# Response to nitrogen addition reveals metabolic and ecological strategies of soil bacteria

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Running head: Community successions in N treated soils

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**Abstract**

The nitrogen (N) cycle represents one of the most well studied systems yet the taxonomic diversity of the organisms that contribute to it is mostly unknown, or linked to poorly characterized microbial groups. While progress has allowed functional groups to be refined, they still rely on *a priori* knowledge of enzymes involved, and the assumption of functional conservation, with little connection to the role the transformation plays for specific organisms. Here, we use soil microcosms to test the impact of N deposition on prokaryotic communities. By combining chemical, genomic and transcriptomic analysis we are able to identify and link changes in community structure to specific organisms catalyzing given chemical reactions. Urea deposition led to a decrease in prokaryotic richness, and a shift in community composition. This was driven by replacement of stable native populations, which utilize energy from N-linked redox reactions for physiological maintenance, with fast responding populations that use this energy for growth. This model can be used to predict response to N disturbances and allows us to identify putative life strategies of different functional, and taxonomic, groups thus providing insights into how they persist in ecosystems by niche differentiation.

## Introduction

Modern microbiology techniques have given us unprecedented access to the microbial world (Spiro 2012; Rinke *et al.* 2013), yet soil microbial communities remain poorly understood (Delmont *et al.* 2015). While many studies have focused on the diversity or abundance of key populations (Taylor *et al.* 2012; Wei *et al.* 2015; Gubry-Rangin *et al.* 2015), fewer have looked at the transcriptional profiles over time (Nicol *et al.* 2008; Morales & Holben 2013), and even less have done so for multiple groups at the same time (Liu *et al.* 2010; 2014; Brenzinger *et al.* 2015). This is particularly true of organisms involved in nitrogen (N) cycling in soils. The complexity of the underlying processes combined with the diversity of microbes contributing to each process provides a large challenge to identifying mechanisms active at any given time (Butterbach-Bahl *et al.* 2013). Currently we lack enough information to understand basic ecological concepts linked to N cycling *in situ* such as: i) substrate competition at both inter and intra species level, ii) full diversity of both present and active N cycling populations, iii) and the life strategies of these populations which in turn control their responses (both as observed growth or transcriptional changes).

The initial discovery of ammonia oxidizing archaea (AOA) and recognition as important players in the N cycle (Leininger *et al.* 2006; Hatzenpichler 2012; Stahl & la Torre 2012) highlighted the unexpected gaps in knowledge. Later studies have suggested different life strategies for AOA when compared to ammonia oxidizing bacteria (AOB) (Sterngren *et al.* 2015), but this may be complicated by variance across strains (Bayer *et al.* 2015). One major unknown is whether observations made in studies, or organisms, from one ecosystem translate to others.

It is well established that individual intermediates in the N cycle can be used for specific reasons (i.e. ammonia oxidation provides electrons, while denitrification intermediates accept reducing equivalents), but the purpose of the reactions for any organism is another major unknown. That is, while some organisms carry out these processes for electrogenic purposes that can result in growth, others do it in order to maintain redox homeostasis (e.g. to dissipate excess reductants) (Green & Paget 2004). Unfortunately examples where an organism harbours multiple versions of the same enzyme for completely different purposes (respiration vs. redox balance) exist (Hartsock & Shapleigh 2011), and are likely to limit generalizations.

Despite this, studies focusing on population changes in response to manipulations have consistently recorded conserved patterns (e.g. growth of AOB but not AOA (Jia & Conrad 2009; Di *et al.* 2009; Pratscher *et al.* 2011)) suggesting that responses by specific populations in a given location or ecosystem are predictable. However, the debate continues on whether niche specialization and differentiation can be determined based solely on correlations, without analyzing the wider array of processes that contribute or influence any given N transformation (Prosser & Nicol 2012). This is relevant in ecosystems such as agricultural grassland where an understanding of N cycling is crucial for management of both productivity and greenhouse gases (Herrero *et al.* 2016), of which nitrous oxide ($N_2O$) is a key player (Reay *et al.*, 2012).

In grazed pastures (i.e. agricultural grasslands) N deposition through ruminant urine drives the emissions of $N_2O$ (Saggar *et al.* 2013). In this system a full cascade of transformations begin with urea and can result in accumulation of any intermediate depending on conditions, but with a final end product of $N_2$ or $N_2O$. While the chemical transformations have been explored (Hamonts *et al.* 2013; Baral *et al.* 2014;

de Klein *et al.* 2014a; b), mechanistic understanding of the populations catalyzing the reactions, and the purpose they serve for the organisms is less clear. In this study, we aimed to identify active N-transformation pathways as well as changes in microbial populations/taxa abundance and transcriptional activity for organisms involved in N loss (through gases) in response to urea (simulated ruminant urine deposition event) and varying moisture content. Observed chemical transformations were linked to changes in genotype (functional potential through DNA; a proxy for population changes), expression of genotype (RNA profiles), and total community composition (specific taxonomically defined populations based on the 16S ribosomal rRNA gene). We hypothesized that sequential transformation of nitrogenous intermediates would be coupled to changes in expression of functional genes catalyzing production and consumption of intermediates. Alternatively, transformations not linked to population, or expression changes, would be driven by other (abiotic) pathways. We also hypothesized that changes in transcription, or population size, could serve to determine life strategies of microbes utilizing each intermediate (whether they are used for growth vs. physiological maintenance). To test this we mimicked a ruminant urine-N deposition event using repacked soil cores (soil bulk density= 1.1 Mg $m^{-3}$) on tension tables monitored for 63 days. Soils were treated with urea under two different moisture contents: high (near saturation; -1.0 kPa) and low (field capacity; -10 kPa) moisture. Simultaneous measurements of soil chemistry, gas kinetics, microbial community composition (by 16S rRNA gene amplicon sequencing) and functional gene abundance (for nitrification and denitrification) at DNA (gene) and RNA (transcript) levels were performed to determine the active populations and pathways.

## Materials and methods

*Sample collection and experimental design*

A detailed methodology can be found in (Clough *et. al*., In review). In brief, soil was collected from a permanently grazed agricultural grassland (dairy pasture) in March (early spring) at the Teagasc Moorepark Research Centre, County Cork, Ireland ($8^{o}15$'W, $52^{o}9$'N). The soil is classified as a Typical Brown earth from the Clashmore Series (Gardiner & Radford 1980). Soil was sampled after the turf was removed and a spade was used to randomly sample the A-horizon (5-20 cm depth, excluding grass layer). To avoid fresh N loading, fields had not been grazed for over a month. Field moist samples were immediately shipped to Lincoln University, New Zealand and kept at $4^{o}$C until processed. Prior to use, soil was sieved (≤ 2 mm) to remove any stones, plant roots or earthworms and packed into stainless steel rings (7.3 cm internal diameter, 7.4 cm deep) to a depth of 4.1 cm at *in situ* soil bulk density (1.1 Mg $m^{-3}$ with a gravimetric water content ($\theta g$) of 0.24 g water $g^{-1}$ soil). The resulting cores had a total porosity of 0.58 $cm^3$ pores $cm^{-3}$ soil and were arranged in a factorial experiment replicated four times. Soil cores were maintained at two moisture contents: high (near saturated; -1.0 kPa) and low (field capacity; -10 kPa) moisture using tension tables (Romano et al., 2002). These moisture contents, -1 and -10 kPa respectively, corresponded to 53% and 30% volumetric water content, or 91% and 52% water-filled pore space (WFPS). Nitrogen was applied as a urea solution at 2141 kg urea/ha dry soil (equivalent to a single urination event at the higher rate expected under bovine urine deposition of 1000 kg N $ha^{-1}$). Four treatments in total were carried out (replicated four times each for a total of 112 cores analyzed) representing two levels of urea and two levels of moisture: urea + high moisture (HM +N; Urea _-1.0kPa), urea + low moisture (LM +N; Urea _-10kPa), no urea + high moisture (HM

139 –N; No Urea _-1.0kPa) and no urea + low moisture (LM –N; No Urea_-10kPa). All
140 cores where held at 20$^{o}$C for a period of 63 days.
141
142 *Soil pH, and inorganic-N measurements*

143 Soil pH was monitored throughout the experiment using a flat surface pH
144 electrode (Broadley James Corp., Irvine, California). Inorganic N concentrations
145 ($NH_4^+$, $NO_2^-$, $NO_3^-$) were determined by destructively sampling batches of soil cores.
146 Each core was homogenized and a subsample was extracted (10 g dry soil: 100 ml 2M
147 KCl shaken for 1 hour), filtered (Whatman 42) and analyzed using flow injection
148 analysis (Blakemore et al., 1987). $N_2O$ flux was determined by placing a soil core into
149 a 1-L stainless steel tin fitted with a gas-tight lid and rubber septa. The headspace was
150 sampled after 15 and 30 minutes and analyzed using an automated gas chromatograph
151 (8610; SRI Instruments, Torrance, CA), linked to an autosampler (Gilson 222XL;
152 Gilson, Middleton, WI) as previously described (Clough et al., 2006).

