Rhizosphere and detritusphere habitats modulate expression of soil N-cycling genes during plant development

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Abstract

Interactions between plant roots and rhizosphere bacteria mediate nitrogen (N)-cycling processes and create habitats rich in low molecular weight (growing roots, rhizosphere) and complex organic molecules (decaying root litter, detritusphere) compared to bulk soil. Microbial N-cycling is regulated by a diverse suite of genes from many interconnected metabolic pathways; but most studies of soil N-cycling gene expression have focused on single pathways. Currently, we lack a comprehensive understanding of the interplay between soil N-cycling gene regulation, spatial habitat and time. Here we present an analysis of a replicated time series of soil metatranscriptomes; we followed multiple N transformations in four soil habitats (rhizosphere, detritusphere, mixed rhizo-/detriusphere, bulk soil) over a period of active root growth for the annual grass, *Avena fatua*. The presence of root litter and living roots significantly altered the trajectory of N-cycling gene expression. Across soil habitats, the most highly expressed N-transformation genes were related to extracellular proteases, ammonium assimilation into microbial biomass via glutamate synthase, and ammonium oxidation. Upregulation of bacterial assimilatory nitrate reduction in the rhizosphere suggests that rhizosphere bacteria were actively competing with roots for nitrate. Simultaneously, bacterial ammonium assimilatory pathways were upregulated in both rhizosphere and detritusphere soil, which could have limited N availability to plants. The detritusphere supported dissimilatory processes DNRA and denitrification. Expression of ammonium oxidation genes was almost exclusively performed by three phylotypes of *Thaumarchaeota* and was upregulated in unamended bulk soil. Unidirectional ammonium assimilation and its regulatory genes

42 (glutamine synthetase/glutamate synthase, or GS/GOGAT) were upregulated in soil surrounding
43 relatively young roots and more highly decayed root litter, suggesting N may have been limiting
44 in these habitats (the GS/GOGAT pathway is known to be activated under low N availability).
45 We did not detect expression of N-fixation or anammox genes. Our comprehensive
46 metatranscriptomic time-series of organic and inorganic N-cycling in rhizosphere,
47 detritusphere, and bulk soil, indicates that differences in C and inorganic N availability control
48 contemporaneous transcription of N-cycling pathways in soil microhabitats that exist in close
49 spatial proximity.
50
51 **Contribution to the field**
52 Plant roots modulate microbial nitrogen cycling by regulating the supply of root-derived carbon
53 and nitrogen uptake. These differences in resource availability cause distinct micro-habitats to
54 develop: soil near living roots (rhizosphere), decaying roots (detritusphere), near both
55 (rhizo/detritusphere), or outside the direct influence of roots (bulk). While many genes control
56 the microbial processes involved in the nitrogen cycle, most research has focused on single
57 genes and pathways, neglecting the interactive effects these pathways have on each other. The
58 processes controlled by these pathways determine consumption and production of N by soil
59 microorganisms. We followed the expression of N-cycling genes in the primary four soil
60 microhabitats over a period of active root growth for an annual grass. We found that the
61 presence of root litter and living roots significantly altered gene expression involving in multiple
62 nitrogen pathways. We also found populations with genes for multiple pathways, where
63 expression was likely shaped by available forms of carbon and by competition with plants for

64 inorganic nitrogen. Phylogenetic differences in spatial and temporal expression of the soil
65 microbial N-pathway genes ultimately regulate N-availability to plants.
66
67 **Introduction**
68
69 Nitrogen (N) is a key limiting nutrient for plant growth, since in soil, N mostly occurs in
70 organic forms within large, complex molecules that are unavailable to plants. Soil
71 microorganisms have suites of extracellular enzymes capable of degrading these molecules,
72 freeing small organic and inorganic N to the surrounding soil and benefitting nearby plants.
73 Other bacteria and archaea transform N to nitrate—an inorganic form that is more available to
74 many plants. Meanwhile, microorganisms also take up inorganic N for a variety of growth-
75 supporting assimilatory and dissimilatory metabolic processes. This can lead to some
76 competition for N between soil bacteria and plants. All of these processes likely occur
77 simultaneously in soil, but we have an incomplete understanding of how they vary with time
78 and in distinct soil habitats.
79 Soils contain numerous microhabitats with heterogeneous distributions of substrates and
80 resources (Sokol et al., 2022). The influence of growing roots can dominate (rhizosphere) or
81 dead root litter may dominate (detritusphere); alternatively these two habitats may co-occur
82 when live roots regrow into previously colonized areas (rhizo/detritusphere). In previous
83 analyses, we have shown that distinct bacterial guilds operate in rhizosphere, detritusphere,
84 and combined rhizosphere-detritusphere soil habitats (Nuccio et al., 2020). The spatial
85 organization of these soil habitats may be particularly important for nutrient transfers that

create distinct N microhabitats that enable or limit N-cycling. For example: (1) in the rhizosphere, exudate-driven blooms of microbial growth are followed by predation that liberates the nutrient capital held in bacterial, fungal, and metazoan bodies, this redistribution of microbial biomass causes conversion to lower molecular weight compounds (*e.g.*, amino acids) that are deaminated to NH_4^+ by diverse bacteria and fungi (Clarholm, 1985; Koller et al., 2013); (2) in the detritusphere, organic N in root litter (e.g., lignoproteins (Myrold, 2021)) is mineralized to make inorganic N available soil microbes, and also serves as a carbon source for soil microbes (Pepe-Ranney et al., 2016), creating a demand for N to maintain cellular stoichiometry; (3) in bulk soils, microbes may express more macromolecule degradation genes relative to more resource rich sites near roots, where degradation enzymes for low molecular weight compounds are more prevalent (Shi et al., 2018).

Rates of microbial N transformations in soil can be significantly impacted by the presence of both growing and decaying roots. Gross N-mineralization, immobilization, and nitrification rates vary as a function of proximity to plant roots, root age and concentration of organic material in grassland soil (Personeni and Loiseau, 2005; Herman et al., 2006). Roots can significantly deplete ammonium in the rhizosphere within days of root introduction into sterile soil (Trofymow et al., 1987). However, in wild soils with intact microbial communities, rhizosphere-associated bacteria can transiently compete with plants (e.g., *A. barbata* roots) for inorganic nitrogen (Jackson et al., 1989a). As part of this competition, plants can inhibit rhizosphere nitrogen transformations such as biological nitrification inhibition (BNI) via production of certain root exudate compounds (Nardi et al., 2020). Plants have also been shown to inhibit both nitrification and denitrification processes in the rhizosphere—both processes lead to loss

108 of N from the plant-available pool (Moreau et al., 2019)—and litter amendment has been
109 shown to increase denitrification (Che et al., 2018). Understanding the N-cycling tradeoffs
110 between different soil habitats is key for our ability to better model and predict the controls of
111 soil N cycling.

112 Soil metatranscriptome analysis is a powerful tool for linking nitrogen cycling genes to
113 specific microbial populations, and correlating transcription levels to environmental conditions
114 (Nicol et al., 2008; Liu et al., 2010; Placella and Firestone, 2013; Nuccio et al., 2020; Sieradzki et
115 al., 2020; Tosi et al., 2020). In comparison, qPCR can target only single genes, and primer biases
116 may leave some taxa undetected. A recent metatranscriptomics study demonstrated a short-
117 term coupling of nitrogen cycling pathways and availability of simple carbons simulating
118 priming by root exudates (Chuckran et al., 2021). Otherwise, there is little knowledge on the
119 contemporaneous feedback through gene expression between carbon availability and nitrogen
120 cycling in naturally complex soil habitats and over annual plant-relevant time frames (Yergeau
121 et al., 2014, 2018; Žifčáková et al., 2016).

