA amplicons (41). The Ribosomal Database Project (RDP) Classifier (version 11.5) was used for taxonomic identification of each observed ASV depending on the sequence similarity to the representatives in the current database. ASVs were removed if no more than 10 total observations were observed in a sample. All statistical analyses were performed in R (version 4.1.0). Two diversity indices, Chao1 and Shannon, were used to compare the alpha diversity of bacteria using the *vegan* package (version 2.5 - 7). Multivariate homogeneity of group dispersions, calculating the average distance of members to the centroid of the group, was used to analyze the dispersion of each sample using *betadisper* function from the *vegan* package (version 2.5 - 7). Significant differences in alpha diversity and homogeneity between DNA and RNA microbial communities were evaluated using the Kruskal-Wallis test with Dunn's post hoc test. Permutational multivariate analysis of variance (PERMANOVA) was performed with the *adonis* function of the *vegan* package using the Bray-Curtis dissimilarity matrix (version 2.5 - 7). PERMANOVA was performed to identify significant differences between centroids of each microbial community, and the R^2 statistic represents the proportion of the variance for the separation of the microbial community that was explained by experimental and field environmental factors (i.e., origin of the sequencing library, stand age, N fertilization amount,

fertilization history, and time since fertilization). PERMANOVA was performed using the "strata" argument for the planted block, which was identified as one of the major factors to structure the microbial composition in the previous study, to better identify the effects of stand age and N fertilization amount, fertilization history, and time since fertilization. This analysis restricted permutations to the dataset within each block and was used to quantify variations between and within treatments (41). The comparison between the two groups within the three (stand age, N fertilization amount) or four (time since fertilization) groups was accomplished using pairwise PERMANOVA. The level of significance in the statistical analysis was defined as $p < 0.05$.

Results

DNA and RNA microbial communities differ in microbial composition and alpha diversity

The 16S rRNA amplicons from DNA and RNA from soil samples representing three ages and three fertilization rates of *M* x *giganteus* were compared. The origin of the sequencing library, either DNA or RNA, was found to have the greatest influence on the separation of the microbial community ($R^2_{PERMANOVA} = 0.117$, $p_{PERMANOVA} = 0.001$, Table S1). Their influence was 3.9 times higher than that of stand age ($R^2_{PERMANOVA} = 0.030$, $p_{PERMANOVA} = 0.001$) and 15 times higher than that of N fertilization amount ($R^2_{PERMANOVA} = 0.008$, $p_{PERMANOVA} = 0.001$). DNA and RNA microbial communities were observed to separate into clear clusters using constrained analysis of principal coordinates (CAP) along the first axis of the CAP (CAP1, 11.8%, $F = 77.56$, $p_{ANOVA} = 0.001$, Figure 1). The microbial composition (ADONIS, $p_{ADONIS} = 0.001$) and homogeneity (betadisper, $p_{betadisper} = 0.001$) of DNA and RNA communities were also observed to be significantly different.

192 Table S1. Comparison of microbial community dissimilarity using permutational multivariate
193 analysis of variance (PERMANOVA) for bacterial communities in *M.* x *giganteus* soil samples.
194
195 Figure 1. Similarities, assessed with Bray-Curtis indices, between DNA and RNA microbial
196 communities from *M.* x *giganteus* soils. Constrained analysis of principal coordinates (CAP) was
197 used to ordinate Bray-Curtis indices calculated with ASVs. Blue dot and red triangle represent the
198 DNA and RNA microbial communities, respectively.
199
200 Alpha diversity of soil microbial communities was compared using the Shannon index,
201 which evaluates both microbial richness and evenness, and Chao1, which evaluates the abundance
202 of observed species. Both alpha diversity indices showed significant differences between DNA
203 and RNA microbial communities, with higher alpha diversity observed in DNA microbial
204 communities ($p_{Shannon} < 0.001$, $p_{Chao1} < 0.001$, Figure 2). On average 32% and 12% higher Chao1
205 and Shannon indices, respectively, were observed in DNA compared to RNA microbial
206 communities. The average value of alpha diversity was higher in DNA, but the variation in alpha
207 diversity indices was larger between RNA samples. Specifically, the DNA Chao1 index was in the
208 range of 1,667 to 9,170, and RNA was associated with a much wider range of 195 to 9,343. Similar
209 results were observed with Shannon indices, with DNA ranging from 4.88 to 7.85 and RNA from
210 2.69 to 7.72.
211
212 Figure 2. Alpha diversity indices of DNA and RNA microbial communities. Richness ((A) Chao1,
213 (B) Shannon index) were estimated for microbial communities with ASVs. Letters "***" denote

214 significant differences of alpha diversity indices between DNA and RNA microbial communities
215 at a p-value < 0.05 as assess by Kruskal-Wallis with post hoc Dunn's test.
216
217 **Taxa distributions varied between DNA and RNA microbial communities**
218 The total number of taxa in DNA and RNA microbial communities was estimated by
219 observations of ASVs, where a total of 39,898 and 32,171 ASVs were identified in DNA and RNA,
220 respectively. We compared the ASVs between DNA and RNA samples and found that 17,779
221 ASVs were shared between DNA and RNA microbial communities (32% and 58%, respectively);
222 22,119 and 14,392 ASVs were unique in DNA and RNA, respectively. Unique ASVs were
223 generally low abundance (average < 0.000003%) and low prevalence (average < 0.022%) in their
224 respective libraries (Figure 3). ASVs that were identified in both DNA and RNA were found to be
225 identified at increased though still low abundance (average < 0.00005%) and higher prevalence
226 (average > 0.16%).
227 ASVs commonly identified between DNA and RNA libraries were further classified based
228 on their enrichment in DNA or RNA, specifically using the ratio of RNA:DNA relative abundances.
229 The RNA:DNA ratio of shared ASVs ranged from 0.0023 to 1,300. The majority of shared ASVs
230 (58%) were more enriched in RNA relative to DNA (Figure 4). For ASVs enriched in DNA
231 (RNA:DNA ratio < 1), the average RNA:DNA ratio was 0.44; the average RNA:DNA ratio for
232 ASVs enriched in RNA (RNA:DNA ratio > 1) was 4.82. Additionally, more variation was
233 observed in shared ASVs which were enriched in RNA relative to those enriched in DNA.
234
235 Figure 3. Abundance-occupancy comparison of ASVs in the DNA and RNA microbial
236 communities. Abundance-occupancy distributions were assessed to identify the dynamics of the

237 DNA and RNA microbial community memberships. Each point is an ASV. The ASVs were
238 classified as (A) unique in DNA or (B) unique in RNA, respectively, when it was detected only in
239 the DNA or RNA microbial communities. ASVs detected in both DNA and RNA microbial
240 communities were classified as "shared" and further classified by the average relative abundance
241 based on its enrichment in (C) DNA or (D) RNA.
242
243 Figure 4. RNA/DNA ratio comparison of the shared ASVs. The ratio of average relative abundance
244 in DNA and RNA microbial communities of ASVs detected in both microbial communities was
245 compared to identify the biased in the DNA and RNA-based microbial community analysis results.
246 Shared - higher in DNA (blue) and Shared - higher in RNA (red).
247
248 **Phylogenetic composition varied between DNA and RNA microbial communities**
249 The phylogenetic composition of DNA and RNA microbial communities was compared,
250 with 20 phyla identified in both libraries. Soils were dominated by *Actinobacteria* (26%) and
251 *Proteobacteria* (33%) in DNA and mainly *Proteobacteria* (49%) in RNA (Figure 5). While DNA
252 and RNA had similar membership at the phylum-level, the relative abundance of every phylum
253 significantly differed (Table S2). Thirteen out of 20 phyla were more enriched in DNA than RNA,
254 and seven phyla were more enriched in the RNA microbial communities.
255 We evaluated whether the phyla observed to be significantly different between DNA and
256 RNA were comprised of ASVs unique to DNA or RNA or shared between the two methods (Figure
257 S1). ASV shared by DNA and RNA microbial communities showed more pronounced variations
258 in microbial community structures differences. *Actinobacteria* and *Bacteroidetes* were more
259 enriched in DNA ($p_{Kruskal\text{-}Wallis} < 0.05$), while *Firmicutes* and *Proteobacteria* were more enriched

in the RNA microbial community ($p_{Kruskal\text{-}Wallis} < 0.05$). Differentiating ASVs unique in DNA included sequences associated with *Actinobacteria*, *Gemmatimonadetes*, *Latescibacteria*, and *Parcubacteria*; in contrast, sequences associated with *Firmicutes* were unique in RNA.

Figure 5. Phylum level differences in DNA and RNA microbial communities. Relative abundances of annotated ASVs are shown, identified to their closest match in the RDP classifier. See Supplementary Table 2 for a different perspective on the dynamics of numerical relative abundance.

Table S2. Kruskal-Wallis with post hoc Dunn's test comparing the average relative abundances of phyla between DNA and RNA microbial communities.

Figure S1. Phylum level differences in DNA and RNA microbial communities based on unique ASVs in DNA or RNA only (A) or shared ASVS and their enrichment in either DNA or RNA (B). Relative abundances of annotated ASVs are shown, identified to their closest match in the RDP classifier.

