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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Summary

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 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 microbiological techniques have given us unprecedented access to the microbial world (Spiro, 2012; Rinke *et al.*, 2013), yet soil 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; Gubry-Rangin *et al.*, 2015; Wei *et al.*, 2015), fewer have looked at the transcriptional profiles over time (Nicol *et al.*, 2008; Morales and 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).

Where progress has been made, groundbreaking findings have followed. The initial discovery of ammonia oxidizing archaea (AOA) and its subsequent rise to major player in the N cycle (Leininger *et al.*, 2006; Hatzenpichler, 2012; Stahl and 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.

65 It is well established that individual intermediates in the N cycle can be used
66 for specific reasons (i.e. ammonia oxidation provides electrons, while denitrification
67 intermediates accept reducing equivalents), but the purpose of the reactions for any
68 organism is another major unknown. That is, while some organisms carry out these
69 processes for electrogenic purposes that can result in growth, others do it in order to
70 maintain redox homeostasis (Simon and Klotz, 2013). Unfortunately examples where
71 an organism harbours multiple versions of the same enzyme for completely different
72 purposes (respiration vs. redox balance) exist (Hartsock and Shapleigh, 2011), and are
73 likely to limit generalizations.

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

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

2014; de Klein, Shepherd, *et al.*, 2014; de Klein, Luo, *et al.*, 2014), 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 biochemical pathways involved in N loss (through gases) in response to urea (simulated ruminant urine deposition event) and varying moisture content. Observed phenotypes (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 16S). 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 (non-biochemical) 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. maintenance). To test this we mimicked a ruminant urine-N deposition event using repacked soil cores (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 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.

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Results:

Soil pH and N transformation dynamics in response to urea

Soil pH increased from acidic (pH = 5.5) to alkaline reaching a maximum (pH = 8.5) at day 3 in urea treated soils. Return to baseline pH was modulated by soil moisture with high moisture (HM; -1.0kPa) soil reaching baseline at day 35 and low moisture soils (LM; -10kPa) doing so at day 53 (Fig. 1). This shift in pH was linked to a successive N transformation process initiated with urea hydrolysis and leading to nitrification and denitrification: urea → NH_4^+ → NO_2^- → NO_3^- → N_2O → N_2 (Fig. 1). Sequential peak activity was observed for each transformation with the response modified by moisture. Maximum production (mean 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

136 Significant changes (ANOVA, $p<0.05$) in relative activity (mRNA copy
137 numbers/16S rRNA gene copy numbers) were observed promptly between day 0 & 3
138 for all functional groups (except AOA and N-fixers in HM soil) in response to urea
139 (Fig. 1). However, maximum relative transcription did not match maximum
140 production peaks for corresponding substrates, or products, for each functional group.
141 Nitrifiers (ammonia oxidizers) displayed niche differentiation, with time, length and
142 strength of response differing between bacterial (AOB) and archaeal ammonia
143 oxidizers (AOA). Relative activity of AOA increased (4.6-fold for LM and 1.6-fold
144 for HM) under urea treatments at day 3 only, with a subsequent decrease (-19.3-fold
145 for LM and -7-fold for HM) resulting in lower expression than in untreated soils (Fig.
146 1). AOB relative activity also increased but was sustained for a much longer period
147 (3-63 days), with maximum activity (>11-fold change) seen at 21 and 35 days for
148 HM+N and LM+N respectively (Fig. 1). Denitrifiers (both nitrite and nitrous oxide
149 reducers) showed similar responses as AOA, with peak activity at day 3 and a rapid
150 return to baseline, in the case of nitrite reducers decreasing to levels below those
151 observed in non-urea treated soils (Fig. 1). To account for endogenous sources of N,
152 N_2 fixers were monitored through the activity of the nitrogenase gene (*nifH*). No
153 significant changes were observed except for day 3 (LM +N only), with a subsequent
154 decrease in activity below background. This decrease below background was
155 observed for all N treated samples.