122 Here, we used soil metatranscriptomics analysis to build a comprehensive time-resolved
123 representation of both organic and inorganic nitrogen transformations in an annual grassland
124 soil. We characterized a period of active root growth, as well as root decomposition, of wild oat
125 grass (*Avena fatua*), a common species in Mediterranean grasslands. We hypothesized that
126 different N cycle pathways would be prevalent in different soil microhabitats that vary in
127 available C and N resources. We analyzed 48 metatranscriptomes from rhizosphere and bulk
128 soil collected over a 3-week time series in the presence or absence of root litter; this allowed us
129 to identify the dominant N-cycling pathways associated with each habitat and untangle the

130 effects of carbon supply by live or dead roots from competition with the plant for N. We
131 previously analyzed a different subset of this data to assess expression of genes coding for
132 carbohydrate degradation (Nuccio et al., 2020) and organic nitrogen degrading enzymes
133 (Sieradzki et al., 2020). While these prior studies provide important context, our analysis here is
134 unique in its comprehensive exploration of how N transformation gene expression is influenced
135 by the distinct environmental conditions of different soil habitats. The use of
136 metatranscriptomics (as opposed to qPCR) also facilitated identification of specific microbial
137 populations involved in each pathway, and helped us test for multiple N transforming pathways
138 expressed within populations found in mutiple soil habitats. Finally, we measured changes in N-
139 pathway gene expression over time as the rhizosphere aged and as root litter was degraded.
140
141 **Methods**
142
143 *Experimental design, sample collection and sequencing*
144
145 The experimental procedures and initial data processing for our 48 soil
146 metatranscriptome dataset are described in detail by (Nuccio et al., 2020). Briefly, *Avena fatua*
147 was grown in a fine loam Alfisol complex (Ultic Haploxeralf mixed with a Mollic Palexeralf) from
148 the Hopland Research and Extension Center (Hopland, CA); pH 5.6, 2% total C) in microcosms
149 with a sidecar with transparent walls. After six weeks, roughly halfway through the plant life
150 span, the solid divider to the sidecar was removed and replaced with a slotted divider so that
151 roots could grow into the sidecar. Root growth was marked on the sidecar wall. All sidecars

152 contained bulk soil bags that were inaccessible to roots. Half of the bulk soil bags as well as the
153 soil in half of the sidecars were amended with dried *A. fatua* root detritus (litter C:N = 13.4).
154 Once the sidecar was opened, paired rhizosphere and bulk soil triplicates were harvested
155 destructively after 3, 6, 12 and 22 days from amended and unamended microcosms for a total
156 of 48 samples. Lifeguard Soil Preservation Reagent (MoBio) was added to 1g subsample of the
157 harvested soil. The supernatant and roots were removed, and the samples were stored at -
158 80°C.

159 DNA/RNA co-extraction was performed with phenol-chloroform. DNA and RNA were
160 separated with an AllPrep kit (Qiagen) and RNA was DNase treated (TURBO DNase, Thermo-
161 Fisher Scientific). Ribosomal RNA was depleted (RiboZero, Illumina) and the mRNA was
162 retrotranscribed. cDNA was sequenced with an Illumina HiSeq 2000 2x150 (TruSeq SBS v3)
163 protocol at the Joint Genome Institute. In addition, the V4 hypervariable region of the 16S gene
164 (primers 515F and 806R) was amplified from the extracted DNA and sequenced via Illumina
165 MiSeq v3 2x300. Amplicons were analyzed by the Joint Genome Institute. Operational
166 taxonomic units (OTUs) were clustered at 97% identity with USEARCH (Edgar, 2010) and
167 taxonomy was assigned at 95% id by RDP (Wang et al., 2007).

168

169 *Expression of genes from a curated collection of Hopland-soil genomes*

170

171 As described previously (Nuccio et al., 2020), raw reads were quality-trimmed (Q20) and
172 rRNA and tDNA were removed. Library size, evenness, richness and Shannon diversity were
173 comparable between experimental groups, with a mean library size of 43 M paired end reads.

174 Reads were mapped at 80% identity with BBsplit (Bushnell, 2014; Nuccio et al., 2020) against a
175 dereplicated reference set of 282 Hopland CA soil genomes including stable isotope probing
176 metagenome assembled genomes (SIP-MAGs) (Starr et al., 2018), metagenomic assembled
177 genomes (MAGs, NCBI PRJNA517182), soil isolates (Starr et al., 2018; Zhalnina et al., 2018) and
178 single amplified genomes (SAGs) (Nuccio et al., 2020). A mean of 12.3% of the reads per sample
179 mapped unambiguously to the reference set.
180 Prodigal (Hyatt et al., 2010; Nuccio et al., 2020) was used to predict open reading frames
181 (ORFs) in the genomes. The ORFs were annotated using KEGG (Kanehisa and Goto, 2000) and
182 ggKbase (http://ggkbase.berkeley.edu). Gene counts were calculated with R package Rsubread
183 function featureCounts (Liao et al., 2014). Three samples were removed from further analysis
184 due to an exceptionally low number of features: H3_Rhizo_NoLitter_39, H1_Rhizo_Litter_2 and
185 H2_Rhizo_Litter_9.
186 All features and their metatranscriptomic read recruitment were analyzed with the R
187 package DESeq2 (Liao et al., 2014; Love et al., 2014) requiring an adjusted p-value < 0.05.
188 Ordination and visualization were conducted using R package ggplot2 (Wickham, 2016) and
189 vegan (Oksanen et al., 2008; Wickham, 2016); the vegan function Adonis was used to detect
190 significant factors affecting expression of nitrogen cycling genes.
191
192 *Expression of nitrogen cycling genes identified in assembled metatranscriptomes*
193
194 After quality control, the metatranscriptomes were assembled into contigs within
195 samples. Only contigs larger than 200 bp were clustered at 99% ID with cd-hit-est (Huang et al.,

196 2010). Prodigal (Huang et al., 2010; Hyatt et al., 2010) was used to predict ORFs from the
197 cluster representatives, and KEGG models (Kanehisa and Goto, 2000) were used to locate
198 nitrogen cycling genes based on the KEGG nitrogen cycle module (see Supplemental Table S1
199 for a list of KEGG profiles, gene lengths and thresholds used). Some ORFs were identified by
200 several HMMs due to homology (e.g. nitrate reductases *nxrA*, *narG* and *napA*). In those cases,
201 the highest scoring (by bitscore) hit from all HMMs was selected. Extracellular proteases were
202 called by reciprocal blast to proteases from the MEROPS database that contained signal
203 peptides (Rawlings et al., 2016; Nguyen et al., 2019). Reads were mapped to nitrogen cycling
204 ORFs at a minimum identity 95% and minimum breadth 75% using bbmap (Bushnell, 2014;
205 Nuccio et al., 2020). Normalization to sequencing depth was done using DESeq2, and when
206 comparing between different genes expression was also divided by the relevant mean
207 assembled gene length to control for gene-length biases in recruitment. Heat maps were
208 generated in R using gplots (Warnes et al., 2015).
209 Phylogeny of the ammonia monooxygenase subunit A (*amoA*) was determined by
210 aligning *amoA* assembled transcripts to curated full amoA gene sequences from RefSeq (Jun 1,
211 2019) using mafft-linsi (sensitive mode) (Katoh and Standley, 2013) and removing positions
212 represented by less than 50% of the aligned sequences with GBlocks version 0.91 (Dereeper et
213 al., 2008) with parameters -b3=50 -b4=5 -b5=h. RAxML version 8.2.12 (Kozlov et al., 2019) with
214 parameters set to matrix LG and seed=13 was used to build a Newick tree which was visualized
215 in iTOL (Letunic and Bork, 2019). The phylogeny of *nirK* transcripts was determined in the same
216 manner. The aggregated coverage of *amoA*/*nirK* variants from all samples was superimposed
217 onto the tree in iTOL. Additionally, since nitrate reductases that participate in multiple