DNA and RNA microbial community compositions were variably changed by stand age, N fertilization amount, and time since fertilization

Previously, the response of the soil microbial community at this site to plant stand age, fertilization history, and time since fertilization was studied based on DNA (41). In this study, subsets of these samples were studied to directly compare DNA and RNA 16S rRNA gene characterization. Based on DNA, community composition responded significantly to the stand age

283 and N fertilization amount. The community response based on RNA was similar, with the notable
284 exception that time since fertilization showed a significant effect only in RNA (Table 1). The effect
285 of stand age and N fertilization amount was generally larger in DNA than RNA, and time since
286 fertilization had a larger effect on the RNA microbial community.
287
288 Table 1. Permutational multivariate analysis of variance (PERMANOVA) for comparing DNA
289 and RNA microbial community dissimilarity.
290
291 Next, pairwise comparisons of DNA and RNA microbial communities between stand ages
292 were performed (pairwise PERMANOVA, Table S3). The stand ages of *M.* x *giganteus* included
293 were 2-, 3-, and 4-year-old, and the microbial community of each stand age was significantly
294 different based on both DNA and RNA microbial communities ($p_{pairwisePERMANOVA} < 0.05$). Similar
295 patterns were observed for the response to N fertilization amount in both libraries, and both DNA
296 and RNA microbial communities were found to have different microbial community compositions
297 for three varying N fertilization amount ($p_{pairwisePERMANOVA} < 0.05$). Pairwise comparison of DNA
298 and RNA based on sampling day or the time since fertilization resulted in no significant differences
299 observed in DNA, but significant differences between pre-fertilization (10 days before fertilization)
300 and 69 days since fertilization in RNA (Table S4, $p_{pairwisePERMANOVA} < 0.05$).
301
302 Table S3. Pairwise permutational multivariate analysis of variance (PERMANOVA) for
303 comparing the effect of stand age and fertilization on the DNA and RNA microbial community
304 dissimilarity.
305

306 Table S4. Pairwise permutational multivariate analysis of variance (PERMANOVA) for
307 comparing the effect of time since fertilization on the DNA and RNA microbial community
308 dissimilarity.
309
310 Stand age was consistently observed to explain the most variation between experimental
311 factors, regardless of DNA or RNA methods (Table 1). We next evaluated if the specific phyla
312 found to be different between stand ages was consistent between DNA and RNA microbial
313 communities. A total of 20 identical phyla were detected in both sequencing libraries. Every
314 phylum showed significant relative abundance differences between DNA and RNA ($p_{Kruskal\text{-}Wallis}$
315 < 0.05). The dominant phyla differed between DNA and RNA (Figure 6), with *Acidobacteria*
316 (>17%), *Actinobacteria* (>24%), and *Proteobacteria* (>32%) dominant in DNA, and *Firmicutes*
317 (>12%) and *Proteobacteria* (>43%) in RNA. We subsequently selected these phyla to evaluate
318 genera level differences between DNA and RNA methods.
319
320 Figure 6. Phylum level differences in DNA and RNA microbial communities according to stand
321 age differences. Relative abundances of annotated ASVs are shown, identified to their closest
322 match in the RDP classifier. The letters "***" denote significant differences of relative abundance
323 between different stand ages of *M.* x *giganteus* at a p-value < 0.05 as assessed by Kruskal-Wallis
324 with post hoc Dunn's test.
325
326 A total of 569 genera were detected among *Actinobacteria*, *Proteobacteria,* and *Firmicutes*
327 and 308, 316, and 337 genera in 2-, 3-, and 4-year-old *M.* x *giganteus*, respectively, showed
328 significant differences between the DNA and RNA microbial communities. We selected the genera

329 with greater than 0.1% relative abundance and compared differences between taxonomic profiles
330 in DNA and RNA (Figure S2). Sequences associated with *Bacillus*, *Clostridium*, *Paenibacillus*,
331 *Sporosarcina* of *Firmicutes* and *Bradyrhizobium*, *Methyloversatilis*, *Nitrosomonas*, *Nitrosospira*,
332 and *Steroidobacter* of *Proteobacteria* were more enriched in RNA than in DNA. On the other hand,
333 *Gaiella* and *Solirubrobacter* of *Actinobacteria* were more enriched in DNA.
334
335 Figure S2. Comparison of dominant genus (> 0.1% relative abundance) in *Actinobacteria*,
336 *Firmicutes*, and *Proteobacteria* between DNA and RNA microbial communities. (A) 4-year-old
337 *M.* x *giganteus*, 73 genera. (B) 3-year-old *M.* x *giganteus*, 77 genera. (C) 2-year-old *M.* x *giganteus*,
338 74 genera. All genera included this analysis were significantly different between DNA and RNA
339 microbial communities ($p_{Kruskal\text{-}Wallis} < 0.05$).
340
341 Differences in response to fertilization were also observed between DNA and RNA
342 microbial communities. Both DNA- and RNA-based methods identified that soil microbial
343 communities showed different responses to N fertilization amount (Table 1, Table S3), though the
344 phylogenetic profile observed under fertilized conditions differed based on the two methods
345 (Figure S3). Overall, a greater number of phyla in RNA relative to DNA were significantly affected
346 by differences in N fertilization amount (Table S5). Seven phyla in RNA (*Actinobacteria*,
347 *Firmicutes*, *Gemmatimonadetes*, *Hydrogenedentes*, *Latescibacteria*, *Nitrospirae*, and
348 *Proteobacteria*) showed significant differences between N fertilization amount differences
349 compared to four phyla in DNA (*Acidobacteria*, *Chloroflexi*, *Latescibacteria*, and *Proteobacteria*).
350 *Actinobacteria* (>26%) was more enriched in DNA microbial communities ($p_{Kruskal\text{-}Wallis} < 0.05$),

and *Firmicutes* (>12%) and *Proteobacteria* (>47%) were significantly more enriched in RNA microbial communities ($p_{Kruskal\text{-}Wallis} < 0.05$).

Figure S3. Phylum level differences in DNA and RNA microbial communities according to N fertilization amount differences. Relative abundances of annotated ASVs are shown, identified to their closest match in the RDP classifier. The letters "***" denote significant differences of relative abundance between different stand age of *M.* x *giganteus* at a p-value < 0.05 as assess by Kruskal-Wallis with post hoc Dunn's test.

Table S5. Kruskal-Wallis with post hoc Dunn's test comparing the average relative abundances of phyla between DNA and RNA microbial communities by different amounts of N fertilization. Denote N0, N224, and N448 are N fertilization amount of 0 kg N ha^{-1}, 224 kg N ha^{-1}, and 448 kg N ha^{-1}, respectively.

Genus-level analysis was performed on the *Actinobacteria*, *Firmicutes*, and *Proteobacteria* and among the 569 genera detected, 330, 323, and 309 genera showed significant differences between DNA and RNA microbial communities at N fertilization amount of 0, 224, and 448 kg N ha^{-1}, respectively (Figure S4). Sequences associated with *Bacillus*, *Clostridium*, *Paenibacillus*, *Sporosarcina* of *Firmicutes* and *Bradyrhizobium*, *Methyloversatilis*, and *Nitrosomonas* of *Proteobacteria* more enriched in RNA than DNA. On the other hand, *Gaiella* from *Actinobacteria* and *Sphingomonas* of *Proteobacteria* were more abundant in DNA.

Figure S4. The dynamics of the 88 major genus (> 0.1% relative abundance) in the *Actinobacteria*, *Firmicutes*, and *Proteobacteria* between DNA and RNA microbial communities. (A) 0 kg N ha^{-1} of fertilizer applied *M.* x *giganteus* soil including 79 genera. (B) 224 kg N ha^{-1} of fertilizer applied *M.* x *giganteus* soil including 76 genera. (C) 448 kg N ha^{-1} of fertilizer applied *M.* x *giganteus* soil including 74 genera. All genera included this analysis were significantly different between DNA and RNA microbial communities ($p_{Kruskal\text{-}Wallis} < 0.05$).

Taxa associated with nitrogen cycle-related bacteria showed a short-term response since fertilization only in RNA microbial communities.

In comparing pre- and post-fertilization soil samples, differences in soil microbial communities were observed only in RNA libraries (Table 1, Table S4). Taxa that were significantly different before and since fertilization were associated with 10 phyla (Figure 7). Additionally, these differences were only observed 69 days since fertilization, where the relative abundances of *Acidobacteria*, *Armatimonadetes*, *Firmicutes*, and *Planctomycetes* were increased compared to before fertilization, and the relative abundances of *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, and *Latescibacteria* decreased.

Figure 7. Phylum-level responses to time since fertilization in RNA microbial communities. The average relative abundances of phyla over time since fertilization were summarized. Letters "***" denote significant differences of relative abundance between pre- and 69 days since fertilization at a p-value < 0.05 as assess by Kruskal-Wallis with post hoc Dunn's test.

The most enrichment since fertilization was observed in the *Firmicutes*, in which relative abundance was increased 10 folds, and *Planctomycetes* also increased by about 1.7 folds. These phyla are notable because they are known to contain known nitrogen cycling taxa (54, 55). To better explore the response to fertilization of nitrogen cycling taxa, we obtained taxa that are associated with nitrogen fixation, nitrification, and denitrification from the Fungene database. These taxa include 51 genera associated with *Firmicutes*, *Nitrospirae*, and *Planctomycetes*. We compared the differences of these genera between DNA and RNA libraries.

Overall, the total relative abundance of these genera comprised 1.18% and 8.51% in the DNA and RNA microbial communities, respectively (Figure 8). The large majority of these genera (with the exception of four genera) showed significant differences between DNA and RNA, and among them, *Bacillus, Paenibacillus*, and *Sporosarcina* were the most abundant (> 20%) in the RNA microbial community.

Figure 8. Comparison of nitrogen cycling-related bacteria in the DNA and RNA microbial communities. The average relative abundances of genus associated with nitrogen fixation, nitrification, and denitrification were summarized.