156 Changes in the relative contribution to total community composition were
157 calculated by normalizing functional gene copy numbers to total 16S rRNA gene copy
158 numbers per sample for each functional group (Fig. 1). The maximum observed
159 relative abundance of each functional group differed for each group (HM and LM
160 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) and richness (-1.5-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) and richness (-1.9-fold change). Moisture was found to play a modifier role, 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 as shown in a non-metric multidimensional scaling (NMDS) plot using a Bray-Curtis dissimilarity

185 matrix (Fig. 2b and Supplementary Fig. S4). At both DNA and RNA level community
186 changes along the first axis corresponded with changes in response to urea treatment,
187 with the second axis accounting for changes in moisture. A pvclust analysis
188 (hierarchical clustering with p-values calculated via multiscale bootstrap resampling,
189 Supplementary Fig. S5) confirmed two major clusters [100% AU (Approximately
190 Unbiased) and 100% BP (Bootstrap Probability)] formed by urea treated (HM+N and
191 LM+N samples, excluding day 0), vs. untreated soils (HM-N, LM-N, field samples,
192 and HM+N & LM+N at Day 0). Temporal variance within each cluster was
193 confirmed using a Mantel correlogram analysis (Fig. 2c). Urea treated samples had
194 significant changes in community composition immediately upon treatment (Day 0 to
195 7), with no return to baseline conditions by the end of the experiment. In contrast,
196 untreated samples did not change significantly over time (Supplemental Fig. S6)

197 Changes in community structure were associated with shifts in major
198 taxonomic lineages (Fig. 3). In general, phylum level changes in abundance and
199 transcription where correlated to each other (Supplementary Table S2 and Fig. S7,
200 S8). Urea deposition induced temporal changes in phylum level abundance with
201 observed maximum fold changes per group (HM & LM at DNA level) being:
202 Acidobacteria, -4.6 & -3.7; Actinobacteria, 2.4 & 5.3; Bacteroidetes, 4.6 & 2.2;
203 Candidate Division WS3, -10.5 & -7; Chloroflexi, -2.9 & -2.6; Firmicutes, 10.8 &
204 16.2; Gemmatimonadetes, 2 & 3.3; Nitrospirae, -3.2 & -2; Planctomycetes, -3.7 & -
205 2.5; Thaumarchaeota, -5.2 & -3.6; Verrucomicrobia, -2.5 & -2; Alphaproteobacteria,
206 1.4 & 1.7; Betaproteobacteria, 4 & 2; Deltaproteobacteria, -2.2 & -1.4;
207 Gammaproteobacteria, 1.5 & 2.6. Normalized transcriptional activity (reads of 16S
208 rRNA/reads of 16S rDNA) identified the Firmicutes and members within classes of
209 the Proteobacteria as the most transcriptionally active. While abundant phyla tended

to have high levels of normalized transcription, less abundant organisms like the Thaumarchaeota, were observed to have high normalized transcriptional activity especially under background conditions (Supplementary Fig. S7). In contrast, groups traditionally considered slow growers (e.g. Nitrospirae and Gemmatimonadetes) had low normalized transcription. It was also noted that while normalized transcription levels remained stable without urea, N deposition induced changes. These changes in normalized activity did not always match trends observed at individual DNA or RNA level (e.g. Firmicutes).

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

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

OTUs within the Proteobacteria identified in the SIMPER analysis did not display a conserved response to urea, however when lower taxonomic levels were examined patterns emerged. A consistent positive response was seen for OTUs within the class Betaproteobacteria and the family Hyphomicrobiaceae, amongst others.

Conserved positive responses to urea where also observed at the phylum level for the Firmicutes, Bacteroidetes, Actinobacteria, Gemmatimonadetes and Planctomycetes, although the level of response varied across lower taxonomic levels. In contrast, with only some exceptions, OTUs within the phyla Acidobacteria, Verrucomicrobia, Nitrospirae, Candidate Division WS3 (also referred to as candidate phylum Latescibacteria) and the Thaumarchaeota all were negatively impacted by urea deposition.

To account for response patterns over time, we focused on OTUs that 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,

285 suggesting niche differentiation driven by time and/or substrate concentration. AOA
286 responded early, and declined, as new N was made available while AOB responded
287 later with population swings spanning from near detection limit to most dominant
288 group. These observations match prior reports showing AOA prefer low N conditions,
289 while AOB respond vigorously to N deposition (Di *et al.*, 2010; Sterngren *et al.*,
290 2015). This has been interpreted as evidence for differing lifestyles for AOB and
291 AOA, with AOA preferring nutrient poor conditions and AOB dominating in rich
292 ones (Sterngren *et al.*, 2015). However, prior assertions that AOB are solely important
293 for driving nitrification might be overstated given that transcriptional activity for both
294 groups is comparable if compared at peak time (Di *et al.*, 2009). This contrasting use
295 of energy between functionally redundant organisms might explain the low
296 correlations between processes and the abundance of their respective functional
297 populations (Rocca *et al.*, 2015). When we examine the response of other functional
298 groups benefiting from influxes of N, like denitrifiers, we see no significant change in
299 population sizes suggesting that either energy is being utilized for maintenance or
300 otherwise for redox balance (Hartsock and Shapleigh, 2011; Y. Li *et al.*, 2012;
301 Dietrich *et al.*, 2013). In contrast, organism which benefit from low levels of N, like N
302 fixers, decline in response to exogenous N demonstrating real time selective pressure
303 in a complex ecosystem. These responses also highlight the temporal nature of these
304 relationships and how by following niche differentiation high number of functionally
305 redundant organisms can be maintained (Stempfhuber *et al.*, 2016).