153 *Nucleic acids extraction*

154 Samples for RNA and DNA extraction were collected simultaneously with
155 samples for inorganic N analysis, but only samples at 0, 7, 14, 21, 35, 63 days were
156 processed for nucleic acids. Each biological replicate was extracted and analyzed
157 separately. For each extraction 2 g (wet weight) of soil were processed using the
158 PowerSoil Total RNA Isolation and DNA Elution Accessory Kits (MoBio, Carlsbad,
159 CA) as per manufacturer's instructions, with slight modifications. Bead beating was
160 done in a Geno/Grinder 2010 (SPEX SamplePrep, LLC, Metuchen, NJ) using two
161 rounds of beating (1750 strokes/min) for 15 s with a 1 min pause in between. The
162 total elution volume for RNA and DNA was 60 µl and 100 µl respectively. RNA was

163 treated with DNase I (RNase-Free) (New England Biolabs, USA) as per the
164 manufacturer's protocol. RNA quality was assessed by denaturing gel electrophoresis.
165 RNA and DNA concentration, purity and humic acid contamination were determined
166 using a Nanodrop Spectrophotometer, ND-1000 (Thermo Scientific). All extractions
167 were stored at -80 $^{o}$C until downstream analyses.

168

169 *Reverse transcription (RT)*

170 Triplicate cDNA conversions (technical replicates) were performed for each
171 RNA extraction using the Maxima H Minus First Strand cDNA Synthesis Kit
172 (Thermo Scientific) according to manufacturer's protocol. Each 20 µl reaction
173 contained: 13 µl of RNA (208 ng Total RNA), 1 µl of random hexamers (100 pmol),
174 1µl of dNTP mix (0.5 mM final conc.) and 5 µl of master mix (4 µl of 5X RT buffer
175 and 1 µl Maxima H Minus reverse transcriptase). All technical replicates for a sample
176 were combined and stored at -80$^{o}$C until further analysis. All further analyses were
177 performed on the same cDNA pool for each sample.

178

179 *16S rRNA gene amplicon sequencing*

180 16S rRNA gene amplicon sequencing was performed using primers
181 515F/806R (V4 region of the 16S gene) and the Earth Microbiome Project conditions
182 (Version 4_13) (Caporaso *et al.* 2012). All samples were run simultaneously on a
183 single Illumina MiSeq run. Sequences were first processed in Qiime (version 1.9.1)
184 using default parameters (Caporaso *et al.* 2010). Sequences were clustered into
185 Operational Taxonomic Units (OTUs) at 97% sequence similarity using the SILVA

(version 119) reference library (Quast *et al.* 2012) and UCLUST (Edgar 2010) following the open-reference Operational Taxonomic Unit (OTU) picking protocol. Taxonomic identification was done using BLAST against the SILVA database (max-e value = 0.001) (Altschul *et al.* 1990). Subsampling and rarefactions (10 times) were performed to equal read depths of 7,400 per sample, and samples below that threshold were removed. After rarefaction, all 10 OTU tables were merged and exported for further processing in R (R Development Core Team 2008). The 16S amplicon sequences are available in the NCBI SRA database (accession numbers SRP091980).

*Quantification of gene and transcript abundance*

Quantitative PCR (qPCR) was performed in 384-well plates using the ViiA7 real-time PCR system (Applied Biosystems, Carlsbad, CA). Absolute quantification was done using a 10-fold serial dilution ($10^8$ to $10^1$) of known copy numbers of pGEM-T easy (Promega, Madison, Wisconsin, USA) cloned templates as standards. For all targets qPCR runs included cloned standards, no template control and no reverse transcription controls (RNA) run in triplicate. No inhibition or positive amplification on negative controls was observed for any target. All DNA and cDNA samples were run in quadruplicates to determine abundance of: prokaryotes (16S rRNA gene), ammonia oxidizers (archaeal [AOA] & bacterial [AOB] ammonia monooxygenase gene; *amoA*), denitrifiers (cytochrome cd1-type nitrite reductase gene; *nirS*, and Clade I nitrous oxide reductase gene; *nosZI*) and nitrogen fixers (nitrogenase gene; *nifH*).

All reactions were performed in 10 µl volumes containing: 1× Master Mix (Fast SYBR Green Master Mix, ABI), 0.2-0.6 µM of each primer [0.2 µM for AOA

(Tourna *et al.* 2008), 0.6 µM for AOB (Rotthauwe *et al.* 1997; Avrahami *et al.* 2003); 0.5 µM for 16S rRNA (Hartman *et al.* 2009); *nirS* (Throbäck *et al.* 2004; Yergeau *et al.* 2007), *nosZI* (Henry *et al.* 2006) & *nifH* (Rösch & Bothe 2005)], 2 µl of template [DNA (1 ng total) or cDNA (80× diluted RT reaction, i.e. total 0.13 ng RNA)] and autoclaved Milli-Q $H_2O$ to a final volume of 10 µl. Primers and qPCR conditions are summarized in Table S1. A melt curve analysis (95$^{o}$C for 15 s, 60$^{o}$C for 1 min then increasing 0.05$^{o}$C/s (data acquisition) until 95$^{o}$C) was performed to test for specificity and to confirm no amplification in the negative controls.

*Statistical analyses*

All statistical analyses were performed in R (R Development Core Team 2008) using the phyloseq (McMurdie & Holmes 2013), pvclust (Suzuki & Shimodaira 2006), vegan (Oksanen *et al.*) and mpmcorrelogram packages. Detailed descriptions can be found in supplemental methods.

*Growth rate estimation and prediction of rRNA operon (rrn) copy numbers*

*rrn* copy numbers for identified OTUs were predicted using the ribosomal RNA operon copy number database (rrnDB) (Stoddard *et al.* 2015). For each OTU, information from the closest strain available was selected. In instances where a closely related organism was not available, the mean copy number for the closest taxonomic group (i.e. genus, class, etc.) was used. Copy numbers where then compared to the maximum observed abundance and the maximum observed fold change (calculated based on lowest observed abundance for the same organism in a preceding time point for OTUs showing growth or succeeding time points for those

234 decreasing in abundance). An estimated growth rate was calculated for OTUs
235 showing increases in population size in response to N using the following formula:

236 $$N_t = N_{0*}e^{rt}$$

237

238 where: $N_t$: The amount at time t; $N_0$: The amount at time 0; r: exponential growth rate;
239 t: Time passed

240 *Fit model for rrn copy numbers*

241 Both non-linear (Michaelis-Menten) and linear regressions were used to fit *rrn*
242 copy numbers and population changes (i.e. maximum abundance and fold-change),
243 and growth rate (per day). The fit model was performed in R using "drc" and
244 "ggplot2" packages.