The taxa that showed distinct responses in RNA compared to DNA were classified by their known nitrogen cycling functions (excluding taxa with multiple functional annotations). Only taxa associated with denitrification in the RNA microbial communities showed a significant difference (Figure 9, Table S6) between pre- and post-fertilization, consistent with the observation that denitrifying bacteria were consistently enriched since fertilization (56).

Figure 9. Comparison of nitrogen cycling-related bacteria in the DNA and RNA microbial communities according to time since fertilization. The average relative abundances of bacteria associated with nitrogen fixation, nitrification, and denitrification function were summarized.

Table S6. Kruskal-Wallis with post hoc Dunn's test comparing the average relative abundances of nitrogen cycling functions in RNA microbial communities by time since fertilization.

Discussion

In direct comparisons of *M.* x *giganteus* soil microbiomes from DNA and RNA extractions, we found that the most significant factor in explaining variation between microbiomes was its sequencing library of origin, even more so than experimental factors of stand age, N fertilization amount, or sampling day (Table S1). DNA and RNA microbiomes also had significantly different alpha diversity, with increased diversity and less variation observed in DNA relative to RNA. These results are consistent with what is known about DNA and RNA. DNA represents the potential genes or membership that may be active and thus is expected to represent more diverse membership with the potential to become metabolically active. RNA, which is actively transcribed, represents growing members, and its higher variability is consistent with its dynamic responses. Previous studies have shown that the RNA microbial community may also have lower alpha diversity because it does not contain the sequences of dormant or dead cells and also has greater variability in response to the environment (47, 57–59).

Overall, the large majority of membership between DNA and RNA was shared (greater than 90%), suggesting that both methods identify the similar presence of taxa. The abundance of these shared taxa, however, could be significantly different between DNA and RNA, and most of

the shared taxa were more enriched in RNA. Based on the assumption that taxa observed in both methods are the most reliable, it is likely that DNA-based methods are underestimating the relative abundance of taxa. Further, these differences between DNA and RNA methods contributed to differences in estimated alpha diversity and varying observations of the microbial community response to plant host stand age and fertilization.

In response to both stand age and N fertilization amount, significant differences were observed in both DNA and RNA communities. While the overall pattern and ranking of differences were similar, the magnitude of this change and taxonomic membership driving these differences varied between DNA and RNA approaches. The most significant difference we observed in *M.* x *giganteus* soil microbial communities between the two library methods was in response to nitrogen fertilization. Only RNA microbial communities showed differences pre- and post-fertilization and only at day 69. RNA is able to show more rapid changes in response to changes in environmental conditions than DNA (60, 61), and here, we show the ability of RNA to capture a relatively short-term response over the course of one growing season in *M.* x *giganteus*, which is not observed in DNA. These results are consistent with RNA's short half-life of several minutes to several hours (62) and also justify its usage for measuring short-term seasonal responses in bioenergy soils. Our results showed that it was not until over two months that a response different to pre-fertilization conditions was observed in RNA, providing some insight into the metabolic response of soil microbes to fertilization in these soils.

Among the taxa which were found to be uniquely identified in RNA libraries were members associated with nitrogen-cycling, including members of *Firmicutes*, *Nitrospirae*, and *Planctomycetes* which were enriched with both the presence of fertilizer and increasing nitrogen fertilizer. These results are consistent with previous studies which have shown that *Firmicutes* are

465 enriched when nitrogen fertilizers are used (63–67). In the context of taxa associated with nitrogen
466 cycling, it was confirmed that the RNA-based approach could better detect the denitrification
467 function among the nitrogen cycling functions. This result is consistent with the results of previous
468 studies that the application of nitrogen fertilizers suppressed the activity of nitrogen-fixing bacteria
469 and enhanced denitrifying bacteria (56, 68). These results also emphasize that DNA may
470 underestimate or miss the contribution of nitrogen-cycling taxa, which are highly relevant for
471 nitrogen management in bioenergy systems. In addition to these taxa, we also found that members
472 of dominant soil phyla, *Actinobacteria* and *Proteobacteria* are underestimated using DNA
473 methods alone.

474 In summary, we found that DNA and RNA methods for characterizing the general response
475 of microbial communities varied. With relevance to developing sustainable bioenergy crops and
476 understanding the role of microbes in nutrient cycling, RNA appears to capture better the response
477 of taxa known to be involved in nitrogen cycling and is also more sensitive to seasonal shifts in
478 microbiomes. To better link microbial communities to ecosystem processes, we need to move
479 towards characterizing the functional response of microbial communities. Due to costs, the first
480 step in this characterization is often phylogenetic characterization of SSU genes based on DNA.
481 Our results indicate that this method alone may bias against the composition results of the relevant
482 microbial membership.

483 Notably, the integration of RNA-based methods into an experiment adds significant costs,
484 requiring materials to quickly preserve samples for RNA extraction and typically more time for
485 extraction and library preparation. RNA used for SSU characterization can be a complement to
486 DNA-based studies, as it leverages the advantages and throughput of indicator gene amplification
487 while not being as expensive as metatranscriptomics strategies. Based on our results, we

recommended that DNA can be used for the initial and broad characterization of community membership. The use of RNA for SSU characterization could be used to complement DNA characterization when experimental questions have been developed. In the context of our experiment, DNA-based analyses were used to validate that there was a significant response to stand age and fertilization. RNA-based analyses were more helpful in identifying the specific taxa that respond to fertilization. With these specific taxa now identified, future research will be focused on functional characterization, guided by the result of this study (e.g., microbial responses to fertilization responses are most significant two months since fertilization). More broadly, in our understanding of microbial ecology, increasing numbers of studies are identifying the environments or gradients for which microbial communities are changing. In future work, it will be necessary to emphasize which taxa or what functions are changing, and our results indicate that RNA-based SSU characterization may be a substantial resource.

Acknowledgments

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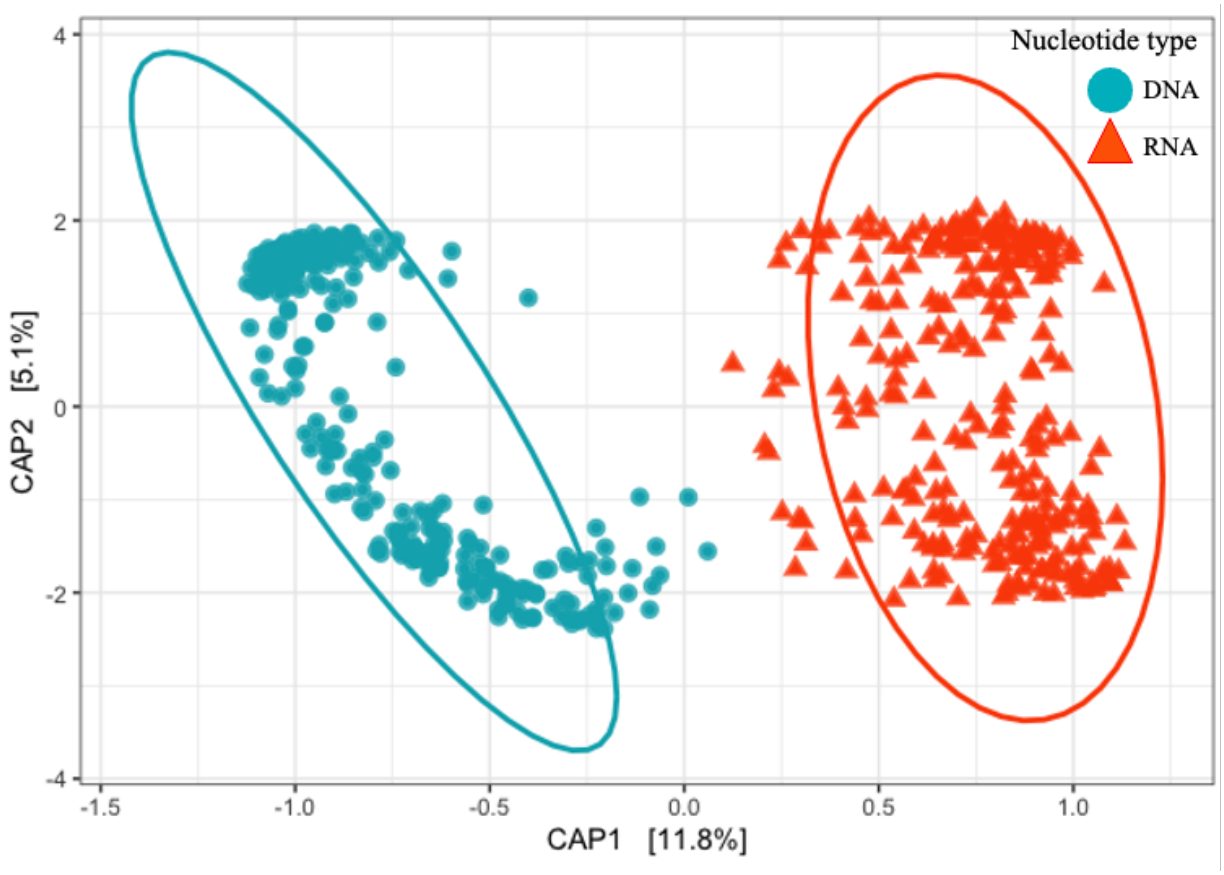


712

713 Figure 1. Similarities, assessed with Bray-Curtis indices, between DNA and RNA microbial
714 communities from *M.* x *giganteus* soils. Constrained analysis of principal coordinates (CAP) was
715 used to ordinate Bray-Curtis indices calculated with ASVs. Blue dot and red triangle represent the
716 DNA and RNA microbial communities, respectively.