306 Observations also highlight how lifestyle preferences for organisms are
307 reflected in their dominance in the ecosystem. Prior work suggests that AOA
308 dominate in soils with low N inputs, but AOB numbers are higher at times of high N
309 loading or in ecosystems with consistent N deposition (Gong *et al.*, 2013; Sterngren *et*

310 *al.*, 2015; Venterea *et al.*, 2015; C. Li *et al.*, 2016). This would suggest that a dynamic
311 ecosystem with varying nutrient levels would select for a higher diversity of
312 organisms that maintain ecosystem processes stable over time and space (Wang and
313 Loreau, 2014). Indeed, our data supports this with alpha diversity (calculated based on
314 16S amplicon analysis at both DNA and RNA) decreasing in response to urea. This is
315 inconsistent with plant responses to nutrient deposition in which multiple resources
316 need to be added to elicit a response (Harpole *et al.*, 2016), although contrasting
317 results have been observed (Suding *et al.*, 2005; Bai *et al.*, 2010; Song *et al.*, 2011;
318 2012). For microbes, high site to site variance is reported (De Schrijver *et al.*, 2011;
319 Leff *et al.*, 2015), but similar negative responses are suggested and could be linked to
320 increased competition in the absence of natural ecosystem variability. However, links
321 between microbial and plant response suggest interplay between the response of
322 macro and microbiota (Zeng *et al.*, 2016). While previous work suggests an important
323 role for moisture in controlling community composition (Waldrop and Firestone,
324 2006), we only observed a modifier role in our experiment.

325 Although broad observations align with ecological theory, precise
326 identification of responsive organisms is rarely carried out. Here we note that while at
327 phylum level clear responses (+/- fold change) are observed, variance is seen at the
328 OTU level suggesting the intra-taxonomic diversity we hypothesized reflected the life
329 history strategies of the organisms. Attempts to link specific transformations to
330 organisms failed, potentially due to the succession of functionally redundant
331 organisms that respond at different time with non-overlapping optima. That is, while
332 functional gene abundance provides the population size of organisms capable of
333 carrying out a process, the group may be composed of many OTUs with divergent life
334 strategies or metabolic potentials that affect when they can respond. This makes

335 functional gene measurement an average of all OTU subpopulations carrying that
336 gene. However, community response allows us to identify OTUs responsive to N
337 deposition, which when analyzed independently, provides insights into metabolic
338 preferences (i.e. aerobic vs. anaerobic, nitrifier vs. denitrifier) based on time and
339 response to treatments. Taxonomic groups regularly recognized as native to, or
340 abundant in, oligotrophic conditions declined in the presence of urea. Most of these
341 groups are still poorly understood, and included the Acidobacteria, Verrucomicrobia,
342 Nitrospirae, Candidate Division WS3 (also referred to as candidate phylum
343 Latescibacteria) and the Thaumarchaeota. These organisms are predicted to be slow
344 growers with the Thaumarchaeal response confirming the AOA patterns observed at
345 the functional level. In contrast, positively responding organisms are those generally
346 associated with groups considered eutrophic or capable of fast response. This
347 discrepancy based on life history strategies has been proposed and applied to
348 microbes previously, and suggests that an organisms' ability to grow, utilize carbon,
349 generate proteins and efficiently transform resources to biomass, amongst others, is
350 related to its rRNA operon copy number (Klappenbach *et al.*, 2000; Stevenson and
351 Schmidt, 2004; Dethlefsen and Schmidt, 2007; Roller *et al.*, 2016). When applied to
352 communities, it is associated with microbial successions in which decreases in copy
353 numbers are associated with later stages of succession including in soils (Nemergut *et*
354 *al.*, 2015). For example, two OTUs matching the Verrucomicrobial OTU DA101
355 where found to be negatively affected by urea, and at least one was found to be highly
356 abundant under background conditions. DA101 seems to be a common soil (and
357 grassland) organism identified throughout the world (Felske and Akkermans, 1998;
358 O'Farrell and Janssen, 1999; Brewer *et al.*, 2016). Based on growth (Sangwan *et al.*,
359 2005) and genome reconstructions (Brewer *et al.*, 2016), these organisms are