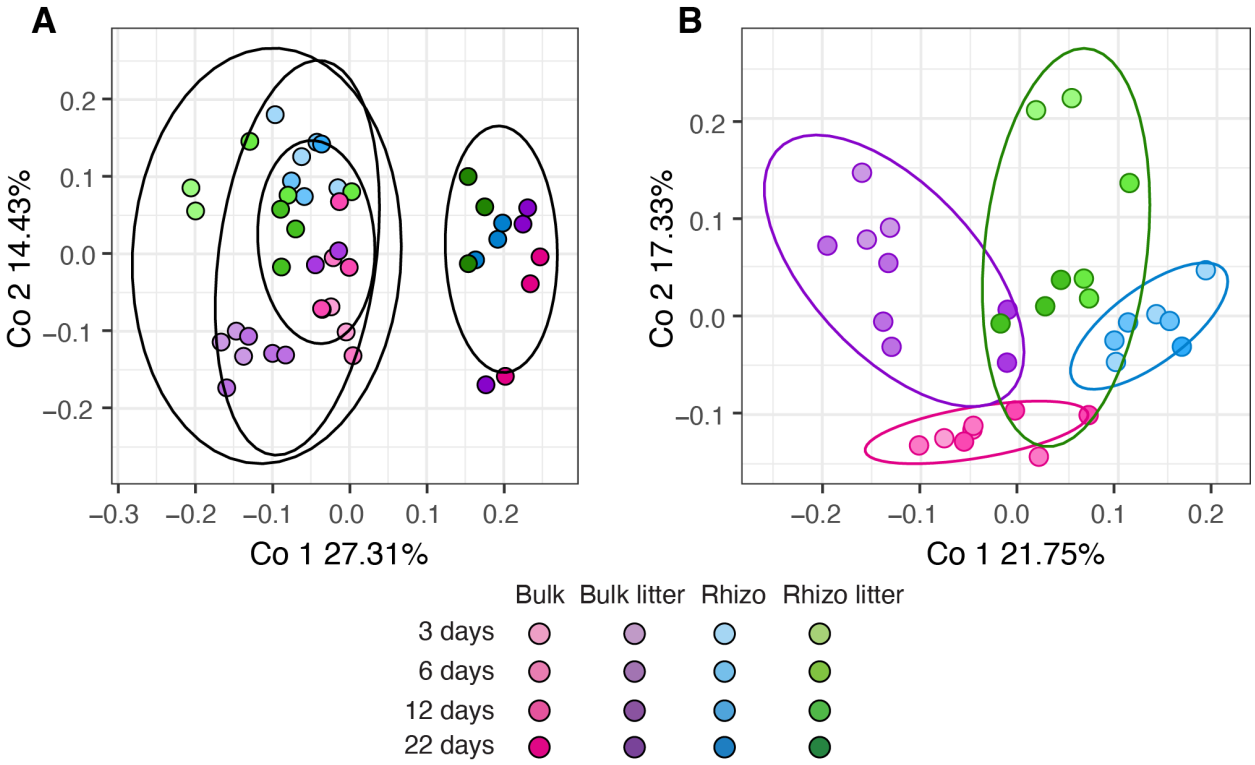
218 pathways are homologous, we wanted to make sure they were correctly identified by the
219 HMMs. Therefore, we placed the assembled sequences on a protein reference tree (Matheus
220 Carnevali et al., 2019) and corrected the annotations according to tree clustering.
221 Normalized expression per gene per time point was compared between groups using
222 ANOVA and Tukey HSD tests, and p-values were corrected for multiple comparisons (time,
223 location, amendment and all interactions between them). Upregulation was determined as
224 log2-fold values calculated by DESeq with corrected *p* values < 0.05.
225
226 **Results**
227
228 To assess whether expression of organic and inorganic nitrogen cycling genes was
229 affected by the presence of root litter in the rhizosphere or in nearby bulk soil, we tested for
230 significant differences in N transformation gene expression over a three-week time series (3, 6,
231 12, 22 days). The PCoA based on Bray-Curtis dissimilarity of expression of N cycling genes
232 normalized to sequencing depth revealed clustering by location (rhizosphere/bulk soil)
233 throughout the whole time-series; the last time point (T4; 22 days) notably distinct from the
234 others (fig. 1A). Analysis of the first three time points only (3, 6, and 12 days) revealed an
235 additional clustering by litter amendment (fig. 1B). In both analyses, the first two principal
236 components explained 40% of the variation. PERMANOVA analysis using all time points
237 indicated a significant effect of location (rhizosphere vs. bulk soil), time and litter amendment
238 (p-value = 10^{-4}) (see sup. table 1 for all values). The combined R^2 of all significant factors was
239 63%. When we conducted the same analysis but excluded the last time point (T4), the effects of

240 single factors remained significant (p-value = 10^{-4}). The combined R^2 of all significant factors

241 was 61%, and the variability explained by time decreased from 28% with T4 to 10% without T4,

242 whereas the variability explained by location and amendment increased by 5-8% each.

243



244

245 **Figure 1: Principal coordinates analysis (PCoA) of nitrogen cycling gene expression**. Influence of

246 living roots and root detritus on soil microbial gene expression during 3 weeks of *Avena fatua*

247 root growth (harvests at 3, 6, 12, 22 days) in a CA annual grassland soil. Input data are Bray-Curtis

248 dissimilarity of expression of N cycling genes normalized to sequencing depth, determined by

249 mapping reads to ORFs assembled from metatranscriptomes followed by log ratio

250 transformation, in (A) all time points with the last time point circled and (B) excluding the last

251 time point (22 days). Groups are indicated in the ellipses: B=Bulk, BL=Bulk Litter, R=Rhizosphere,

252 RL=Rhizosphere Litter. n=3 for each habitat and timepoint. Ellipses represent a 90% confidence
253 interval of the ordination coordinates (calculated by stat_ellipse).
254
255 *Expression of nitrogen cycling genes by pathway*
256
257 To identify the dominant N-cycling pathways in four soil microhabitats (rhizosphere,
258 detritusphere, rhizo/detritusphere, bulk soil), we compared differential expression of each
259 nitrogen cycling gene in the presence or absence of roots and litter amendment. Expression and
260 upregulation trends of N cycling genes (defined in Table S1) are shown in fig. 2. Several genes
261 involved in assimilatory pathways were significantly upregulated in the rhizosphere:
262 assimilatory nitrate reduction genes *nasA*, *NR* and *nirA*, glutamate dehydrogenase *gdh2* and
263 GS/GOGAT genes *glt1* and *gltB* (fig. 2). The first step of GS/GOGAT, *glnA*, was upregulated both
264 in the rhizosphere and in the presence of litter (fig. 2), and the GS/GOGAT regulatory proteins
265 *glnD* and *glnG*, triggered under N limitation, were upregulated in the rhizosphere, whereas *glnB*
266 was upregulated in the presence of litter (sup. fig. 1 B,C). In the detritusphere, the presence of
267 litter also triggered macromolecular organic nitrogen degradation (extracellular protease and
268 chitinase), as well as dissimilatory pathways; dissimilatory nitrate reduction (DNRA) and
269 denitrification (fig. 2). However, not all genes in these pathways were upregulated and some
270 were not detected at all (*nirS* and *norBC* in denitrification), whereas others were not
271 significantly upregulated (*nirK*, *narGHI*, *napB*) (fig. 2). Finally, nitrification, DNRA and the first
272 step of denitrification were upregulated in bulk soil compared to the rhizosphere (sup. fig. S1).