245

246 **Results:**

247 *Soil pH and N transformation dynamics in response to urea*

248 Soil pH increased from acidic (pH = 5.5 ± 0.1, i.e. mean ± SD) to alkaline
249 reaching a maximum (pH = 8.7 ± 0.2) at day 3 in urea treated soils. Return to baseline
250 pH was modulated by soil moisture with high moisture (HM; -1.0kPa) soil reaching
251 baseline at day 35 and low moisture soils (LM; -10kPa) doing so at day 53 (Fig. 1).
252 This shift in pH was linked to a successive N transformation process initiated with
253 urea hydrolysis and leading to nitrification and denitrification: urea → $NH_4^+$ → $NO_2^-$
254 → $NO_3^-$ → $N_2O$ → $N_2$ (Fig. 1). Sequential peak activity was observed for each
255 transformation with the response modified by moisture. Maximum production (mean
256 µg N $g^{-1}$ soil) for each transformation was observed at day 3, 21 and 35 respectively

for $NH_4^+$ (HM+N = 1758; LM+N= 1730), $NO_2^-$ (HM+N = 79.2; LM+N= 39.7) and $NO_3^-$ (HM+N = 429.2; LM+N= 335). Two distinct production peaks were observed for $N_2O$, with a short pulse (0 to 5 days) reaching a maximum at day 2 for HM soils (11602.8 µg $m^{-2}$ $h^{-1}$) and day 3 for LM soils (46.8 µg $m^{-2}$ $h^{-1}$) (Fig. 1 and Supplementary Fig. S1). A second, longer duration (10 to ~50 days), $N_2O$ pulse reached a maximum at day 28 for HM soils (6405.1 µg $m^{-2}$ $h^{-1}$) and day 30 for LM soils (448.9 µg $m^{-2}$ $h^{-1}$). The large $N_2O$ spike (first peak) between days 0 to 5 in the HM+N treatment was about 11.6% of the total $N_2O$ cumulative flux over 63 days, whereas in the LM+N treatment the 0 to 5 day periods accounted for 22.3% of the total $N_2O$ cumulative flux over 63 days.

*Population and transcription dynamics for nitrogen related functional groups*

Significant changes (ANOVA, $p<0.05$) in relative activity (mRNA abundance/16S rRNA gene abundance) were observed promptly between day 0 & 3 for all functional groups (except AOA and N-fixers in HM soil) in response to urea (Fig. 1). However, maximum relative transcription did not match maximum production peaks for corresponding substrates, or products, for each functional group. Nitrifiers (ammonia oxidizers) displayed niche differentiation, with time, length and strength of response differing between bacterial (AOB) and archaeal ammonia oxidizers (AOA). Relative activity of AOA increased (4.6-fold for LM and 1.6-fold for HM) under urea treatments at day 3 only, with a subsequent decrease (-19.3-fold for LM and -7-fold for HM) resulting in lower expression than in untreated soils (Fig. 1). AOB relative activity also increased but was sustained for a much longer period (3-63 days), with maximum activity (>11-fold change) seen at 21 and 35 days for

HM+N and LM+N respectively (Fig. 1). Denitrifiers (both nitrite and nitrous oxide reducers) showed similar responses as AOA, with peak activity at day 3 and a rapid return to baseline, in the case of nitrite reducers decreasing to levels below those observed in non-urea treated soils (Fig. 1). To account for endogenous sources of N, $N_2$ fixers were monitored through the activity of the nitrogenase gene (*nifH*). No significant changes were observed except for day 3 (LM +N only), with a subsequent decrease in activity below background. This decrease below background was observed for all N treated samples.

Changes in the relative contribution to total community composition were calculated by normalizing functional gene abundance to total 16S rRNA gene abundance per sample for each functional group (Fig. 1). The maximum observed relative abundance of each functional group differed for each group (HM and LM respectively): AOB, 19 & 12 %; AOA, 8 & 13 %; *nirS*, 6.3 & 2.9 %; *nosZI*, 3.3 & 3.4 %; *nifH*, 4.7 & 4.32 %. Further, large population changes over time were mostly limited to AOB. Generally, AOB comprised <1 % of the total community, but in response to urea increased up to 29-fold to make up 19 % (day 21 for HM) and 20-fold to make up 12 % (day 35 for LM) of the community in urea treated soils. In contrast, AOA were found at consistently high levels (median=4.2 %) in untreated soils, but numbers decreased >7-fold in response to urea (~1.3 % at least 63 day). Similarly, other functional groups (*nosZI*, *nifH*) decreased or remained stable (*nirS*) in response to urea. Similar patterns for both activity and population changes were observed when absolute values were analyzed (Supplementary Fig. S2).

*N deposition induces both a genotypic and a transcriptional response at the community level that is modified by soil moisture content*

Urea deposition imposed a general negative selective pressure leading to decreases in OTU level prokaryotic diversity (Shannon, -1.2-fold change), richness (-1.5-fold change) and evenness (-1.1-fold change) at DNA level (Fig. 2a, Supplementary Fig. S3). The same pattern was observed when active microbes (based on RNA) were analyzed with decreases in OTU level prokaryotic diversity (Shannon, -1.3-fold change), richness (-1.9-fold change) and evenness (-1.2-fold change). Moisture was found to have a smaller, but significant, effect compared to urea, with LM samples consistently resulting in lower diversity and richness when compared to their HM pairs. Richness and diversity losses were not recovered even after 63 days. In contrast, samples where no urea was applied remained stable (i.e. constant diversity and richness).

Urea deposition significantly altered community structure (Adonis test: $F= 18.04$, $p< 0.001$ for 16S rDNA and $F= 26.27$, $p< 0.001$ for 16S rRNA) as shown in a non-metric multidimensional scaling (NMDS) plot using a Bray-Curtis dissimilarity matrix (Fig. 2b and Supplementary Fig. S4). At both DNA and RNA level community changes along the first axis corresponded with changes in response to urea treatment, with the second axis accounting for changes in moisture. A pvclust analysis (hierarchical clustering with p-values calculated via multiscale bootstrap resampling, Supplementary Fig. S5) confirmed two major clusters [100% AU (Approximately Unbiased) and 100% BP (Bootstrap Probability)] formed by urea treated (HM+N and LM+N samples, excluding day 0), vs. untreated soils (HM-N, LM-N, field samples, and HM+N & LM+N at Day 0). Temporal variance within each cluster was

328 confirmed using a Mantel correlogram analysis (Fig. 2c). Urea treated samples had
329 significant changes in community composition immediately upon treatment (Day 0 to
330 7), with no return to baseline conditions by the end of the experiment. In contrast,
331 untreated samples did not change significantly over time (Supplemental Fig. S6)

332 Changes in community structure were associated with shifts in major
333 taxonomic lineages (Fig. 3). In general, phylum level changes in abundance and
334 transcription where correlated to each other (Supplementary Table S2 and Fig. S7,
335 S8). Urea deposition induced temporal changes in phylum level abundance with
336 observed maximum fold changes per group (HM & LM at DNA level) being:
337 Acidobacteria, -4.6 & -3.7; Actinobacteria, 2.4 & 5.3; Bacteroidetes, 4.6 & 2.2;
338 Candidate Division WS3, -10.5 & -7; Chloroflexi, -2.9 & -2.6; Firmicutes, 10.8 &
339 16.2; Gemmatimonadetes, 2 & 3.3; Nitrospirae, -3.2 & -2; Planctomycetes, -3.7 & -
340 2.5; Thaumarchaeota, -5.2 & -3.6; Verrucomicrobia, -2.5 & -2; Alphaproteobacteria,
341 1.4 & 1.7; Betaproteobacteria, 4 & 2; Deltaproteobacteria, -2.2 & -1.4;
342 Gammaproteobacteria, 1.5 & 2.6. Normalized transcriptional activity (reads of 16S
343 rRNA/reads of 16S rDNA) identified the Firmicutes and members within classes of
344 the Proteobacteria as the most transcriptionally active. While abundant phyla tended
345 to have high levels of normalized transcription, less abundant organisms like the
346 Thaumarchaeota, were observed to have high normalized transcriptional activity
347 especially under background conditions (Supplementary Fig. S7). In contrast, groups
348 traditionally considered slow growers (e.g. Nitrospirae and Gemmatimonadetes) had
349 low normalized transcription. It was also noted that while normalized transcription
350 levels remained stable without urea, N deposition induced changes. These changes in
351 normalized activity did not always match trends observed at individual DNA or RNA
352 level (e.g. Firmicutes).

353

354 *Shifts in N and moisture status trigger OTU response linked to divergent life*
355 *strategies*

356

357 Since Fig. 3 only represents a taxonomic summary of all OTUs (irrespective
358 of their response to treatments), it does not provide a clear indication of who is
359 changing and why. To account for this, urea responsive OTUs were identified
360 independently in RNA and DNA profiles (under each treatment) through a SIMPER
361 analysis. OTUs accounting for 50% of the variance were analyzed (Fig. 4). Response
362 patterns for detected OTUs were conserved between RNA and DNA profiles.
363 However, while some OTUs responded similarly to urea under varying moisture
364 conditions, marked differences were observed with no detectable pattern based on
365 taxonomy.