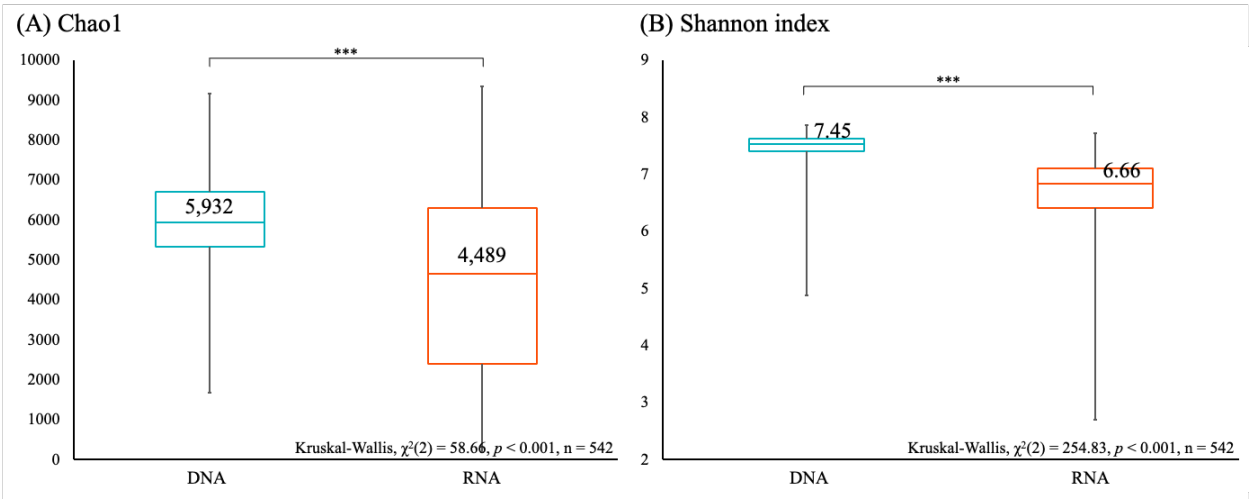


Figure 2. Alpha diversity indices of DNA and RNA microbial communities. Richness ((A) Chao1, (B) Shannon index) were estimated for microbial communities with ASVs. Letters "***" denote significant differences of alpha diversity indices between DNA and RNA microbial communities at a p-value < 0.05 as assess by Kruskal-Wallis with post hoc Dunn's test.

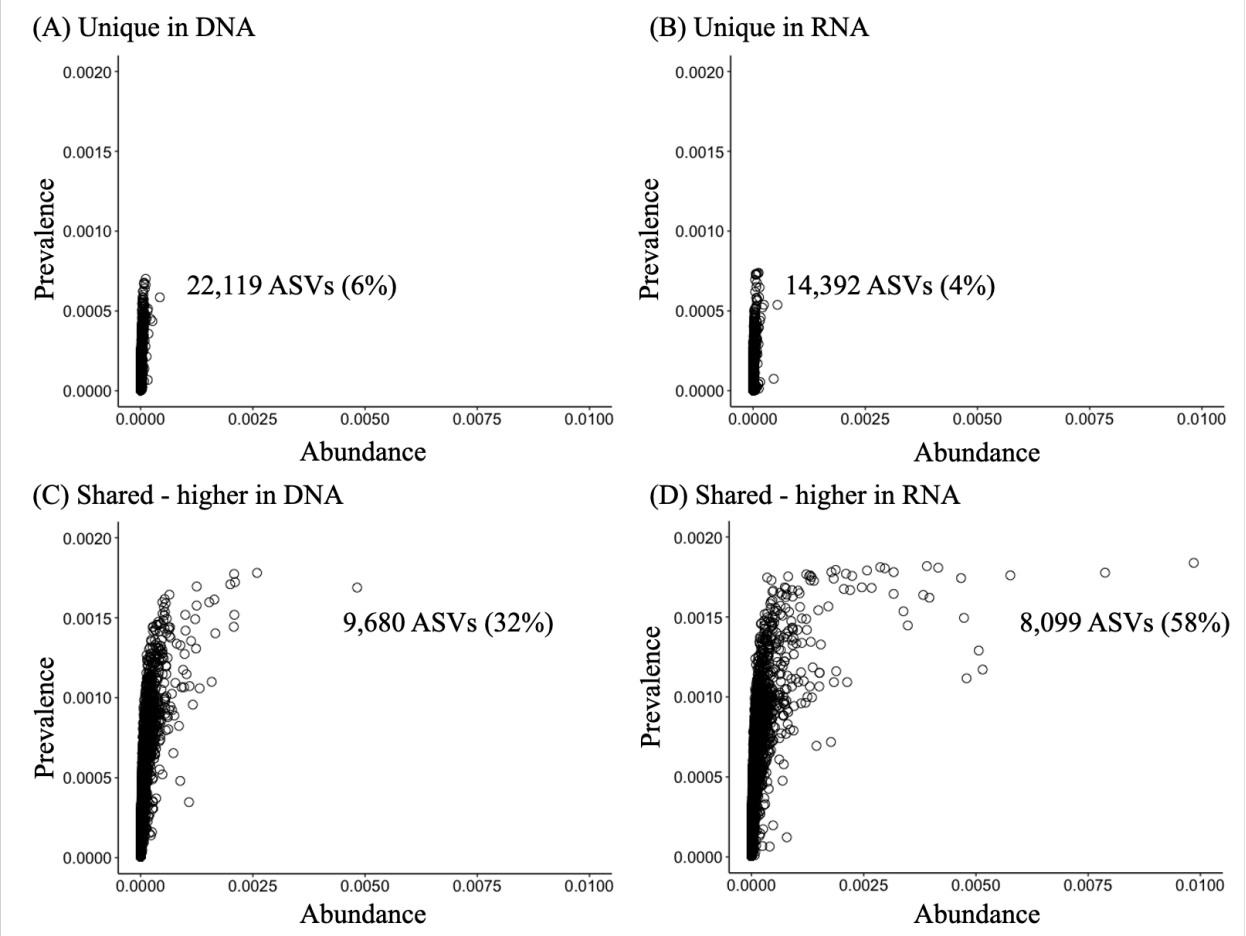


Figure 3. Abundance-occupancy comparison of ASVs in the DNA and RNA microbial communities. Abundance-occupancy distributions were assessed to identify the dynamics of the DNA and RNA microbial community memberships. Each point is an ASV. The ASVs were classified as (A) unique in DNA or (B) unique in RNA, respectively, when it was detected only in the DNA or RNA microbial communities. ASVs detected in both DNA and RNA microbial communities were classified as "shared" and further classified by the average relative abundance based on its enrichment in (C) DNA or (D) RNA.

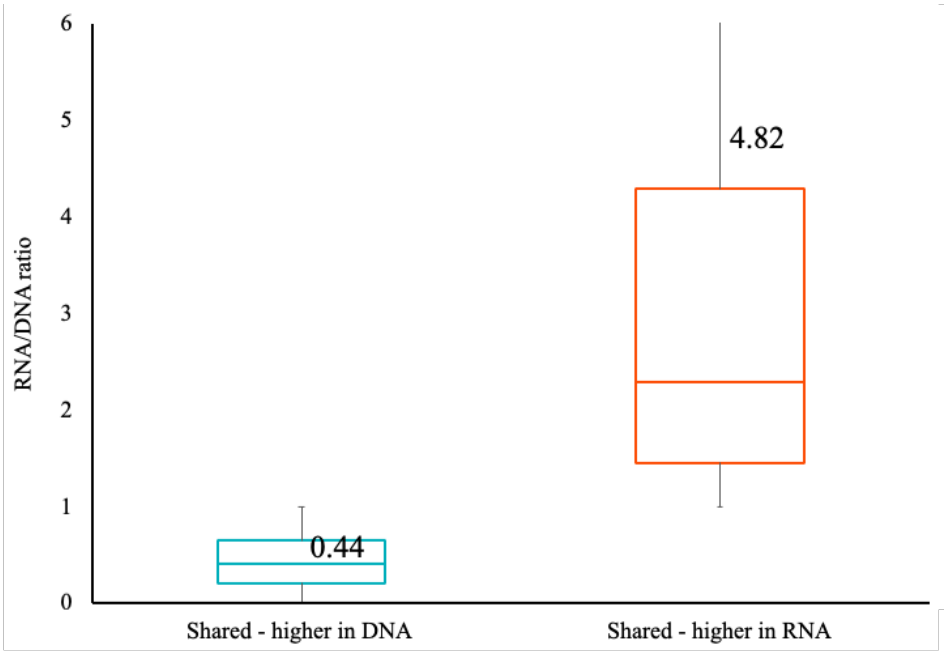


Figure 4. RNA/DNA ratio comparison of the shared ASVs. The ratio of average relative abundance in DNA and RNA microbial communities of ASVs detected in both microbial communities was compared to identify the biased in the DNA and RNA-based microbial community analysis results. Shared - higher in DNA (blue) and Shared - higher in RNA (red).