273 Several genes were particularly highly expressed in our dataset (e.g., ammonia
274 monooxygenase subunits *amoA* and *amoC*, and nitrate reductase *nirK* (sup. fig. S1 A,B)) but did
275 not show clear patterns of differential expression by either soil habitat treatment or time. We
276 did not detect any genes involved in nitrogen fixation or anaerobic ammonia oxidation.
277 Expression of fungal N cycle genes such as *cyp55* (nitric oxide to nitrous oxide) and *nit-6* (nitrite
278 to ammonium) was very low (sup. fig. 1C).
279

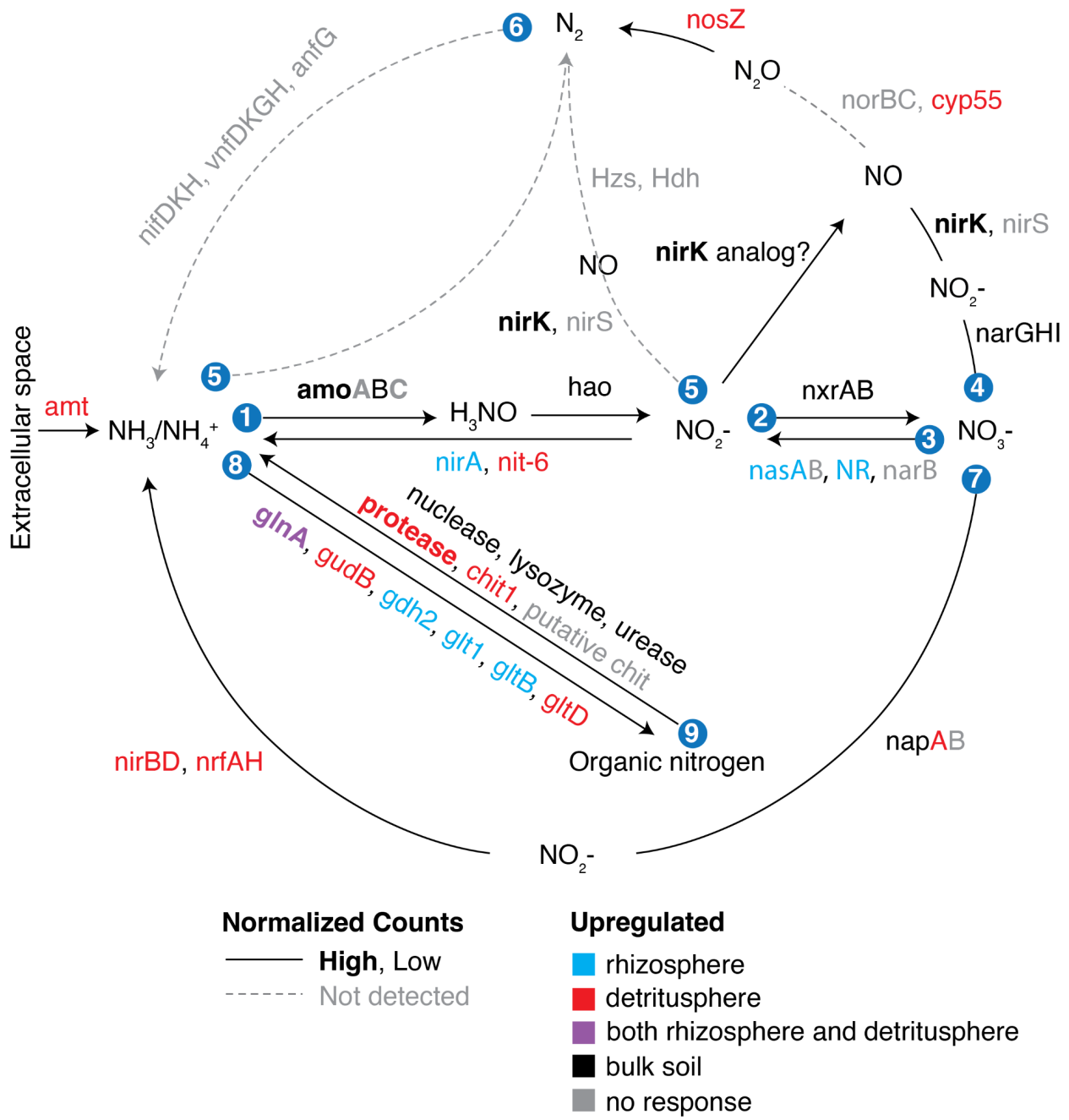


Figure 2: Expression level and upregulation by soil habitat of nitrogen cycling genes from assembled transcripts. Bold font indicates high expression levels (mean counts in each time point >5 after normalization to gene length and sequencing depth). Significant upregulation was determined by ANOVA and Tukey post hoc test (adjusted $p<0.05$). Numbers denote pathways: 1=Ammonia oxidation, 2=Nitrite oxidation, 3=Assimilatory nitrate reduction,

286 4=Denitrification, 5=Anammox, 6=Nitrogen fixation, 7=DNRA, 8=Ammonium assimilation (GDH
287 and GS/GOGAT), 9=Macromolecular N mineralization, amt = Ammonium transporter. The
288 functional role of each enzyme is explained in Table S2. The nirK analog is an archaeal enzyme
289 which participates in archaeal nitrification, but unlike its bacterial analog, it does not generate
290 nitrate (Stein, 2019).
291
292 *Archaeal nitrification*
293
294 Two of the most highly expressed genes we identified were subunits of ammonium
295 monooxygenase, the first enzyme in the nitrification pathway. Nitrification genes were not
296 affected by the presence of litter, but *nirK*, *amoB*, *hao*, *nxrA* and *nxrB* were significantly
297 upregulated in bulk soil compared to rhizosphere soil (ANOVA: $p<0.05$, Tukey HSD test:
298 adjusted $p<0.05$) (sup. table S2).
299 Ammonium oxidation can be performed either by ammonium oxidizing archaea (AOA)
300 or ammonium oxidizing bacteria (AOB). The *amoA* subunit of ammonia monooxygenase is
301 commonly used as a taxonomic marker to differentiate between AOA and AOB. To determine
302 which taxa were responsible for the high expression of ammonia oxidation genes, we placed
303 the 13 *amoA* ORFs identified in assembled transcripts into a phylogenetic tree with full-length
304 *amoA* reference sequences from the RefSeq database (fig. 3A). The cumulative coverage per
305 *amoA* variant in all samples was superimposed onto the tree. Read recruitment to AOA was
306 orders of magnitude higher than to AOB, accounting for 98% ± 0.007% (N=16) of the reads