366 OTUs within the Proteobacteria identified in the SIMPER analysis did not
367 display a conserved response to urea, however when lower taxonomic levels were
368 examined patterns emerged. A consistent positive response was seen for OTUs within
369 the class Betaproteobacteria and the family Hyphomicrobiaceae, amongst others.
370 Positive responses to urea where also observed at the phylum level for the Firmicutes,
371 Bacteroidetes, Actinobacteria, Gemmatimonadetes and Planctomycetes, although the
372 level of response varied across lower taxonomic levels. In contrast, with only some
373 exceptions, OTUs within the phyla Acidobacteria, Verrucomicrobia, Nitrospirae,
374 Candidate Division WS3 (also referred to as candidate phylum Latescibacteria) and
375 the Thaumarchaeota all were negatively impacted by urea deposition.

376 To account for response patterns over time, we focused on OTUs that
377 accounted for 30% of the variance in the SIMPER analysis (36 total), with individual

OTU contributions ranging from 5 to 0.1 percent at the DNA level and 5 to 0.06 percent at the RNA level (Table S3). Temporal patterns were conserved between DNA and RNA profiles (Supplementary Fig. S9, S10), despite differences in absolute abundance. Once again, moisture acted as a modulator of response with the extent of impact dependent on the OTU (Fig. 5 and 6). While most functional groups responded immediately (at both DNA and RNA level), positively affected OTU responses were observed along all time points creating a succession of positively selected organisms. In contrast, negatively affected OTUs all responded within the first 2 time points indicating an immediate negative selective pressure (Fig. 6). Large variances in absolute changes were observed, even within similar organisms (e.g. Pedobacter), with fold changes ranging from -10.5 to 410 across both positively and negatively affected OTUs. Despite this, OTU response was noted to correspond to taxonomy, with both the effect (positive or negative) and the extent of response (fold change or total abundance) in line with predicted ecological growth strategies (r vs. k) predicted for different taxa. To test this, we predicted rRNA operon copy numbers (rrn) for all 36 OTUs and compared them to the observed maximum abundance, max fold change in population or observed growth rate per day. We consistently observed a non-linear response with an asymptote reached at higher copy numbers (Fig. 7). These trends were consistent independent of which moisture conditions were present at the time of response. To account for preferential response due to moisture, we selected the highest response for each organism and saw no clear difference in patterns. To account for potential biases due to uneven representation, OTUs were grouped into low (1-2 copies of rrn) or high (>2) copy number organisms (Supplementary Table S4). While significant changes ($p < 0.05$, Supplementary Fig. S11) were observed in most instances, exceptions were noted (e.g. growth rate under HM).

## Discussion

Functional profiling (identification and quantification of specific functional genes/transcripts) is normally utilized to link chemical transformations to specific microbial populations capable of catalyzing reactions. However, functional groups are comprised of taxonomically diverse species of microbes with different lifestyle strategies that are unlikely to share a conserved response to an ecosystem disturbance (Ho *et al.* 2012). While functional profiling allows us to measure the net response of a functional group, and could serve as a proxy for determining the importance of the group in a sample, it does not identify how specific organisms benefit from a catalyzed transformation. Here we used a controlled microcosm experiment to measure the response of soil communities to a disturbance in the form of changes in moisture and nitrogen (urea) deposition. Functional analysis (qPCR) demonstrated a biological response to urea, but differing responses to moisture depending on group (Fig. 1). Responses are potentially linked to different life strategies amongst these groups. Ammonia oxidizers displayed contrasting population and expression profiles, suggesting niche differentiation driven by time and/or substrate concentration. AOA responded early, and declined as new N was made available while AOB responded later with population swings spanning from near detection limit to most dominant group. These observations match prior reports showing AOA prefer low N concentrations, while AOB respond vigorously to N deposition (Di *et al.* 2010; Sterngren *et al.* 2015). This has been interpreted as evidence for differing lifestyles for AOB and AOA, with AOA preferring nutrient poor conditions and AOB dominating in rich ones (Sterngren *et al.* 2015). However, prior assertions that AOB are solely important for driving nitrification might be overstated given that transcriptional activity for both groups is comparable if compared at peak time (Di *et*

428 *al.* 2009). This contrasting use of energy between functionally redundant organisms
429 might explain the low correlations between processes and the abundance of their
430 respective functional populations (Rocca *et al.* 2015). When we examine the response
431 of other functional groups benefiting from influxes of N, like denitrifiers, we see no
432 significant change in population sizes suggesting that either energy is being utilized
433 for physiological maintenance or otherwise for redox balance/homeostasis (Hartsock
434 & Shapleigh 2011; Li *et al.* 2012; Dietrich *et al.* 2013). The distinction here being that
435 we use the term physiological maintenance as it refers to the state of energetics in a
436 cell where the energy consumed is used for functions other than the production of new
437 cell material (i.e. growth) (van Bodegom 2007; Lipson 2015). Alternatively, redox
438 balance reactions are used to maintain viable metabolic processes by controlling the
439 redox state of all the cellular components (Green & Paget 2004). In contrast, organism
440 adapted to low N concentrations, like N fixers, decline in response to exogenous N
441 demonstrating real time selective pressure in a complex ecosystem. These responses
442 also highlight the temporal nature of these relationships and how by following niche
443 differentiation high number of functionally redundant organisms can be maintained
444 (Stempfhuber *et al.* 2016). However, the use of very high concentrations of urea
445 (leading to rapid hydrolysis to ammonium followed by substantial nitrification) has
446 major consequences for soil pH, physicochemical parameters, and potentially other
447 factors (e.g. osmolarity). Without accounting for those it is unclear what the direct
448 mechanism causing an increase or decrease in the relative abundance of a specific
449 population is.

450 Despite this, our observations highlight how lifestyle preferences for
451 organisms may be reflected in their dominance in the ecosystem. Prior work suggests
452 that AOA dominate in soils with low N inputs, but AOB numbers are higher at times

of high N loading or in ecosystems with consistent N deposition (Gong *et al.* 2013; Venterea *et al.* 2015; Sterngren *et al.* 2015; Li *et al.* 2016). This would suggest that a dynamic ecosystem with varying nutrient levels would select for a higher diversity of organisms that maintain ecosystem processes stable over time and space (Wang and Loreau, 2014). Indeed, our data supports this with alpha diversity (calculated based on 16S amplicon analysis at both DNA and RNA) decreasing in response to urea. This is inconsistent with plant responses to nutrient deposition in which multiple resources need to be added to elicit a response (Harpole *et al.* 2016), although contrasting results have been observed (Suding *et al.* 2005; Bai *et al.* 2010; Song *et al.* 2011; 2012). For microbes, high site to site variance is reported (De Schrijver *et al.* 2011; Leff *et al.* 2015), but similar negative responses are suggested and could be linked to increased competition in the absence of natural ecosystem variability. However, links between microbial and plant response suggest interplay between the response of macro and microbiota (Zeng *et al.* 2016). While previous work suggests an important role for moisture in controlling community composition (Waldrop & Firestone 2006), we only observed a modifier role in our experiment.