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

Experimental procedure

Sample collection and experimental design

A detailed methodology can be found in (Clough *et. al*., In review). In brief, soil (8°15'W, 52°9'N) was collected from a permanently grazed agricultural grassland (dairy pasture) in March (early spring) at the Teagasc Moorepark Research Centre, County Cork, Ireland. 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°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 (θg) 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 –N; No Urea _-1.0kPa) and no urea + low moisture (LM –N; No Urea_-10kPa). All cores where held at 20^{o}C for a period of 63 days.

Soil pH, and inorganic-N measurements

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

Nucleic acids extraction

Samples for RNA and DNA extraction were collected simultaneously with samples for inorganic N analysis, but only samples at 0, 7, 14, 21, 35, 63 days were processed for nucleic acids. Each biological replicate was extracted and analyzed

separately. For each extraction 2 g (wet weight) of soil were processed using the PowerSoil Total RNA Isolation and DNA Elution Accessory Kits (MoBio, Carlsbad, CA) as per manufacturer's instructions, with slight modifications. Bead beating was done in a Geno/Grinder 2010 (SPEX SamplePrep, LLC, Metuchen, NJ) using two rounds of beating (1750 strokes/min) for 15 s with a 1 min pause in between. The total elution volume for RNA and DNA was 60 µl and 100 µl respectively. RNA was treated with DNase I (RNase-Free) (New England Biolabs, USA) as per the manufacturer's protocol. RNA quality was assessed by denaturing gel electrophoresis. RNA and DNA concentration, purity and humic acid contamination were determined using a Nanodrop Spectrophotometer, ND-1000 (Thermo Scientific). All extractions were stored at -80 ^{o}C until downstream analyses.

Reverse transcription (RT)

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

16S rRNA gene amplicon sequencing

16S rRNA gene amplicon sequencing was performed using primers 515F/806R (V4 region of the 16S gene) and the Earth Microbiome Project conditions (Version 4_13) (Caporaso *et al.*, 2012). All samples were run simultaneously on a single Illumina MiSeq run. Sequences were first processed in Qiime (version 1.9.1) using default parameters (Caporaso *et al.*, 2010). Sequences were clustered into 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 (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 and 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 and Holmes, 2013), pvclust (Suzuki and 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,

503 information from the closest strain available was selected. In instances where a
504 closely related organism was not available, the mean copy number for the closest
505 taxonomic group (i.e. genus, class, etc.) was used. Copy numbers where then
506 compared to the maximum observed abundance and the maximum observed fold
507 change (calculated based on lowest observed abundance for the same organism in a
508 preceding time point for OTUs showing growth or succeeding time points for those
509 decreasing in abundance). An estimated growth rate was calculated for OTUs
510 showing increases in population size in response to N using the following formula:

511 $$N_t = N_{0*}e^{rt}$$

512

513 where: N_t: The amount at time t; N_0: The amount at time 0; r: exponential growth rate;
514 t: Time passed