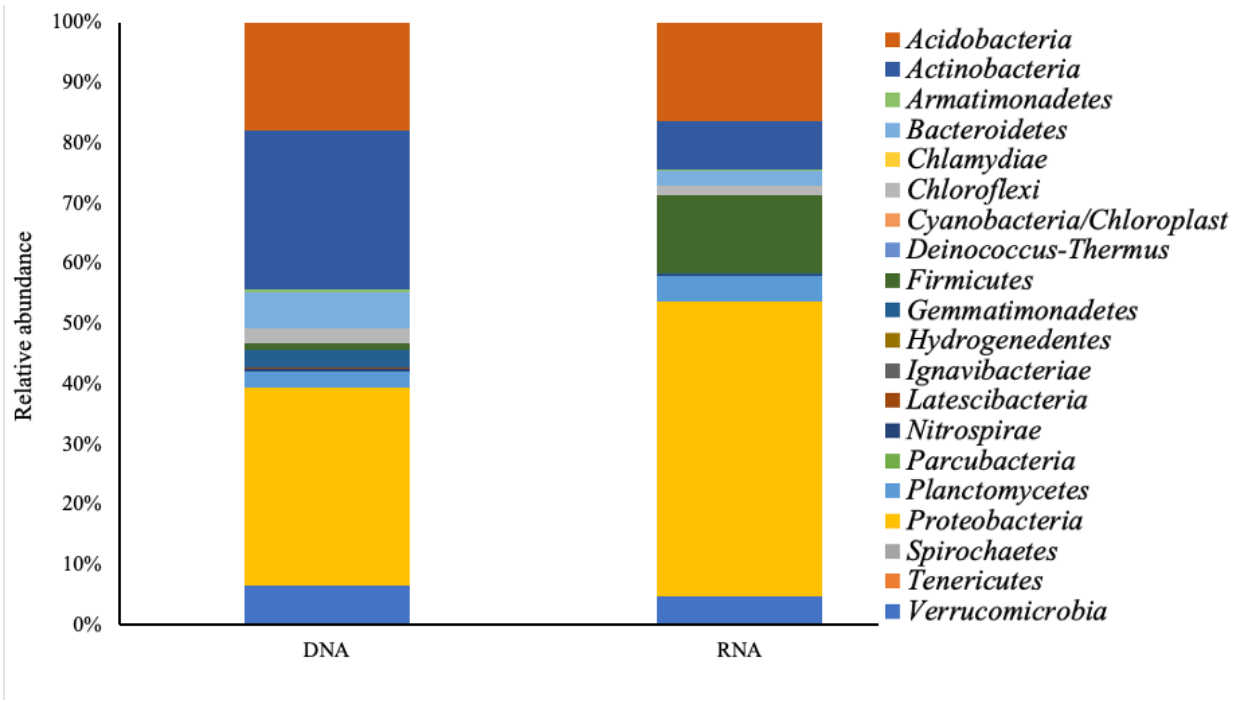


Figure 5. Phylum level differences in DNA and RNA microbial communities. Relative abundances of annotated ASVs are shown, identified to their closest match in the RDP classifier. See Supplementary Table 2 for a different perspective on the dynamics of numerical relative abundance.

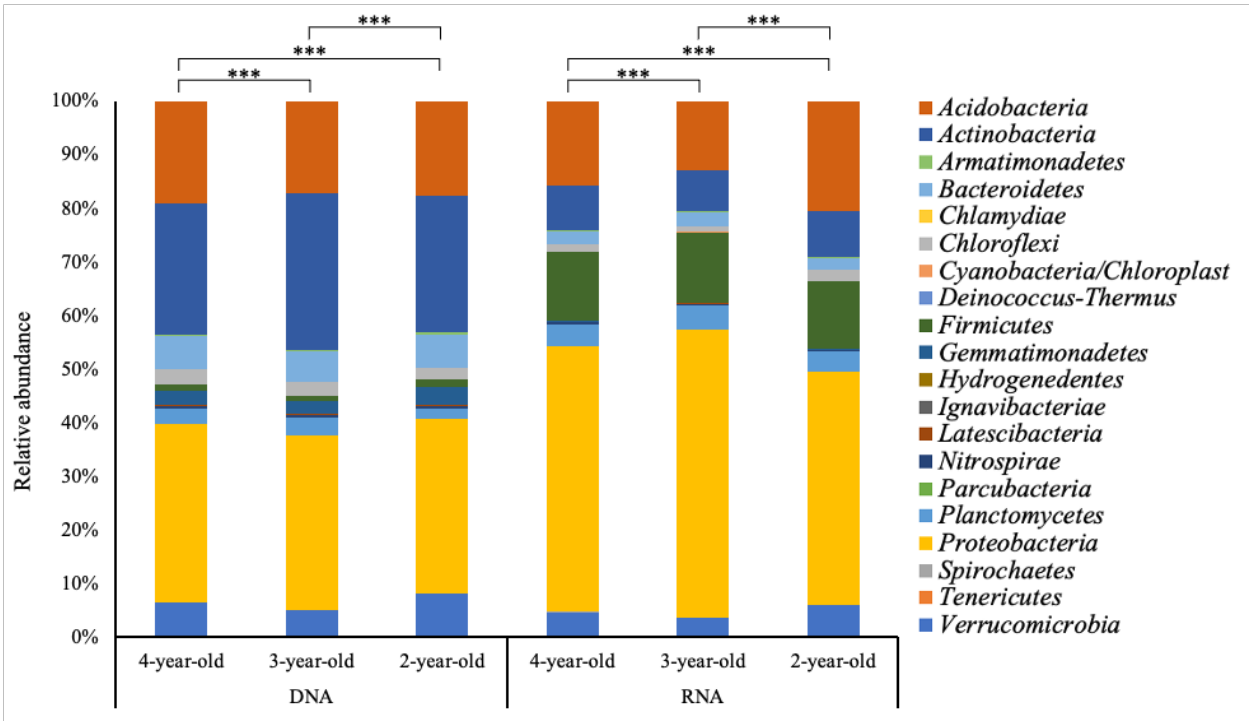


Figure 6. Phylum level differences in DNA and RNA microbial communities according to stand age differences. Relative abundances of annotated ASVs are shown, identified to their closest match in the RDP classifier. The letters "***" denote significant differences of relative abundance between different stand ages of *M.* x *giganteus* at a p-value < 0.05 as assessed by Kruskal-Wallis with post hoc Dunn's test.

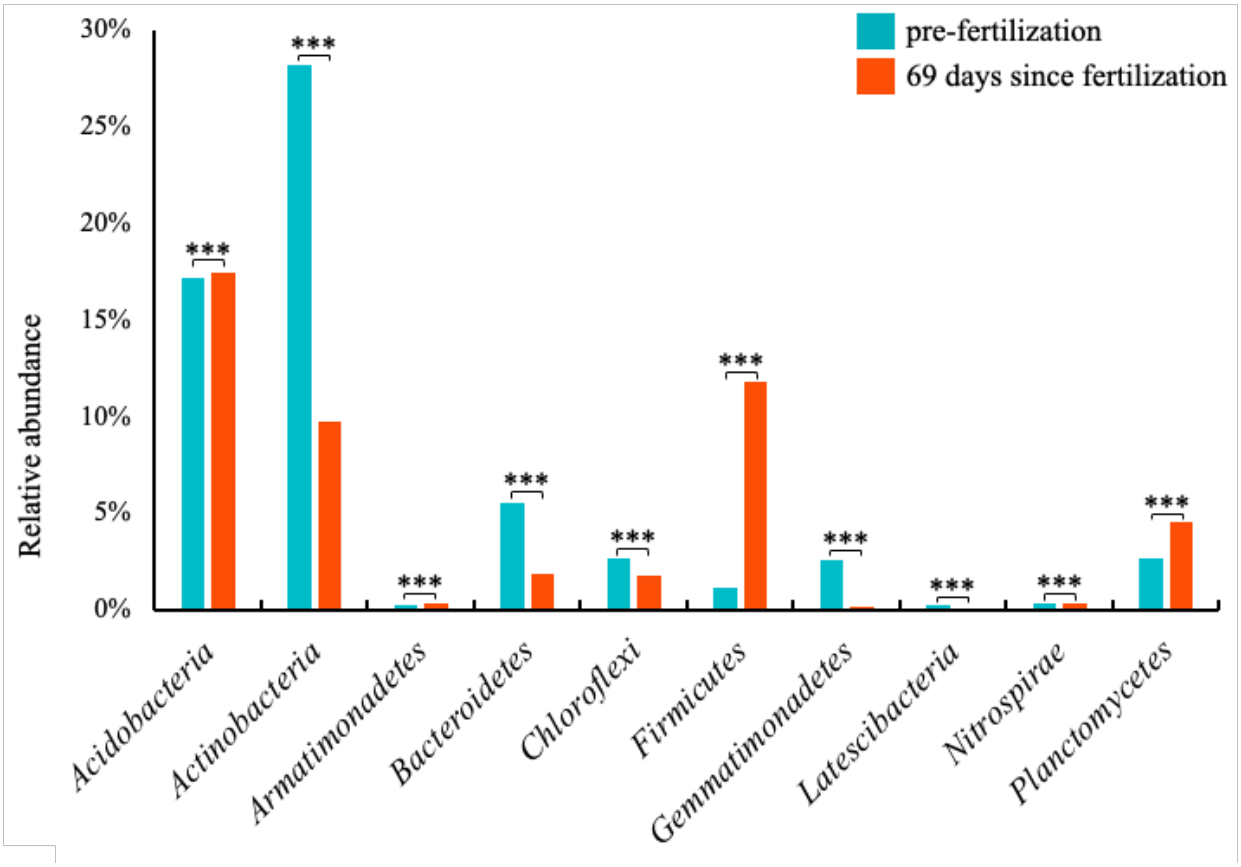


Figure 7. Phylum-level responses to time since fertilization in RNA microbial communities. The average relative abundances of phyla over time since fertilization were summarized. Letters "***" denote significant differences of relative abundance between pre- and 69 days since fertilization at a p-value < 0.05 as assess by Kruskal-Wallis with post hoc Dunn's test.