Although broad observations align with ecological theory, precise identification of responsive organisms is rarely carried out. Here we note that while at phylum level clear responses (+/- fold change) are observed, variance is seen at the OTU level suggesting intra-taxonomic (i.e. same phylum but different species or OTUs) diversity. We hypothesized this reflects the life history strategies of the different organisms. Attempts to link specific transformations to organisms failed, potentially due to the succession of functionally redundant organisms that respond at different time with non-overlapping optima. That is, while functional gene abundance provides the population size of organisms capable of carrying out a process, the group

478 may be composed of many OTUs with divergent life strategies or metabolic potentials
479 that affect when they can respond. This makes functional gene measurement an
480 average of all OTU subpopulations carrying that gene. However, community response
481 allows us to identify OTUs responsive to N deposition, which when analyzed
482 independently, provides insights into metabolic preferences (i.e. aerobic vs. anaerobic,
483 nitrifier vs. denitrifier) based on time and response to treatments. Taxonomic groups
484 regularly recognized as native to, or abundant in, oligotrophic conditions declined in
485 the presence of urea. Most of these groups are still poorly understood, and included
486 the Acidobacteria, Verrucomicrobia, Nitrospirae, Candidate Division WS3 (also
487 referred to as candidate phylum Latescibacteria) and the Thaumarchaeota. These
488 organisms are predicted to be slow growers with the Thaumarchaeal response
489 confirming the AOA patterns observed at the functional level. In contrast, positively
490 responding organisms are those generally associated with groups considered eutrophic
491 or capable of fast response. This discrepancy based on life history strategies has been
492 proposed and applied to microbes previously, and suggests that an organisms' ability
493 to grow, utilize carbon, generate proteins and efficiently transform resources to
494 biomass, amongst others, is related to its rRNA operon copy number (Klappenbach *et*
495 *al.* 2000; Stevenson & Schmidt 2004; Dethlefsen & Schmidt 2007; Roller *et al.*
496 2016). When applied to communities, it is associated with microbial successions in
497 which decreases in copy numbers are associated with later stages of succession
498 including in soils (Nemergut *et al.* 2015). For example, two OTUs matching the
499 Verrucomicrobial OTU DA101 where found to be negatively affected by urea, and at
500 least one was found to be highly abundant under background conditions. DA101
501 seems to be a common soil (and grassland) organism identified throughout the world
502 (Felske & Akkermans 1998; O'Farrell & Janssen 1999; Brewer *et al.* 2016). Based on

503 growth (Sangwan *et al.* 2005) and genome reconstructions (Brewer *et al.* 2016), these
504 organisms are predicted to be slow but efficient growers (k strategists). In contrast,
505 most of the positively affected organisms seemed to posses higher *rrn* copy numbers
506 and included members of the Proteobacteria and Bacteroidetes in line with prior
507 predictions (Fierer *et al.* 2007). Statistical analysis supported this interpretation with
508 low copy numbers (1-2) significantly associated to a negative response to N
509 deposition, while high copy numbers (>2) were linked to increased capacity for
510 growth, growth rate and maximum abundance. However, we found a non-linear
511 relationship between increased *rrn* copy numbers and growth capacity, best fitted by
512 models reaching an asymptote. These are first order models that suggest that while a
513 benefit exists where increased copy numbers lead to increased growth rate, after a
514 certain threshold other variables might limit any benefit. Alternatively, a decrease in
515 growth rate might be observed with increasing copy numbers once a tradeoff
516 threshold is passed (Lipson 2015). However, when *rrn* copy numbers are log2
517 transformed, a significant linear fit was observed as seen in prior studies (Roller *et al.*
518 2016). In our study these predictions are made complicated due to the observed intra-
519 taxonomic variance that can arise from the lack of accurate knowledge of copy
520 numbers for many organisms, or from metabolic plasticity at higher taxonomic levels.
521 In addition, our analysis focused on N responsive organisms only, and with only 38
522 identified it indicates that most organisms were neither positively nor negatively
523 affected. This could explain why certain organisms (e.g. Actinobacteria) expected to
524 be k strategist, based on their ability to produce secondary metabolites (Abdelmohsen
525 *et al.* 2015) and compete with other organisms (Barka *et al.* 2015), showed a positive
526 response to N deposition. Alternatively, the low number of responsive organisms
527 could indicate that our false discovery rate corrections were too restrictive.

These findings help us get closer to understanding not just the metabolic potential of organisms in soils, but the role specific pathways play for an organism. It also allows us to understand the repercussion of disturbances and management of soils on below ground biodiversity. The knowledge gained through these type of observations, and integration of life history strategies into microbial ecology, will get us one step closer to microbiome management as part of soil care.

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765 **Figures**
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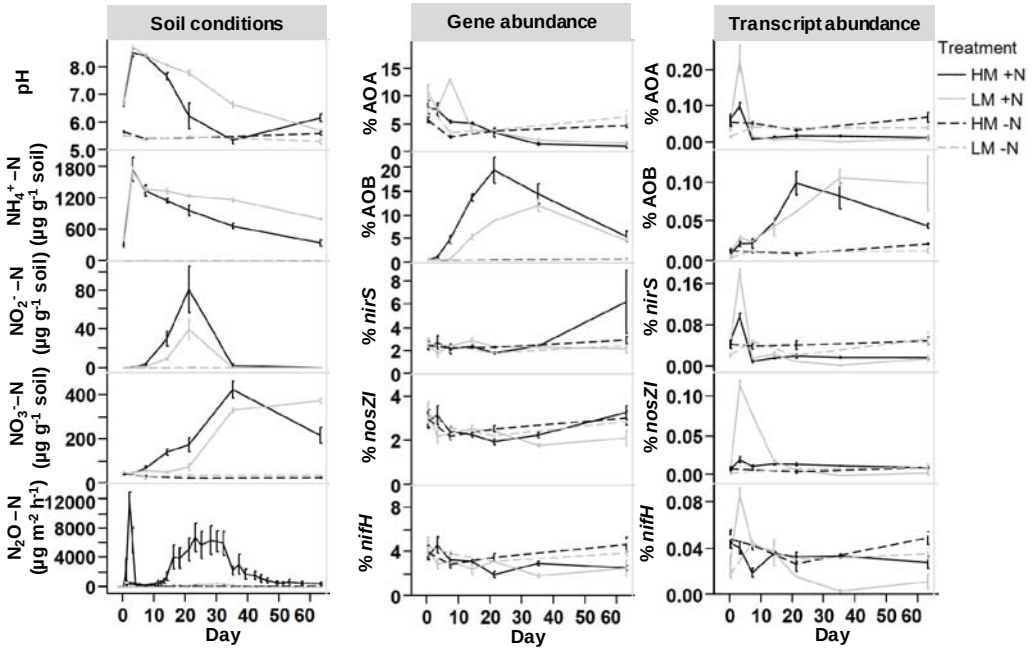