307 mapped to *amoA* per sample. While there were some ORFs placed into comammox clade B,
308 their expression was negligible compared to archaeal *amoA* (fig. 3A).
309 The expression of hydroxylamine oxidoreductase (*hao*), the next step in nitrification
310 after ammonium oxidation, was extremely low across all samples. Similarly, expression of
311 nitrite oxidase (*nxrAB*) was very low. However, the expression of *nirK* was very high (fig. 2). A
312 phylogenetic analysis placed 96 out of 158 *nirK* assembled ORFs in the archaeal clade and, as in
313 the case of *amoA*, revealed that the archaeal variants recruited more than 95% ± 0.07% (N=16)
314 of the reads that mapped to *nirK* (fig. 3B). Three archaeal phylotypes dominated *nirK*
315 expression, with 76% ± 0.07% of all reads mapped to *nirK*.
316 To determine whether the high expression of archaeal nitrification genes can be
317 explained by abundance of AOA compared to AOB, we searched our *16S rRNA* gene amplicons
318 (Nuccio et al., 2020) for known AOA and AOB. The relative abundance of AOA *16S rRNA* genes
319 was 2- to 5-fold higher than for AOB, and the relative abundance of AOB was consistently
320 higher than that of nitrite-oxidizing bacteria (NOB) (fig. 3C). The aggregate expression of *amoA*
321 and *nirK* was well correlated with a ratio of *nirK*:amoA = 1.6 (R^2=0.7, p<0.0001) (fig. 3D). In
322 addition, the aggregated expression of amoA was also highly correlated to that of
323 Thaumarchaeal extracellular proteases (Sieradzki et al., 2020) (adjusted R^2=0.61, p<0.0001).

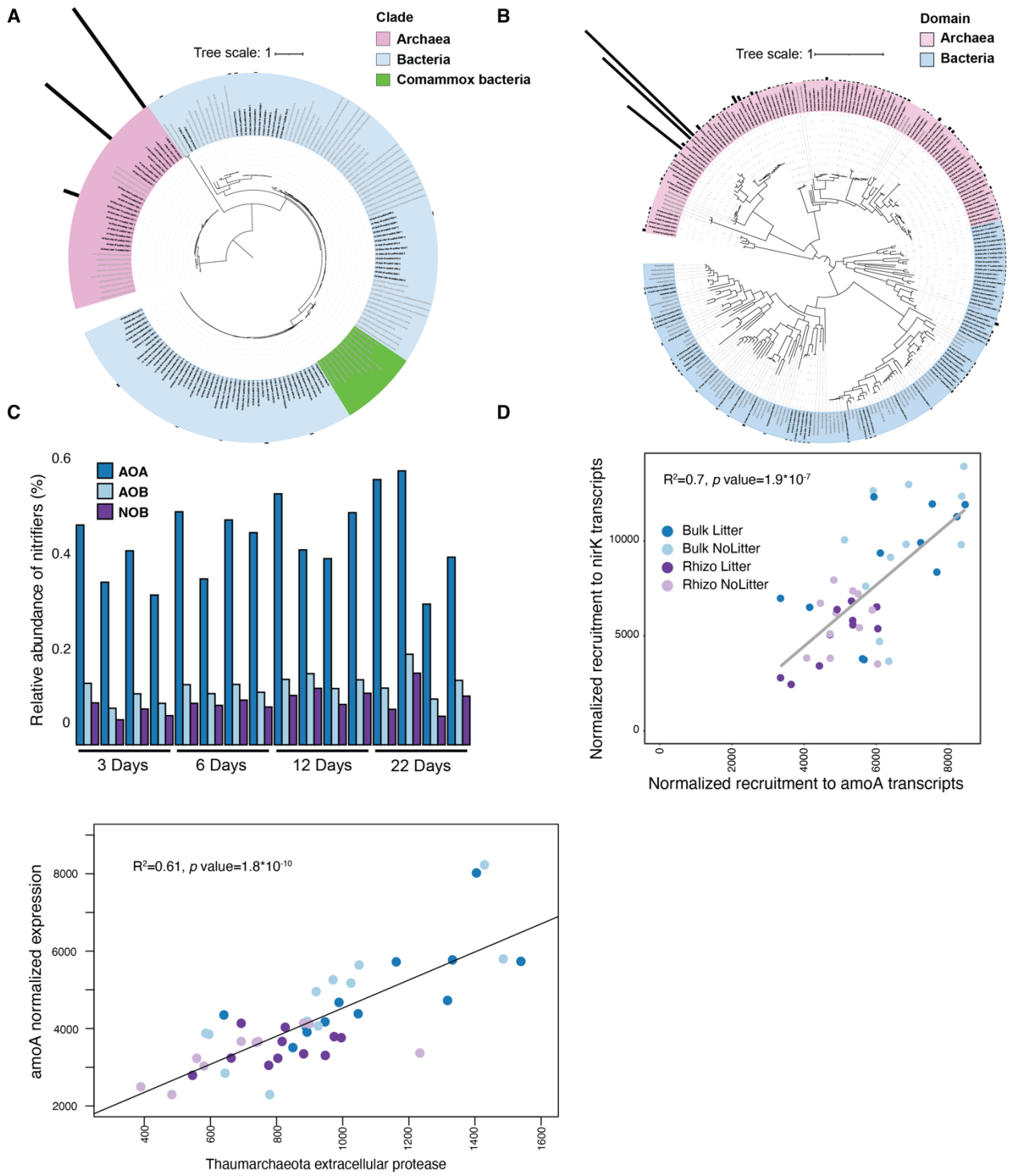
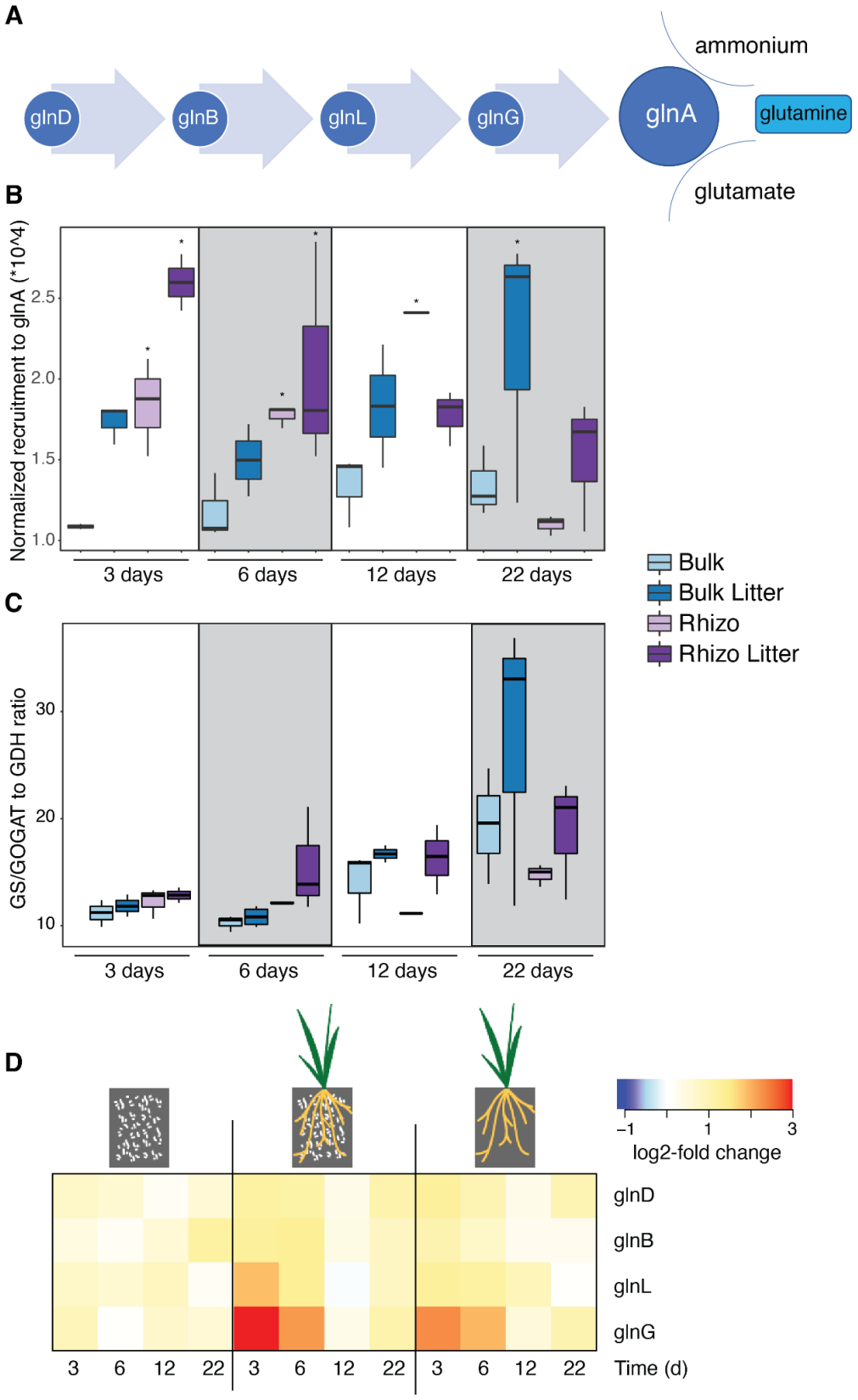