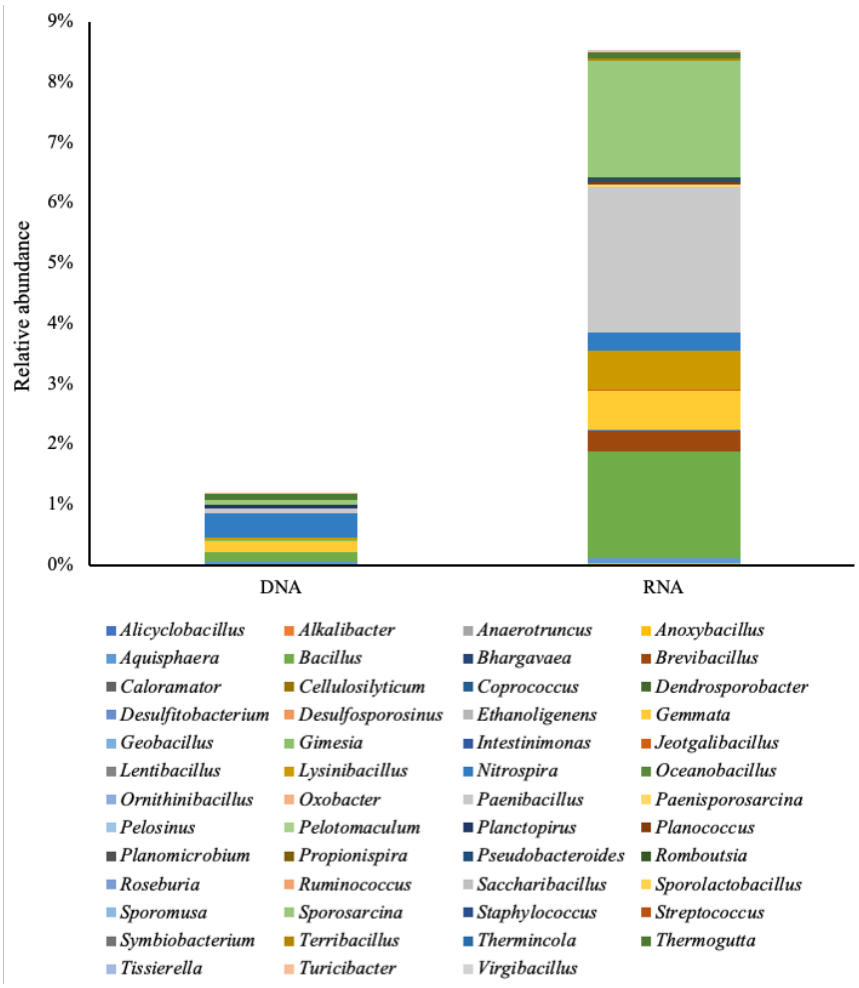


Figure 8. Comparison of nitrogen cycling-related bacteria in the DNA and RNA microbial communities. The average relative abundances of genus associated with nitrogen fixation, nitrification, and denitrification were summarized.

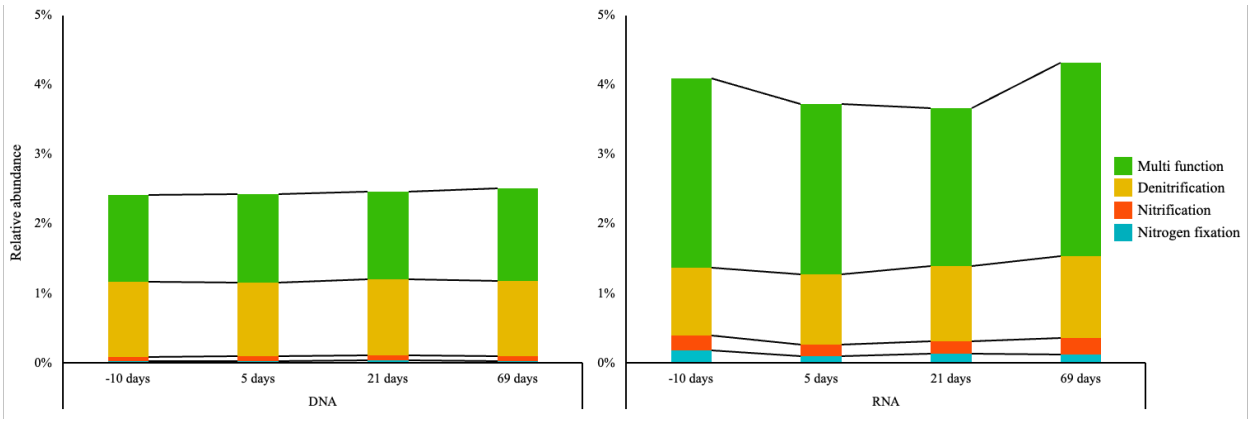
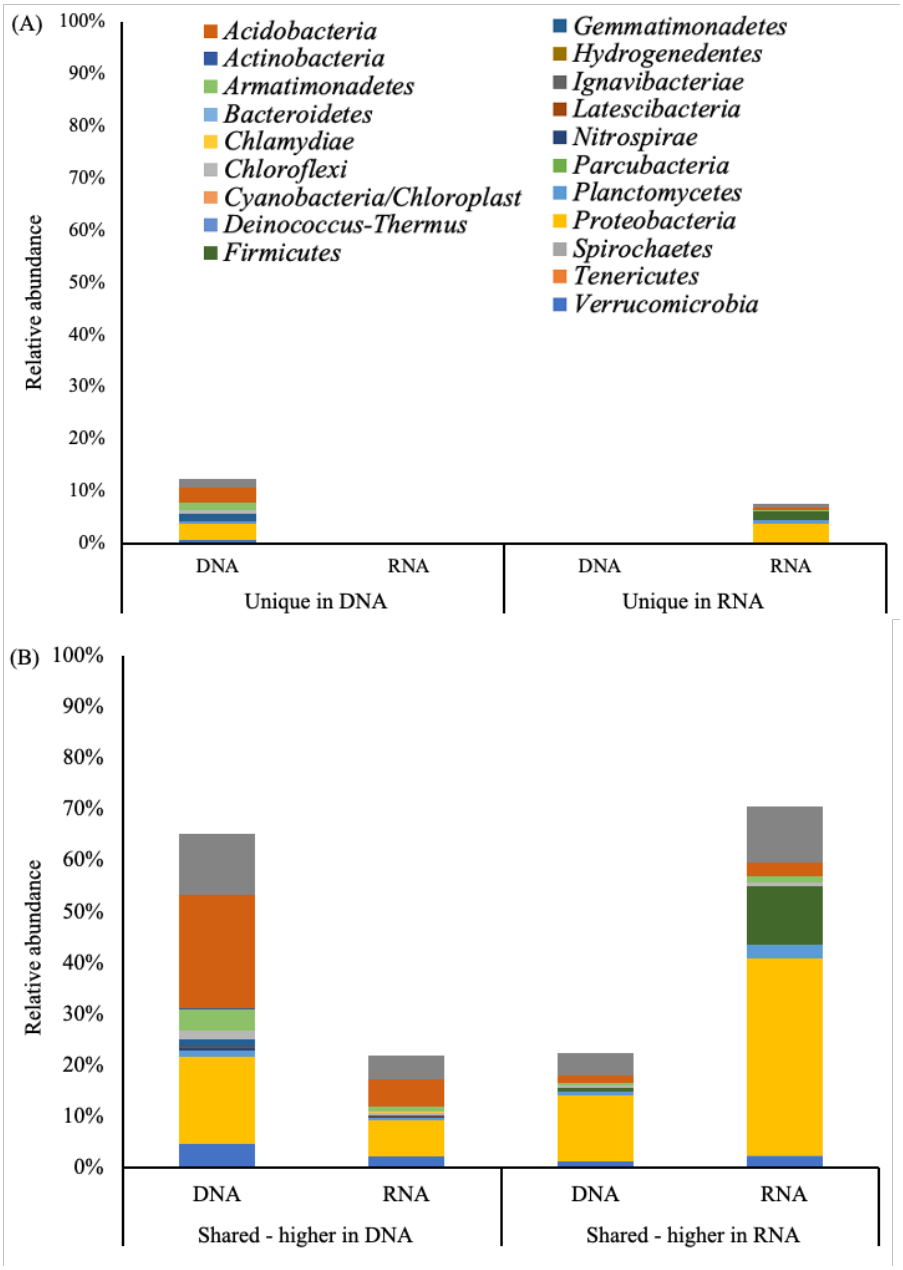


Figure 9. Comparison of nitrogen cycling-related bacteria in the DNA and RNA microbial communities according to time since fertilization. The average relative abundances of bacteria associated with nitrogen fixation, nitrification, and denitrification function were summarized.



759

760 Figure S1. Phylum level differences in DNA and RNA microbial communities based on unique
761 ASVs in DNA or RNA only (A) or shared ASVS and their enrichment in either DNA or RNA (B).
762 Relative abundances of annotated ASVs are shown, identified to their closest match in the RDP
763 classifier.