515 *Fit model for rrn copy numbers*

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

520

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Figures

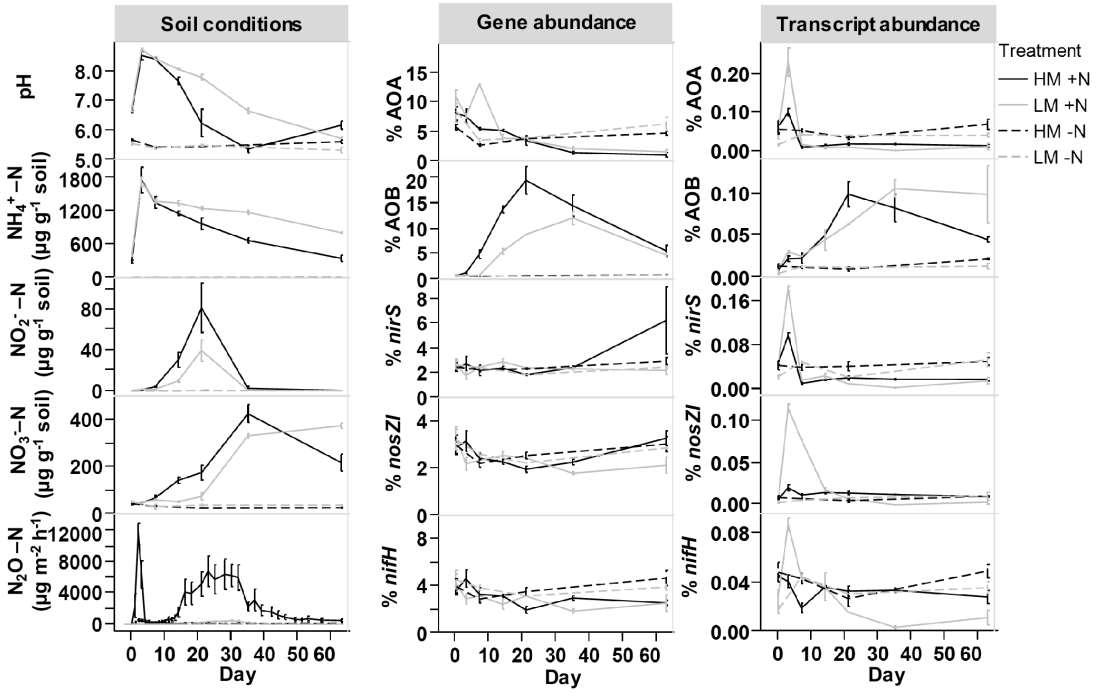


Fig. 1 Chemical transformations and biological (functional group) response in soils treated with urea (+/- 1000 µg N/g dry soil) under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]). Error bars are the standard error of the mean (n ≥ 3, except gene abundance data of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