Figure 3: Phylogenetic determination of nitrifiers by *amoA*, *nirK* and *16S-rRNA*. Phylogeny of assembled ORFs (black) (A) *amoA* and (B) *nirK* placed into phylogenetic trees containing curated reference sequences from RefSeq (grey). Colors represent phylogenetic clades. Bars around the

328 trees represent cumulative coverage from all samples to specific assembled transcripts. (C)
329 Relative abundance of AOA, AOB and nitrite oxidizing bacteria (NOB) of the entire microbial
330 community by *16S-rRNA* gene abundance. (D) Positive correlation between expression of *amoA*
331 and *nirK* transcripts by location and amendment (R^2=0.7). (E) Positive correlation between
332 expression of *amoA* and *Thaumarchaeota* extracellular protease transcripts by location and
333 amendment (R^2=0.61).
334
335 *Ammonium assimilation and transport pathways*
336
337 The most highly expressed ammonia acquisition pathway was glutamate synthase
338 (GS/GOGAT), which can be activated by a cascade of four enzymes under nitrogen limitation
339 (fig. 4A) (MacNeil et al., 1982; Bueno et al., 1985; Leigh and Dodsworth, 2007). As opposed to
340 single-gene dependent transporters (*amt*) and glutamate dehydrogenase (GDH), GS/GOGAT is a
341 two-step pathway: the first step is encoded by the gene *glnA*, and the second step either by
342 *glt1, Glu* or the heterodimer *gltBD*. Both ammonium transporters (sup. fig. 1) and GS/GOGAT
343 (*glnA*, fig. 4B) were significantly upregulated in detritusphere microhabitats within the young
344 rhizosphere (Rhizo Litter, 3 days) and bulk soil at the final timepoint (Bulk Litter, 22 days) (fig.
345 4b). *glnA* was also significantly upregulated in the early rhizosphere compared to the bulk
346 control (Rhizo, fig 4B), and three out of four detected proteins involved in GS/GOGAT were also
347 upregulated in the rhizosphere after 3, 6 and 12 days (sup. fig. S3). We found that expression of
348 *gltBD* was generally much higher than that of *glt1*, whereas *Glu* was not detected (sup. fig. 1).
349 Expression of an additional ammonium assimilation pathway, glutamate dehydrogenase (GDH),

350 was low (sup. fig. S1). GDH consists of a single step performed by one of two enzymes: *gdh2* or
351 *gudB*. *Gdh2* was upregulated in the rhizosphere, whereas *gudB* was upregulated in the
352 presence of litter (sup. fig. S1, sup. table S2).

353 In theory, there is an advantage to using GS/GOGAT for ammonia assimilation over GDH
354 under N limitation, as GS/GOGAT in a one-way pathway, whereas GDH can also catalyze
355 deamination of glutamate which might lead to loss of ammonium via diffusion. Expression of
356 GS/GOGAT was an order of magnitude higher than that of GDH under all experimental
357 conditions (fig. 4C). The ratio of expression (GS/GOGAT to GDH) significantly increased over
358 time (Permanova ratio~group*time, p(time)=0.002). In the young rhizosphere the ratio
359 appeared to be higher in the rhizosphere compared to bulk soil, whereas in the mature
360 rhizosphere, litter seemed to trigger a higher ratio (fig. 4D). Expression of the GS/GOGAT
361 regulatory proteins *glnD, glnG and glnL* was significantly upregulated in the rhizosphere
362 compared to bulk soil, particularly in the young rhizosphere (Table S3). Most regulatory genes
363 were also upregulated in the detritusphere compared to unamended soil at the last time point.


A
ammonium
glnD
glnB
glnL
glnG
glnA
glutamine
glutamate
B
Normalized recruitment to glnA (*10^4)
2.5
2.0
1.5
1.0
3 days
6 days
12 days
22 days
Bulk
Bulk Litter
Rhizo
Rhizo Litter
C
GS/GOGAT to GDH ratio
30
20
10
3 days
6 days
12 days
22 days
D
−1
1
3
log2-fold change
glnD
glnB
glnL
glnG
3 6 12 22 3 6 12 22 3 6 12 22
Time (d)

364

365 **Figure 4: Expression of ammonium assimilation pathways and their regulatory genes.** (A)
366 Simplified conceptual representation of *glnA* activation via the regulatory cascade (B)
367 Normalized expression of *glnA* in amended/unamended rhizosphere and bulk soil. Asterisks
368 denote a significant increase compared to the same litter treatment in bulk soil (e.g.,
369 unamended rhizosphere vs. unamended bulk) (C) ratio of GS/GOGAT to GDH (*glnA*:*gudB)*
370 expression in the metatranscriptomes. T. (D) Mean differential expression compared to
371 unamended bulk soil of regulatory genes that activate GS/GOGAT under N limitation as well as
372 *glnA*.
373
374 **Discussion**
375
376 In this study we used metatranscriptomic gene expression (% of reads mapped to a
377 gene) and upregulation (fold change of expression in a habitat compared to unamended bulk
378 soil) to identify contemporaneous N transformations in the main soil habitats: rhizosphere
379 (near live roots), detritusphere (near dead roots), rhizo-detritusphere (live and dead roots) and
380 bulk soil (neither). We found that expression of genes coding for N-cycling processes varied
381 between soil habitats as well as over time. The use of metatranscriptomic analyses allowed us
382 to track multiple N cycling pathways occurring at the same time. Moreover, as N pools in this
383 grassland soil are relatively constant but fluxes are high, it has been suggested that studying N
384 cycling parameters may be a better proxy than pools (Sudderth et al., 2012).
385 Habitat and temporal patterns of N cycling gene expression were interrelated. Effects of
386 habitat on gene expression were detected at the first three sampling times, but not after 22