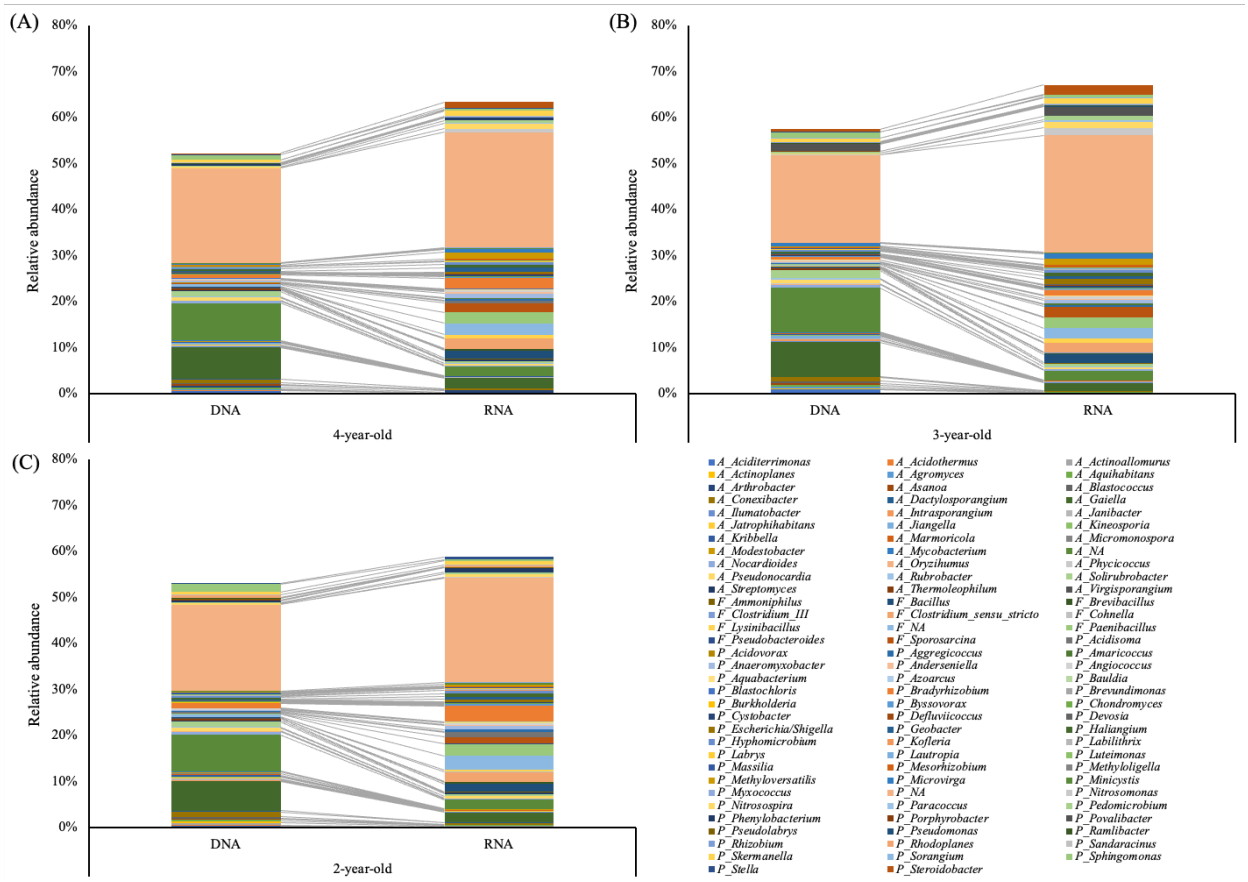


Figure S2. Comparison of dominant genus (> 0.1% relative abundance) in *Actinobacteria*, *Firmicutes*, and *Proteobacteria* between DNA and RNA microbial communities. (A) 4-year-old *M.* x *giganteus*, 73 genera. (B) 3-year-old *M.* x *giganteus*, 77 genera. (C) 2-year-old *M.* x *giganteus*, 74 genera. All genera included this analysis were significantly different between DNA and RNA microbial communities ($p_{Kruskal\text{-}Wallis} < 0.05$).

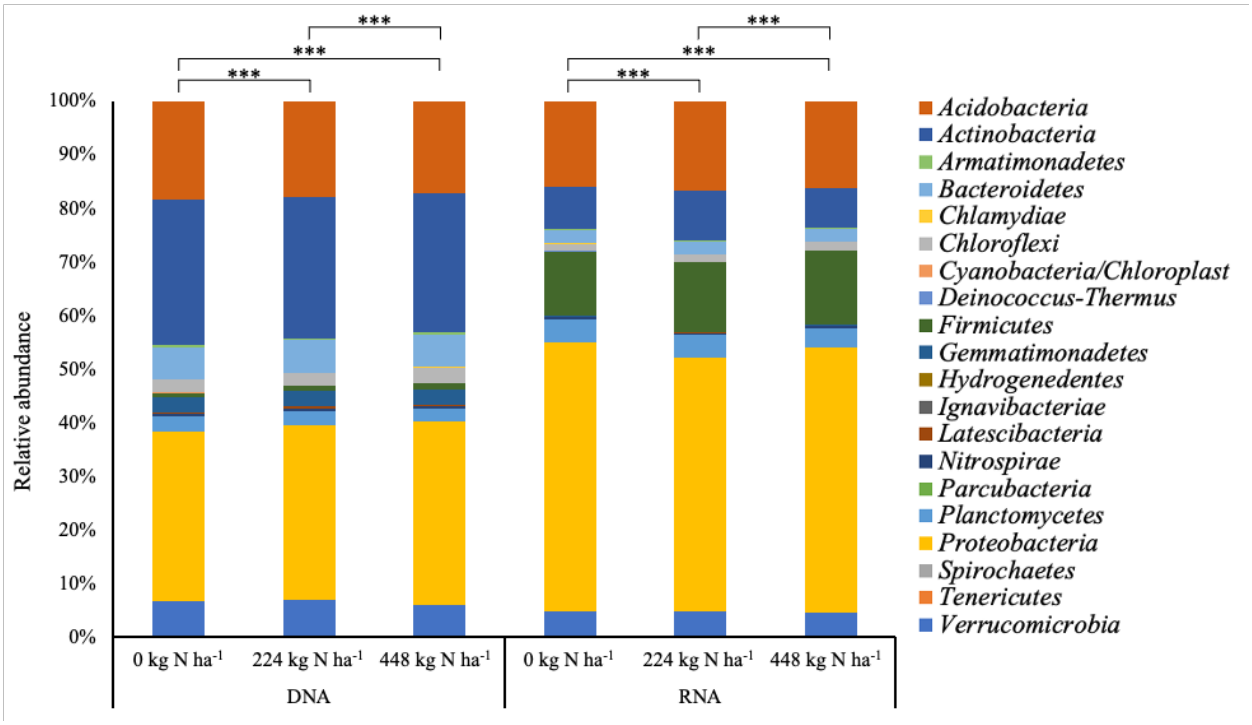


Figure S3. Phylum level differences in DNA and RNA microbial communities according to N fertilization amount differences. Relative abundances of annotated ASVs are shown, identified to their closest match in the RDP classifier. The letters "***" denote significant differences of relative abundance between different stand age of *M.* x *giganteus* at a p-value < 0.05 as assess by Kruskal-Wallis with post hoc Dunn's test.

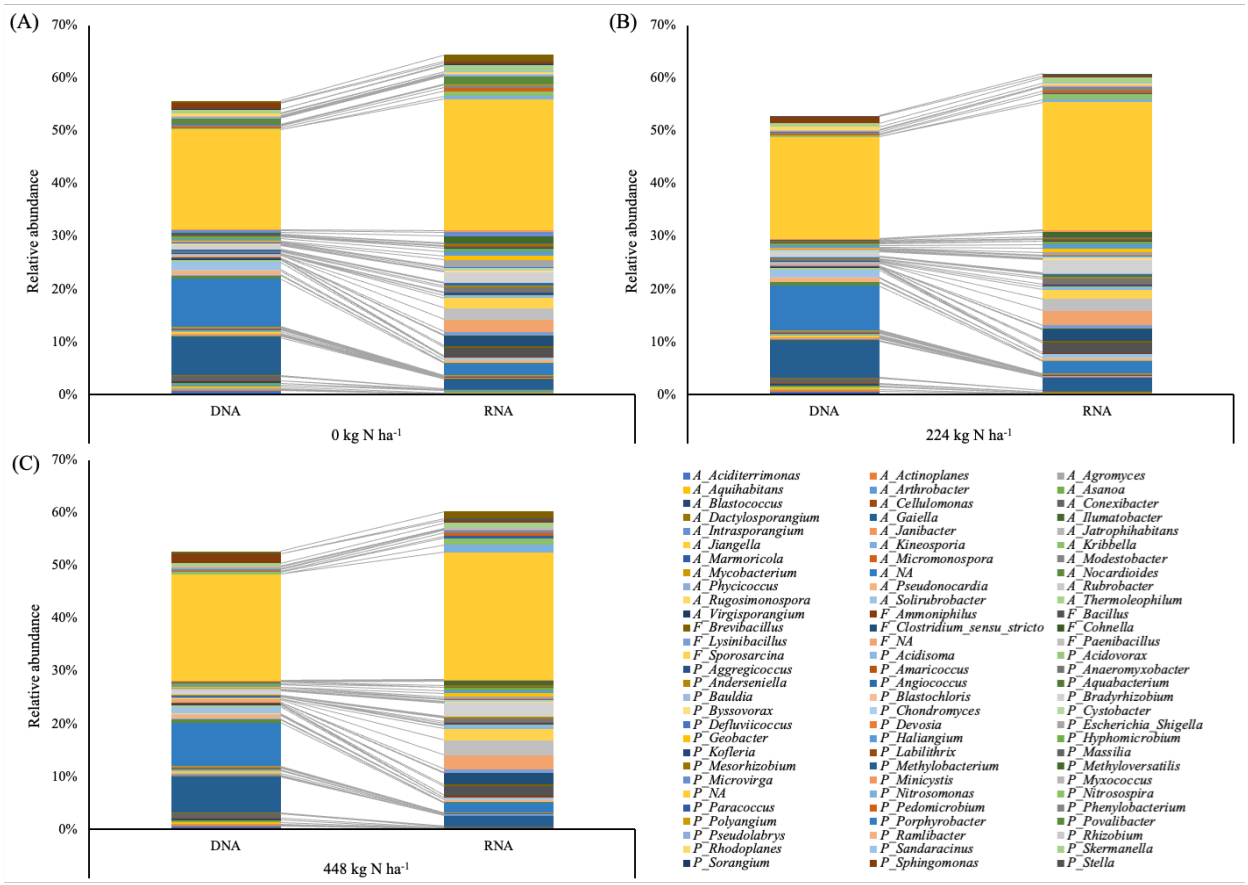


Figure S4. The dynamics of the 88 major genus (> 0.1% relative abundance) in the *Actinobacteria*, *Firmicutes*, and *Proteobacteria* between DNA and RNA microbial communities. (A) 0 kg N ha^{-1} of fertilizer applied *M.* x *giganteus* soil including 79 genera. (B) 224 kg N ha^{-1} of fertilizer applied *M.* x *giganteus* soil including 76 genera. (C) 448 kg N ha^{-1} of fertilizer applied *M.* x *giganteus* soil including 74 genera. All genera included this analysis were significantly different between DNA and RNA microbial communities ($p_{Kruskal\text{-}Wallis} < 0.05$).