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771 **Fig. 1** Chemical transformations and biological (functional group) response in soils
772 treated with urea (+/- 1000 µg N/g dry soil) under two moisture conditions (LM = low
773 moisture [-10kPa]; HM = high moisture [-1.0kPa]). Error bars are the standard error
774 of the mean (n ≥ 3, except gene abundance data of day 7 [n=1; LM soil] and day 21
775 [n=1; LM soil]) for replicate mesocosms. Gene and transcript abundance were
776 measured by qPCR targeting: nitrifiers (AOA, ammonia oxidizing archaea; AOB,
777 ammonia oxidizing bacteria), denitrifiers (*nirS*, cytochrome cd $_1$-containing nitrite
778 reductase*;* *nosZI*, nitrous oxide reductase) and nitrogen fixers (*nifH*, nitrogenase
779 reductase). All qPCR results are normalized to 16S rRNA copy numbers and
780 presented as percent of the nucleic acid pool.
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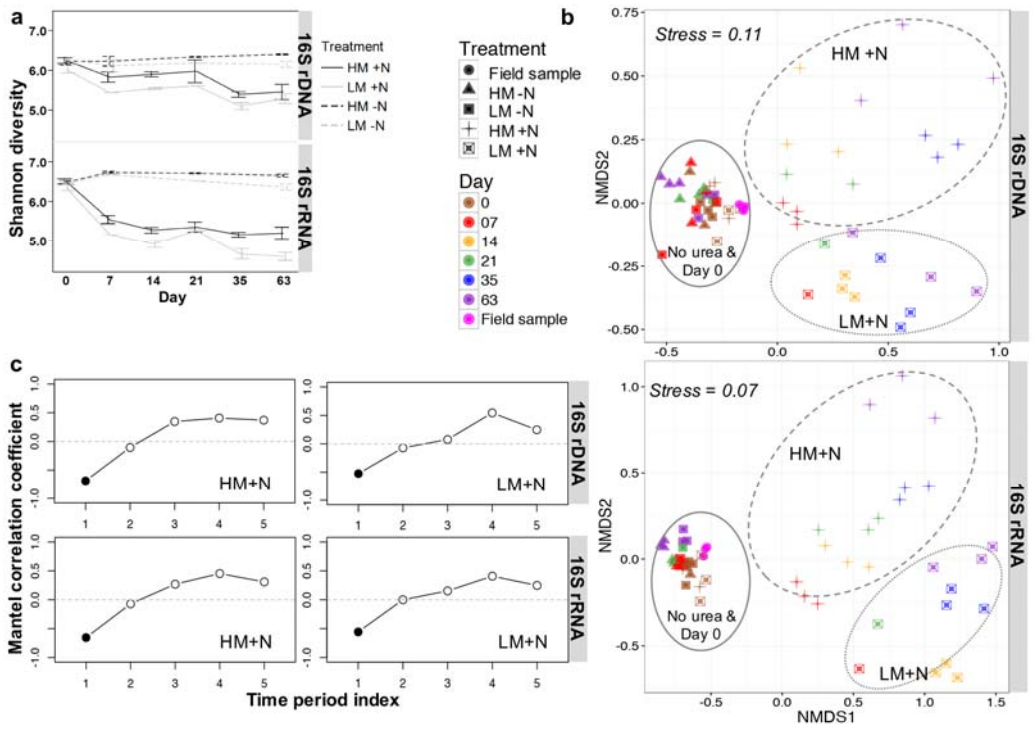
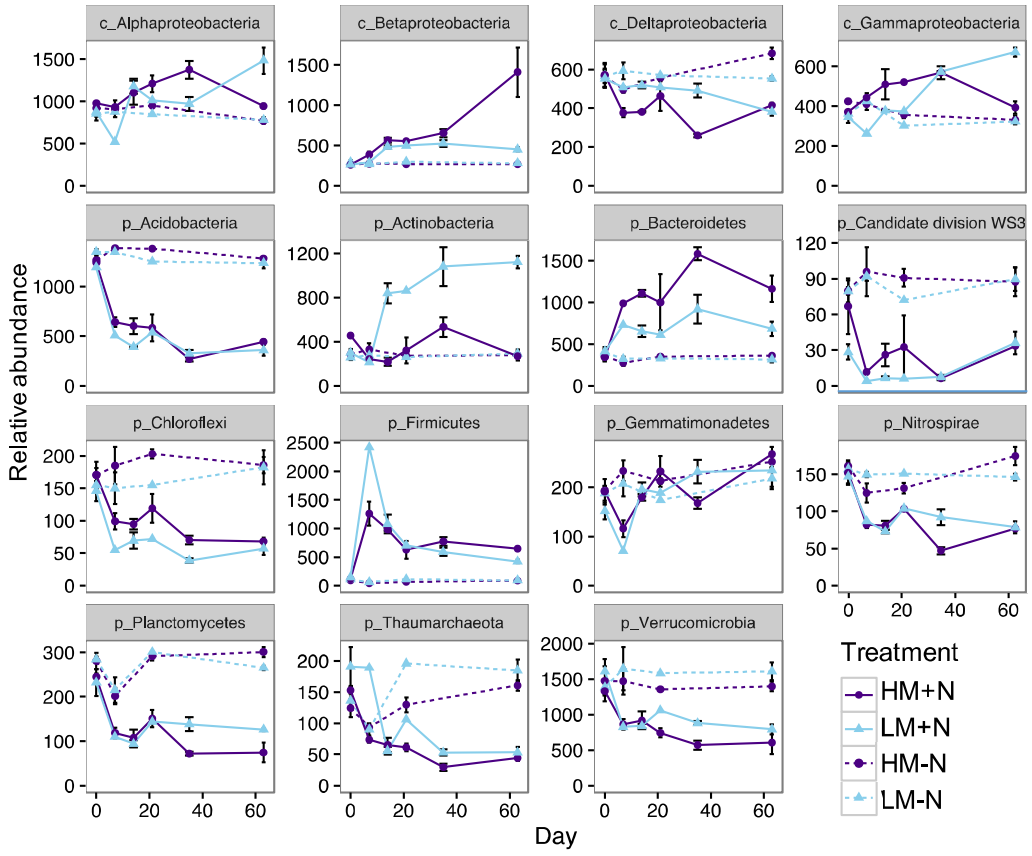



**Fig. 2** Total microbial community response (based on 16S rRNA gene amplicon profiling and clustering of sequences at OTU level (97% sequence similarity)) to urea (+/-1000 µg N/g dry soil) under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]) at both DNA and RNA level. Error bars are the standard error of the mean (n = 3, except day 7 [n=1; LM soil] and day 21 [n=1; LM soil]) for replicate mesocosms. (a) Changes in microbial diversity (Shannon) index over time in response to treatment. (b) Non-metric multidimensional scaling (NMDS) ordination plots based on Bray-Curtis distances showing relationships among samples based on OTU level changes in community composition. (c) Mantel correlogram showing autocorrelation on community composition by performing sequential Mantel tests between the Bray-Curtis dissimilarities and the grouping of samples using a time period index (index 1 represents 0-7 days; 2 represents 7-14; 3 represents 14-21; 4 represents 21-35; 5 represents 35-63). Filled circles represent significant correlation ($p < 0.05$) in community composition at specific time periods, with open circles indicating no significant correlation.

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805 **Fig. 3** Phylum and class level (for Proteobacteria only) changes in abundance (DNA)
806 representing relative contribution >1% of all detected phyla (based on OTUs clustered
807 at 97% sequence similarity). A total of 7,400 sequences were examined per sample.
808 Error bars are the standard error of the mean (n = 3, except day 7 [n=1; LM soil] and
809 day 21 [n=1; LM soil]) for replicate mesocosms. Treatments = +/- N [+/-1000 µg N/g
810 dry soil] under two moisture conditions (LM = low moisture [-10kPa]; HM = high
811 moisture [-1.0kPa]). Abbreviations: c: Class; p: Phylum. See supplemental Fig. S8 for
812 relative abundance
813