387 days. At this time point, the soil was much drier due to plant transpiration (Nuccio et al., 2020).
388 This decrease in soil moisture likely affected rates of substrate diffusion as well as microbial
389 mobility, leading to changes in the environmental milieu experienced by the microbes.
390 Removing the last time point from analyses, revealed strong effects of roots and litter as well as
391 the interaction between the two, implying that rhizosphere bacteria respond to litter
392 amendment differently than bulk soil bacteria. At 22 days, the roots present in the rhizosphere
393 treatment may have begun senescence and degradation processes more similar to that of root
394 litter. . Moreover, root exudation decreases as the root senesces, thus likely reducing the effect
395 of simple carbon sources originating from exudation as well as driving usage of litter, which is a
396 less labile C source (Shi et al., 2018).
397 Several N cycling pathway genes were differentially expressed in different soil habitats.
398 In the rhizosphere we identified upregulation of assimilatory nitrate reduction and ammonium
399 assimilation through glutamate dehydrogenase (GDH; *gdh2*) and glutamine synthetase
400 GS/GOGAT; *glnA, glt1, gltB*). An additional GDH enzyme, *gudB*, was upregulated in the litter-
401 amended rhizosphere. Three of four regulatory enzymes that activate GS/GOGAT under N
402 limitation were also upregulated in the rhizosphere, and the fourth was upregulated in the
403 litter-amended rhizosphere. The upregulation of these pathways likely indicate that the
404 rhizosphere microbial community experiences inorganic N limitation. N limitation can stem
405 from competition over inorganic N with the plant (Jackson et al., 1989b; Schimel et al., 1989), as
406 well as from a higher demand for N as microbial density is ca. 10X higher in the rhizosphere
407 compared to bulk soil (Pett-Ridge et al., 2021). *Avena* plants have been shown to be better

408 competitors than their rhizosphere community for nitrate, potentially explaining why
409 rhizosphere microorganisms would upregulate nitrate reduction to ammonium.
410 Pathways upregulated in the detritusphere compared to bulk soil were dissimilatory
411 nitrate reduction (DNRA), denitrification, ammonium transporters, mineralization of organic N
412 and ammonium assimilation via GDH (*gudB*) and GS/GOGAT (*glnA*, *gltD*). Dissimilatory
413 processes, often overlooked in soil N cycling studies (Moreau et al., 2019), can eventually lead
414 to loss of gaseous nitrogen from the system either as N_2, N_2O or NO. As N is commonly a
415 limiting nutrient in the rhizosphere, coveted by both plants and microbes, dissimilatory
416 processes might be disadvantageous in this habitat. Indeed, plants have been demonstrated to
417 outcompete nitrate reducing bacteria for nitrate (Moreau et al., 2015) and there is some
418 evidence that root exudates can inhibit denitrification (Dassonville et al., 2011). Some of the
419 genes involved in dissimilatory processes were also significantly upregulated in litter-amended
420 bulk soil compared to litter-amended rhizosphere, also suggesting the inhibition of N-loss
421 processes in rhizosphere soil. Expression of assimilatory processes that were upregulated in the
422 detritusphere compared to bulk soil (*gudB, gltD*) was higher than that of any DNRA or
423 denitrification genes. Assimilatory processes are possibly a more common strategy for N
424 acquisition in the detritusphere, and their upregulation compared to unamended soil is likely
425 driven by carbon availability from root litter to maintain C:N cellular stoichiometry. Compared
426 to bulk soil, both detritusphere and rhizosphere have a much higher oxygen consumption due
427 to C utilization and biomass increase. However, in the rhizosphere there is extreme competition
428 over nitrate against the plant, whereas in the detritusphere the driving force is only C and O

429 consumption which may create anaerobic or microaerobic niches that would support DNRA and
430 denitrification.

431 Bulk soil was characterized by upregulation of genes coding for nitrification, another
432 process that leads to loss of N from the system through generation of nitrous oxide and
433 leaching of nitrate from soil (Prosser et al., 2020). Ammonia monooxygenase subunit C (*amoC*)
434 was also one of the most highly expressed genes in this soil. Interestingly, the expression of
435 subsequent steps in bacterial nitrification (*hao, nxrA, nxrB*) was extremely low. However,
436 expression of nirK, traditionally considered a part of the denitrification process, was one of the
437 highest we detected. When we explored the phylogeny of detected amoA and nirK variants, we
438 discovered that all highly transcribed variants were archaeal. nirK has been detected in archaeal
439 genomes and the current leading hypothesis is that archaea oxidize hydroxylamine by
440 combining it with nitric oxide (NO), yielding two nitrite molecules, one of which is reduced back
441 to NO by *nirK* (Prosser et al., 2020).

442 While it is possible that conditions are more favorable for nitrification in bulk soil as
443 there is less competition over ammonium in the absence of plant roots, it is also possible that
444 nitrification was downregulated or inhibited in the rhizosphere. Biological nitrification inhibition
445 (BNI) by the exudation of secondary metabolites from plant roots that can suppress ammonia
446 oxidation by nitrifying microorganisms (Subbarao et al., 2007, 2013), thus increasing the
447 availability of ammonium to plants. Hence it is proposed as one strategy to increase crop yields
448 and reduce fertilizer loss (Coskun et al., 2017). Root exudates of *A. fatua* have been shown to
449 inhibit nitrification by ammonia oxidizing bacterium *Nitrosomonas europea* (O'Sullivan et al.,
450 2017). If *A. fatua* inhibited ammonia oxidizing archaea in order to increase ammonium

451 availability to its roots, it would likely have appeared as upregulation in bulk soil. Given that
452 microbes in the rhizosphere appear to be experiencing N-limitation, our observations from the
453 rhizosphere and bulk soil may be partially explained by competition over ammonium between
454 rhizosphere microorganisms and the plant.

455 Despite the low relative abundance of AOA, archaeal genes for ammonia
456 monooxygenase (*amoABC*) were among the most highly expressed out of all N-cycling
457 transcripts in this study across time, location (bulk soil vs. rhizosphere) and presence or absence
458 of litter amendment. The relative abundance of AOA in our study based on 16S-rRNA gene
459 abundance, while never exceeding 1% of the microbial community, was several-fold higher than
460 that of ammonia oxidizing bacteria (AOB) and matches previous results from other grasslands
461 (Leininger et al., 2006; Nicol et al., 2008; Zeglin et al., 2011). While they may be very slow
462 growers, AOA can be highly metabolically active in soil (Pratscher et al., 2011). Moreover,
463 transcription of archaeal *amoA* has also been shown to be correlated to nitrification rates
464 (Zhang et al., 2010; Pratscher et al., 2011; Orellana et al., 2019). A similar experiment using a
465 different species of *Avena* demonstrated that gross nitrification rates decreased with
466 rhizosphere age (Herman et al., 2006). This pattern further supports correlation between *amoA*
467 expression and nitrification rates, and could reflect increasing competition over labile N as well
468 as reduced root exudation by the plant in aging rhizosphere (Jackson et al., 1988; Hu et al.,
469 2001; Zhalnina et al., 2018).