783 Table 1. Permutational multivariate analysis of variance (PERMANOVA) for comparing DNA
784 and RNA microbial community dissimilarity.

Response variable	Stand age	N fertilization amount	Time since fertilization	Fertilization history
DNA microbial community	$R^2_{PERMANOVA}$: 0.051 $p_{PERMANOVA}$: 0.001	$R^2_{PERMANOVA}$: 0.015 $p_{PERMANOVA}$: 0.002	$R^2_{PERMANOVA}$: 0.005 $p_{PERMANOVA}$: n.s	$R^2_{PERMANOVA}$: 0.003 $p_{PERMANOVA}$: n.s
RNA microbial community	$R^2_{PERMANOVA}$: 0.037 $p_{PERMANOVA}$: 0.001	$R^2_{PERMANOVA}$: 0.009 $p_{PERMANOVA}$: 0.007	$R^2_{PERMANOVA}$: 0.010 $p_{PERMANOVA}$: 0.008	$R^2_{PERMANOVA}$: 0.005 $p_{PERMANOVA}$: n.s

785

786 Table S1. Comparison of microbial community dissimilarity using permutational multivariate
787 analysis of variance (PERMANOVA) for bacterial communities in *M.* x *giganteus* soil samples.

Response variable	Dissimilarity
Nucleic Acid (DNA and RNA)	$R^2_{PERMANOVA}$: 0.117 $p_{PERMANOVA}$: 0.001
Stand ages (2, 3, or 4-year-old)	$R^2_{PERMANOVA}$: 0.030 $p_{PERMANOVA}$: 0.001
N fertilization amount (0, 224, and 448 kg N ha^{-1})	$R^2_{PERMANOVA}$: 0.008 $p_{PERMANOVA}$: 0.001
Time since fertilization (-10, 5, 21, and 69 days)	$R^2_{PERMANOVA}$: 0.003 $p_{PERMANOVA}$: 0.015
Fertilization history (fertilized or unfertilized)	$R^2_{PERMANOVA}$: 0.002 $p_{PERMANOVA}$: n.s

788

789 Table S2. Kruskal-Wallis with post hoc Dunn's test comparing the average relative abundances of

790 phyla between DNA and RNA microbial communities.

Phylum	Average relative abundance		p-value
	DNA	RNA	
Acidobacteria	17.88%	16.29%	6.54E-09
Actinobacteria	26.46%	8.18%	2.22E-87
Armatimonadetes	0.31%	0.23%	1.92E-17
Bacteroidetes	6.02%	2.34%	1.13E-77
Chlamydiae	0.05%	0.10%	1.60E-93
Chloroflexi	2.58%	1.47%	3.04E-90
Cyanobacteria/Chloroplast	0.02%	0.03%	7.67E-93
Deinococcus-Thermus	0.00%	0.02%	4.75E-07
Firmicutes	1.05%	12.90%	1.13E-92
Gemmatimonadetes	2.81%	0.14%	1.33E-87
Hydrogenedentes	0.00%	0.00%	7.75E-83
Ignavibacteriae	0.01%	0.01%	2.61E-11
Latescibacteria	0.28%	0.10%	4.30E-83
Nitrospirae	0.45%	0.33%	1.69E-74
Parcubacteria	0.05%	0.00%	7.05E-68
Planctomycetes	2.64%	4.06%	4.49E-98
Proteobacteria	32.76%	48.97%	3.08E-90
Spirochaetes	0.01%	0.01%	1.50E-94
Tenericutes	0.00%	0.00%	1.93E-33
Verrucomicrobia	6.63%	4.81%	3.18E-15

791

792 Table S3. Pairwise permutational multivariate analysis of variance (PERMANOVA) for
793 comparing the effect of stand age and fertilization on the DNA and RNA microbial community
794 dissimilarity.

Response variable	Stand age		N fertilization amount (kg N ha^{-1})	
DNA microbial community	4-year-old vs 3-year-old	$R^2_{pairwisePERMANOVA} = 0.063$, $p_{pairwisePERMANOVA} = 0.001$	0 vs 224	$R^2_{pairwisePERMANOVA} = 0.014$, $p_{pairwisePERMANOVA} = 0.023$
	4-year-old vs 2-year-old	$R^2_{pairwisePERMANOVA} = 0.073$, $p_{pairwisePERMANOVA} = 0.001$	0 vs 448	$R^2_{pairwisePERMANOVA} = 0.021$, $p_{pairwisePERMANOVA} = 0.018$
	3-year-old vs 2-year-old	$R^2_{pairwisePERMANOVA} = 0.210$, $p_{pairwisePERMANOVA} = 0.001$	224 vs 448	$R^2_{pairwisePERMANOVA} = 0.015$, $p_{pairwisePERMANOVA} = 0.023$
RNA microbial community	4-year-old vs 3-year-old	$R^2_{pairwisePERMANOVA} = 0.039$, $p_{pairwisePERMANOVA} = 0.001$	0 vs 224	$R^2_{pairwisePERMANOVA} = 0.011$, $p_{pairwisePERMANOVA} = 0.031$
	4-year-old vs 2-year-old	$R^2_{pairwisePERMANOVA} = 0.057$, $p_{pairwisePERMANOVA} = 0.001$	0 vs 448	$R^2_{pairwisePERMANOVA} = 0.013$, $p_{pairwisePERMANOVA} = 0.029$
	3-year-old vs 2-year-old	$R^2_{pairwisePERMANOVA} = 0.152$, $p_{pairwisePERMANOVA} = 0.001$	224 vs 448	$R^2_{pairwisePERMANOVA} = 0.012$, $p_{pairwisePERMANOVA} = 0.029$

795

Table S4. Pairwise permutational multivariate analysis of variance (PERMANOVA) for comparing the effect of time since fertilization on the DNA and RNA microbial community dissimilarity.

Response variable	Time since fertilization	
DNA microbial community	-10 vs 5	n.s
	-10 vs 21	n.s
	-10 vs 69	n.s
	5 vs 21	n.s
	5 vs 69	n.s
	21 vs 69	n.s
RNA microbial community	-10 vs 5	n.s
	-10 vs 21	n.s
	-10 vs 69	$R^2_{pairwisePERMANOVA} = 0.018$, $p_{pairwisePERMANOVA} = 0.018$
	5 vs 21	n.s
	5 vs 69	n.s
	21 vs 69	n.s

Table S5. Kruskal-Wallis with post hoc Dunn's test comparing the average relative abundances of phyla between DNA and RNA microbial communities by different N fertilization. Denote N0, N224, and N448 are N fertilization of 0 kg N ha^{-1}, 224 kg N ha^{-1}, and 448 kg N ha^{-1}, respectively.

	Phylum	Difference in average relative abundance		p-value
DNA microbial community	*Acidobacteria*	18.44%	17.23%	3.62E-02 between N0 and N448
	Chloroflexi	2.30%	2.90%	1.24E-02 between N224 and N448
	Latescibacteria	0.32%	0.30%	1.79E-04 between N0 and N448
		0.30%	0.21%	1.30E-03 between N224 and N448
	Proteobacteria	31.54%	34.10%	3.17E-04 between N0 and N448
RNA microbial community	*Actinobacteria*	9.18%	7.39%	1.33E-03 between N224 and N448
	Firmicutes	12.91%	13.78%	2.55E-03 between N224 and N448
	Gemmatimonadetes	0.12%	0.13%	4.52E-02 between N0 and N448
	Hydrogenedentes	0.0054%	0.0047%	2.47E-02 between N0 and N224
		0.0047%	0.0035%	2.62E-02 between N224 and N448
	Latescibacteria	0.10%	0.08%	3.87E-03 between N224 and N448
	Nitropirae	0.30%	0.31%	1.59E-04 between N0 and N448
	Proteobacteria	50.08%	49.46%	3.27E-05 between N0 and N448

804 Table S6. Kruskal-Wallis with post hoc Dunn's test comparing the average relative abundances of

805 nitrogen cycling functions in RNA microbial communities by time since fertilization.

	Function	Days		Relative abundance		p-value
RNA		-10 days	69 days	0.85%	1.15%	1.52E-08
microbial	Denitrification	5 days	69 days	0.95%	1.15%	7.72E-03
community		21 days	69 days	0.94%	1.15%	2.72E-03

806