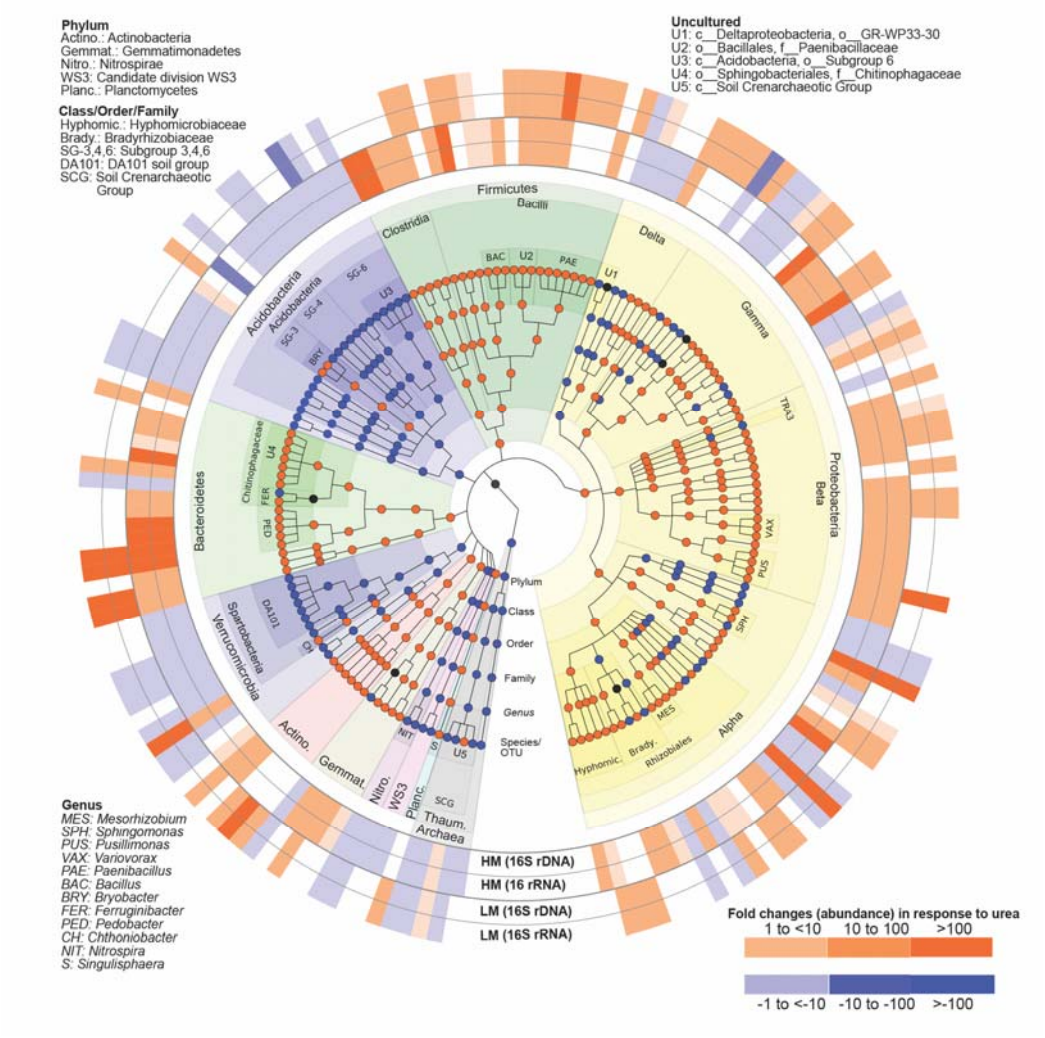


**Fig. 4** Taxonomic summary of OTUs responsive to urea treatment identified through similarity percentages (SIMPER) analysis (representing top 50% cumulative sum). The 4 outer rings represent fold changes in response to urea under high and low moisture content (MH & LM respectively) at either DNA or RNA level, with blank gaps indicating OTUs not identified in SIMPER analysis under the specified ring condition. Nodes on the tree (moving outwards from center) correspond to taxonomic level [Domain, Phylum, Class, Order, Family, Genus and Species/OTUs]. Nodes are colored based on dominant response (>50% conserved fold change response across OTUs within a node) with black notes indicating equal representation of positive and negatively responding OTUs. Shaded areas of branches delineate defined taxonomic groups. See Supplementary file (Table S3) for full classification.

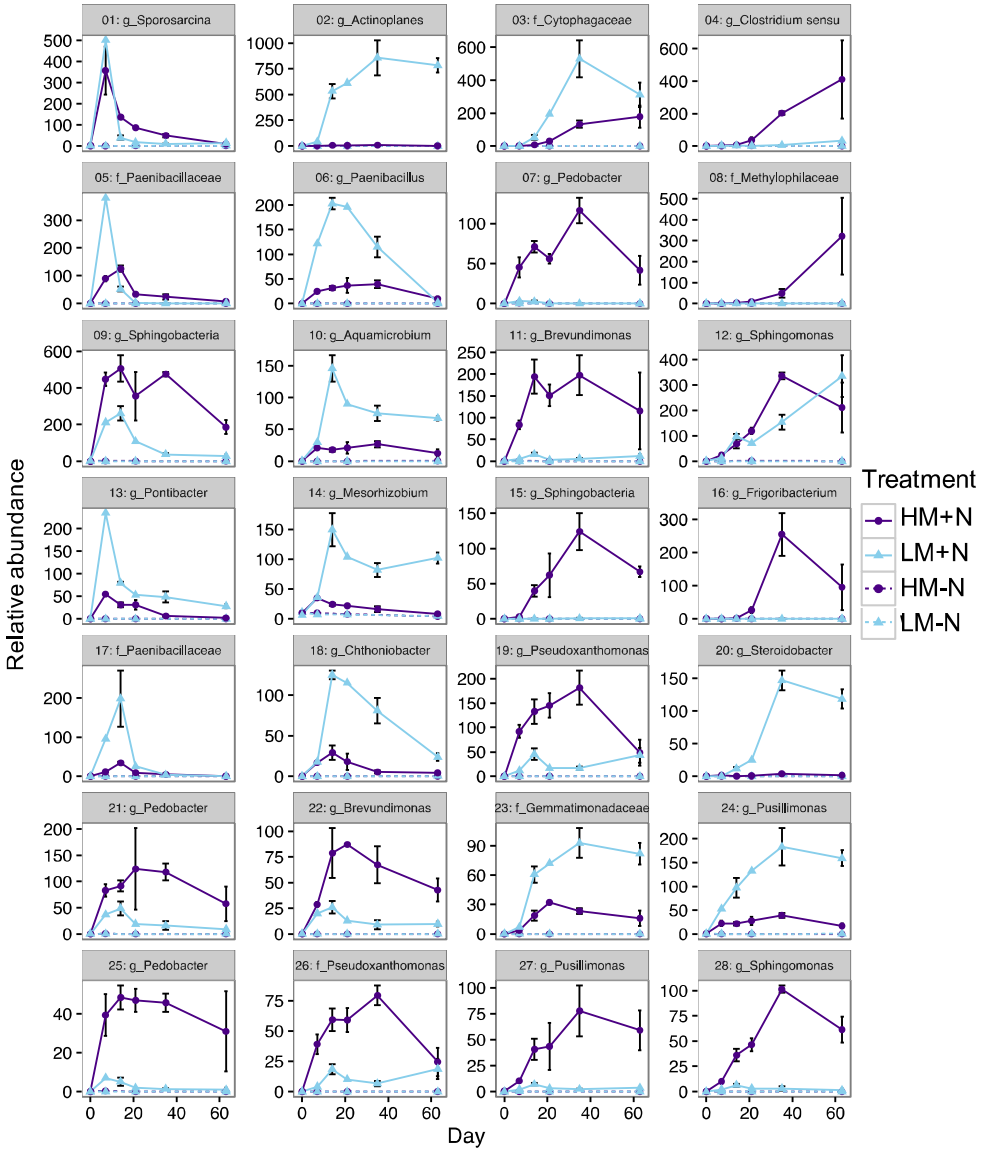


**Fig. 5** Population (16S rDNA) changes (abundance based on 7400 reads per samples) for OTUs identified as positively responsive to urea treatment based on similarity percentages (SIMPER) analysis (representing top 30% cumulative sum). Treatments = +/- N [+/-1000 µg N/g dry soil] under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]).

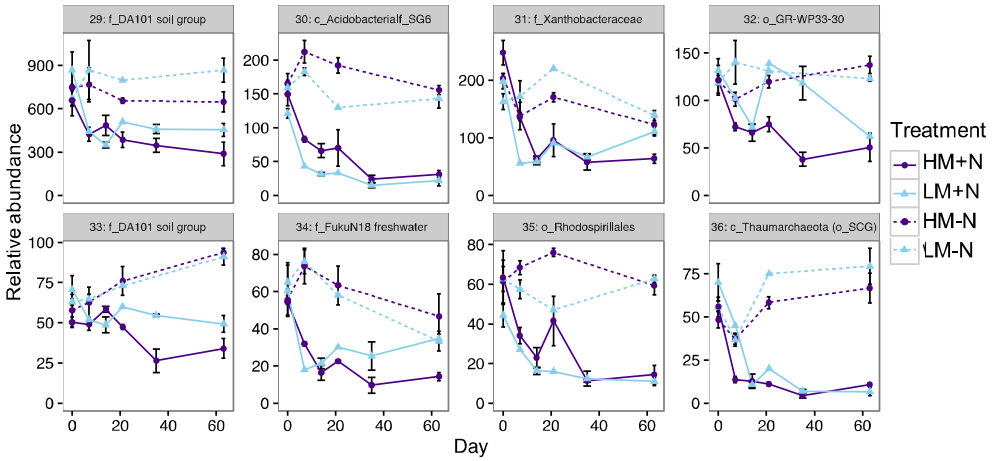


**Fig. 6** Population (16S rDNA) changes (abundance based on 7,400 reads per samples) for OTUs identified as negatively responsive to urea treatment based on similarity percentages (SIMPER) analysis (representing top 30% cumulative sum). Treatments = +/- N [+/-1000 µg N/g dry soil] under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]).