470 Expression of *amoA* has been previously shown to be dominated by few archaeal
471 phylotypes (Tourna et al., 2008; Offre et al., 2009; Gubry-Rangin et al., 2011). Not only do our
472 results support this, but they quantitatively show that on average 98% of the reads mapped to

473 *amoA* were assigned to three variants, all archaeal. All three phylotypes were within the
474 *Nitrososphaera* clade, commonly found in soil (Tolar et al., 2017). Similarly, *nirK* expression was
475 also dominated by three variants, but surprisingly these were most closely related to another
476 soil AOA genus – *Nitrosocosmicus*. Additionally, expression of *amoA* and *nirK* is well correlated.
477 It is possible that Nitrososphaera are performing ammonium oxidation and *Nitrosocosimus*
478 oxidizing the product. While there are ammonia oxidizing and nitrite oxidizing bacteria, such a
479 cooperation is not known in archaea. However, it is also possible that the taxonomic placement
480 of one of the genes is wrong. Both *amoA* and *nirK* have been identified in curated genomes of
481 AOA (Jung et al., 2014; Bayer et al., 2016; Carini et al., 2018) which raises the possibility that the
482 same AOA cell can enact both steps of nitrification (Kozlowski et al., 2016; Reji et al., 2019), but
483 dual expression of amoA and nirK by AOA has not yet been shown experimentally. Our strong
484 correlation suggests that there may be a relationship between these two processes, but further
485 experimentation is required to determine if they are mechanistically linked.
486 Thaumarchaeal extracellular proteases identified in the same dataset were previously
487 found to be upregulated in bulk soil (Sieradzki et al. 2022) and their temporal expression
488 patterns highly correlated with *amoA* expression. Thaumarchaeota have been shown to prefer
489 ammonium from organic nitrogen sources such as urea, amino acids and peptides in forest soil
490 (Levičnik-Höfferle et al., 2012). As AOA have to compete with the plant over ammonium in the
491 rhizosphere, they may turn to organic N instead (Craine et al., 2007; Blagodatskaya and
492 Kuzyakov, 2008), explaining the tight correlation between *amoA* and AOA proteases.
493 Ammonium assimilation via glutamate synthase (GS/GOGAT) is generally thought to be
494 preferred to glutamate dehydrogenase (GDH) under N limitation due to the K_m of GS/GOGAT

495 which is an order of magnitude lower than that of GDH (Sakamoto et al., 1975; Alibhai and
496 Villafranca, 1994). In our study GS/GOGAT gene expression was widespread across treatments
497 and over time, with significant upregulation in response to both roots and litter. Moreover, the
498 expression of GS/GOGAT was consistently at least an order of magnitude higher than that of
499 GDH. The first step of the GS/GOGAT pathway, *glnA* (glutamine synthetase), was one of the
500 most highly expressed genes in our study. The P_{II} regulatory protein *glnB* which is activated
501 during N limitation and activates GS/GOGAT over GDH (Huergo et al., 2013) was also highly
502 expressed under all experimental conditions and upregulated in the presence of live or dead
503 roots. In contrast, expression of single-enzyme pathway glutamate dehydrogenase (GDH) was
504 much lower. Assuming comparable downstream post-transcriptional regulation, a preference
505 for multi-step unidirectional pathway GS/GOGAT (Hochman et al., 1988) as opposed to the
506 bidirectional GDH, which could also perform glutamate deamination (Melo-Oliveira et al.,
507 1996), could result from nitrogen limitation, as it prevents nitrogen loss from the organism
508 (Yuan et al., 2009).

509 We identified differences in expression of GS/GOGAT and GDH between habitats.
510 Expression of *glnA* (GS/GOGAT) was higher in the rhizosphere compared to bulk soil at 3, 6 and
511 12 days, and was higher in litter amended soil compared to unamended both in bulk soil and in
512 the rhizosphere. Its expression was highest in the presence of both live and dead roots (litter-
513 amended rhizosphere) at 3 and 6 days, indicating an additive effect of live roots and root litter.
514 This effect may be due to high resource demand when C is available by both litter and
515 exudates, which exacerbated the demand for N to maintain cellular stoichimetry (Zhu et al.,
516 2014; Moreau et al., 2019). Expression of carbohydrate active enzymes (CAZy) in the same soil

517 was also higher in the young rhizosphere and decreased over time and expression of
518 extracellular protease genes was highest at 3 days (Nuccio et al., 2020; Sieradzki et al., 2020).
519 This additive effect, generally accepted to be short-term (Kuzyakov et al., 2000), disappears by
520 6 days of rhizosphere maturation.

521 Temporal effects of ammonium assimilation gene expression varied by gene, pathway
522 and habitat. For example, the two GDH genes we identified were upregulated in different
523 habitats: rhizosphere (*gdh2*) and detritusphere (*gudB*). The expression of both genes decreased
524 over time, possibly because ammonium concentrations dropped to a concentration too low for
525 the affinity of this enzyme (Alibhai and Villafranca, 1994). The expression of *glnA* (GS/GOGAT)
526 tells a more complicated story. In the litter-amended rhizosphere its expression decreases over
527 time, as does the expression of its regulatory genes. However, in the litter-amended bulk soil
528 the expression of *glnA* increases from day 6 on. The consistently lower expression of this
529 enzyme in unamended bulk soil implies that any C input, be it from rhizodeposition of root
530 litter, creates a demand for N that is at least somewhat alleviated via the GS/GOGAT pathway.
531 Finally, we posited that there is a trade-off between the GDH and GS/GOGAT pathways that
532 corresponds to nitrogen limitation. Therefore, we examined the ratio of gene expression
533 between *glnA* (GS/GOGAT) and the more highly expressed of the GDH genes, *gudB*. This ratio
534 was consistently higher than 10, implying a general preference for GS/GOGAT, possibly due to
535 low N availability at all times with or without the presence of roots or root litter. This ratio also
536 increased over time under all experimental conditions, but this increase began earlier in the
537 litter-amended rhizosphere. However, by 22 days the highest ratio was in litter-amended bulk

538 soil, possibly because growth rates are slower in bulk soil compared to the rhizosphere (Pett-
539 Ridge et al., 2021) and therefore N limitation developed slower.
540
541 **Conclusions**
542 Here we present a comprehensive analysis of expression of all nitrogen cycling pathways
543 over time and in the main soil habitats: rhizosphere, litter-amended rhizosphere, litter-
544 amended bulk soil and unamended bulk soil. We propose several classes of controllers on the
545 expression of nitrogen cycling genes in soil with or without roots and/or root litter. Processes
546 that lead to loss of N from the system were downregulated near live roots. Availability of
547 carbon (C) and the quality of that C , whether from root exudates or root litter, could drive N
548 uptake to maintain cellular stoichiometry, and proximity to live roots can lead to competition
549 for inorganic nitrogen. The use of metatranscriptomics allowed us to track multiple pathways
550 and identify trade-offs between them which are likely based on N availability and limitation.
551
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553
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