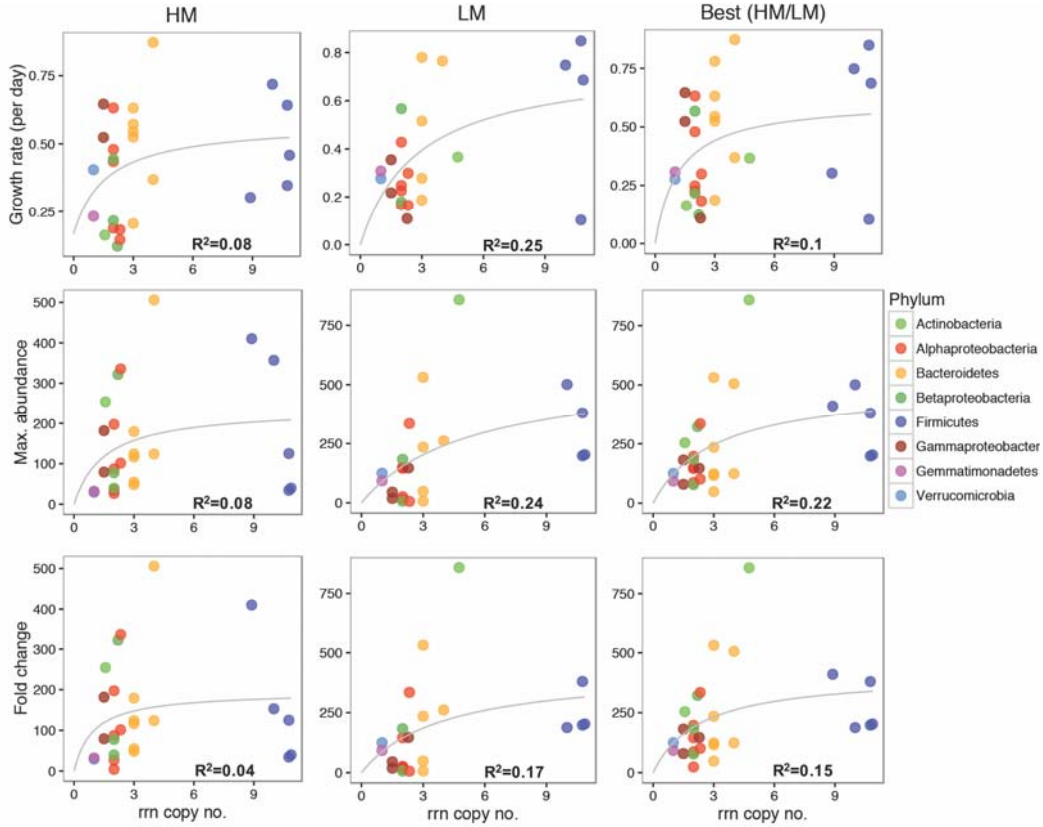


**Fig. 7** Relationship between predicted ribosomal RNA operon (rrn) copy numbers and growth rate (per day), maximum observed population change, or fold change in response to N treatment under both high moisture (HM) content, low moisture (LM) content and best growth either in HM or in LM (based on maximum observed growth). Copy number was estimated using rrn database (Stoddard *et al.* 2015). Copy number values were obtained by finding the closest match (lowest taxonomic level possible) to each OTU and retrieving the mean rRNA copy number for that group.

876 **Supporting information**

877 **Fig. S1** $N_2O$ response in soils treated with urea (+/- 1000 µg N/g dry soil) under two
878 moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]).
879 Error bars are the standard error of the mean ($n \geq 3$) for replicate mesocosms.

880 **Fig. S2** Functional group response (absolute quantification) in soils treated with urea
881 (+/-1000 µg N/g dry soil) under two moisture conditions (LM = low moisture [-
882 10kPa]; HM = high moisture [-1.0kPa]). Gene and transcript abundance were
883 measured from DNA template (1 ng of DNA) and cDNA template (1 ng RNA). Error
884 bars are the standard error of the mean (n = 3, except day 7 [n=1; LM soil] and day 21
885 [n=1; LM soil]) for replicate mesocosms. Absolute gene and transcript abundance
886 were measured by qPCR targeting: 16S (total prokaryotic community), nitrifiers
887 (AOA, ammonia oxidizing archaea; AOB, ammonia oxidizing bacteria), denitrifiers
888 (*nirS*, cytochrome $cd_1$-containing nitrite reductase*;* *nosZI*, nitrous oxide reductase)
889 and nitrogen fixers (*nifH*, nitrogenase reductase).

890 **Fig. S3** Changes in microbial a) Richness and b) Evenness (Pielou's) over time in
891 response to treatment.

892 **Fig. S4** Stress plots for Fig. 2b.

893 **Fig. S5** Pvclust tree displaying sample clustering based on Bray-Curtis distances
894 calculated from 16S rRNA gene community composition and indicating significant
895 clusters based on p values ([AU (approximately unbiased) BP (bootstrap
896 probability)]) for each node. Red boxes mark clusters with 95% confidence. Bootstrap
897 replication (n=1000). Two clusters: with urea (light red box) and no urea + day 0 N
898 treated samples (light green box).

899 **Fig. S6** Mantel correlogram showing autocorrelation on community composition by
900 performing sequential Mantel tests between the Bray-Curtis dissimilarities and the

grouping of samples using a time period index (index 1 represents 0-7 days; 2 represents 7-21; 3 represents 21-63). Opened circles represent no significant correlations ($p > 0.05$) in community composition at specific time periods.

**Fig. S7** Changes in abundance (DNA), activity (RNA) and RNA/DNA ratio for phyla, or classes, representing top 11 phyla (based on OTUs clustered at 97% sequence similarity). A total of 7,400 sequences were examined per sample. Error bars are the standard error of the mean (n = 3, except day 7 [n=1; LM soil] and day 21 [n=1; LM soil]) for replicate mesocosms. Treatments = +/- N [+/- 1000 µg N/g dry soil] under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]). Abbreviations: Firmi., Firmicutes; Verru., Verrucomicrobia; Bact., Bacteroidetes; Acido., Acidobacteria; Actino., Actinobacteria; Planct., Planctomycetes; Gemma., Gemmatimonadetes; Thaum., Thaumarchaeota; Chloro., Chloroflexi, Nitro., Nitrospirae.

**Fig. S8** Phylum level changes (relative abundance) in genome (16S rDNA) and transcript (16S rRNA) levels representing relative contribution >1% of all detected phyla (based on OTUs clustered at 97% sequence similarity). A total of 7,400 sequences were examined per sample. Treatments = +/- N [+/- 1000 µg N /g dry soil] under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]).

**Fig. S9** Transcriptional (16S rRNA) and population (16S rDNA) changes (absolute abundance based on 7400 reads per samples) for OTUs identified as positively responsive to urea treatment based on similarity percentage (SIMPER) analysis (representing top 30% cumulative sum). Treatments = +/- N [+/- 1000 µg N/g dry soil] under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]).

**Fig. S10** Transcriptional (16S rRNA) and population (16S rDNA) changes (absolute abundance based on 7400 reads per samples) for OTUs identified as negatively responsive to urea treatment based on similarity percentage (SIMPER) analysis (representing top 30% cumulative sum). Treatments = +/- N [+/- 1000 µg N (urea)/g dry soil] under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]).

**Fig. S11** Relationship between predicted ribosomal RNA operon (rrn) copy numbers and observed growth rate (per day), maximum observed population change, or fold change in population abundance for OTUs responsive to N treatment under both high moisture (HM) content. Copy number was estimated using rrn database (Stoddard *et al.* 2015). Predicted rrn copy numbers represent the mean rRNA copy number for the closest taxonomic match (at the lowest taxonomic level possible) for each OTU. The rrn copy numbers were log2 transformed before linear regression analysis. Significant "p" value is marked with an asterisk (*p<0.05; **p<0.01; ***p<0.001)

**Table S1** Primer pairs used in this study

**Table S2** Pairwise correlation between observed phylum (or class) abundance at DNA and RNA level for urea (+N) treated soils. Correlation analysis was done between DNA (16S rDNA) and RNA (16S rRNA) samples based on mean absolute abundance (per 7,400 sequence reads) at each time point (day 0, 7, 14, 21, 35, 63). Only Proteobacteria shown at class level.

**Table S3** Top OTUs cumulatively contributing 50% of the variance between groups (+Urea; -Urea) at 16S rDNA and 16S rRNA levels based on SIMPER analsysis.

**Table S4:** Two sample t-test for mean comparison between low copy number rrn (rRNA operon) samples (1-2) and high copy number of rrn samples (>2). The significant correlation (p<0.05) are showed as bold.