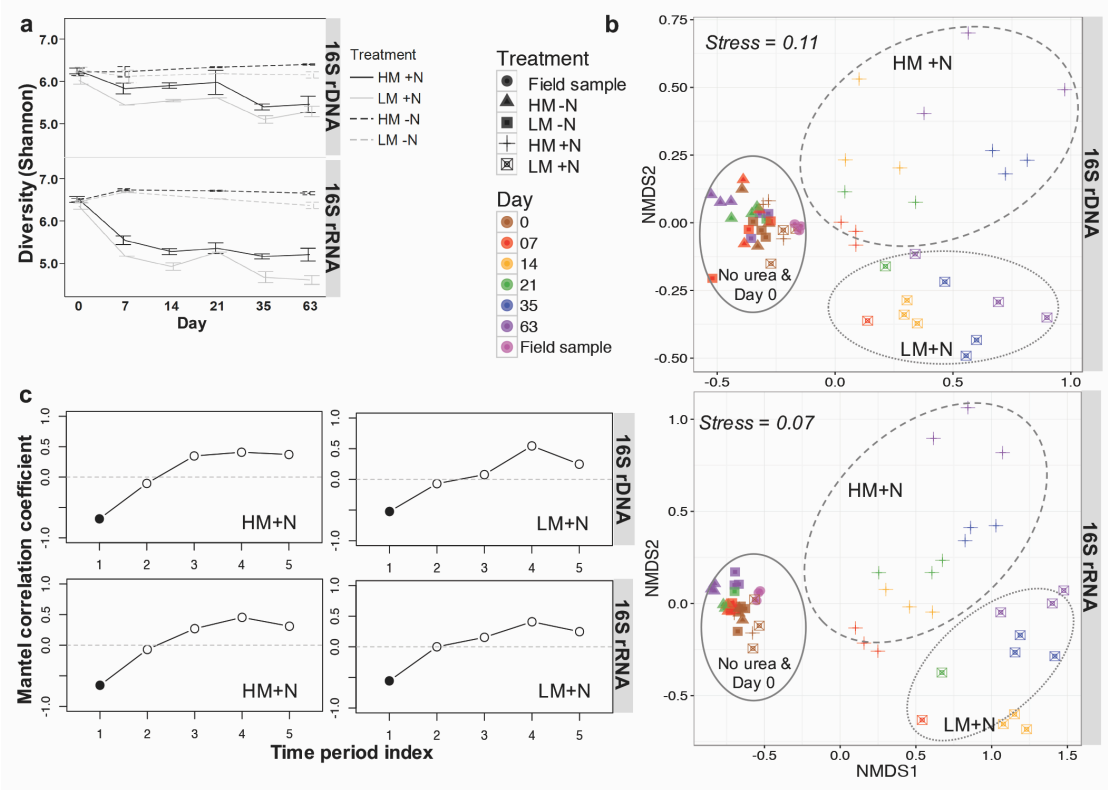
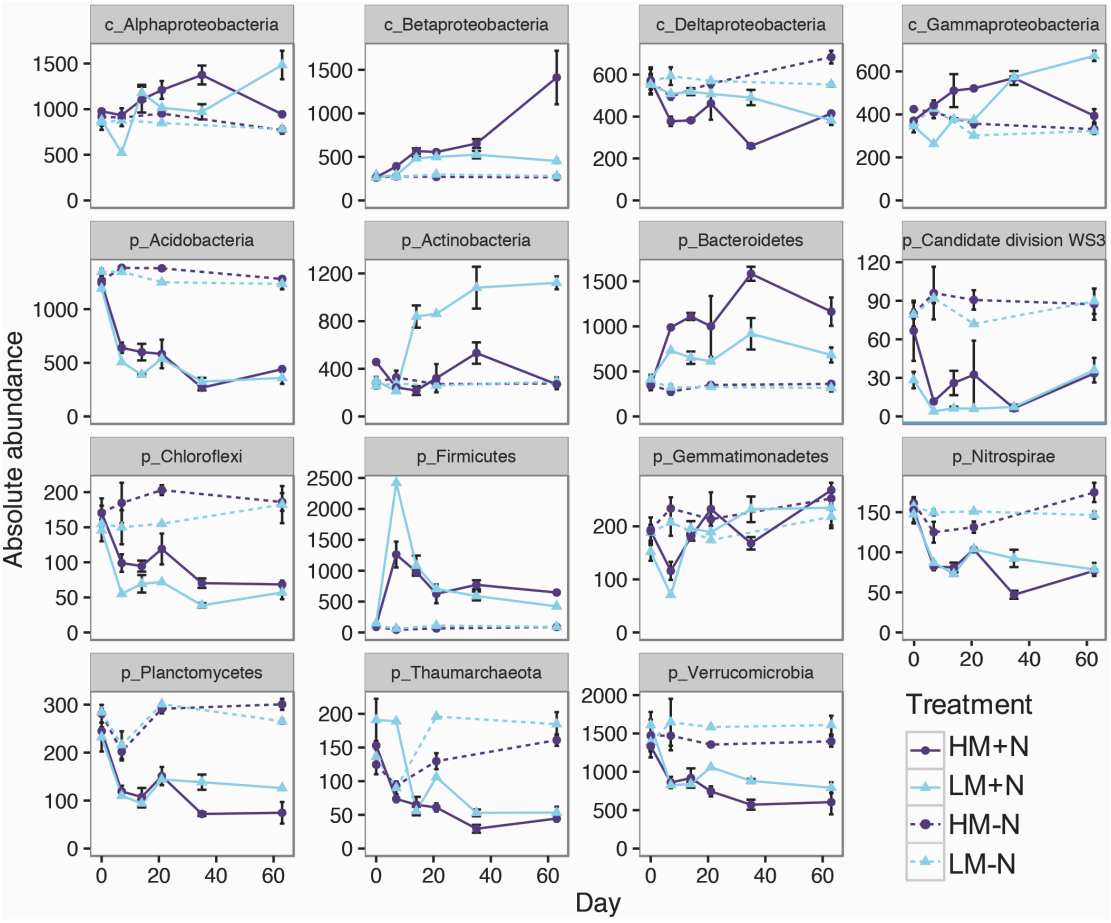


Fig. 2 Total microbial community response (based on 16S rRNA gene amplicon profiling and clustering of sequences at OTU level (97% sequence similarity)) to urea (+/-1000 µg N/g dry soil) under two moisture conditions (LM = low moisture [-10kPa]; HM = high moisture [-1.0kPa]) at both DNA and RNA level. 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

787 significant correlation ($p < 0.05$) in community composition at specific time periods,

788 with open circles indicating no significant correlation.

789



790

791 **Fig. 3** Phylum and class level (for Proteobacteria only) changes in abundance (DNA)

792 representing relative contribution >1% of all detected phyla (based on OTUs clustered

793 at 97% sequence similarity). A total of 7,400 sequences were examined per sample.

794 Error bars are the standard error of the mean (n = 3, except day 7 [n=1; LM soil] and

795 day 21 [n=1; LM soil]) for replicate mesocosms. Treatments = +/- N [+/-1000 µg N/g

796 dry soil] under two moisture conditions (LM = low moisture [-10kPa]; HM = high

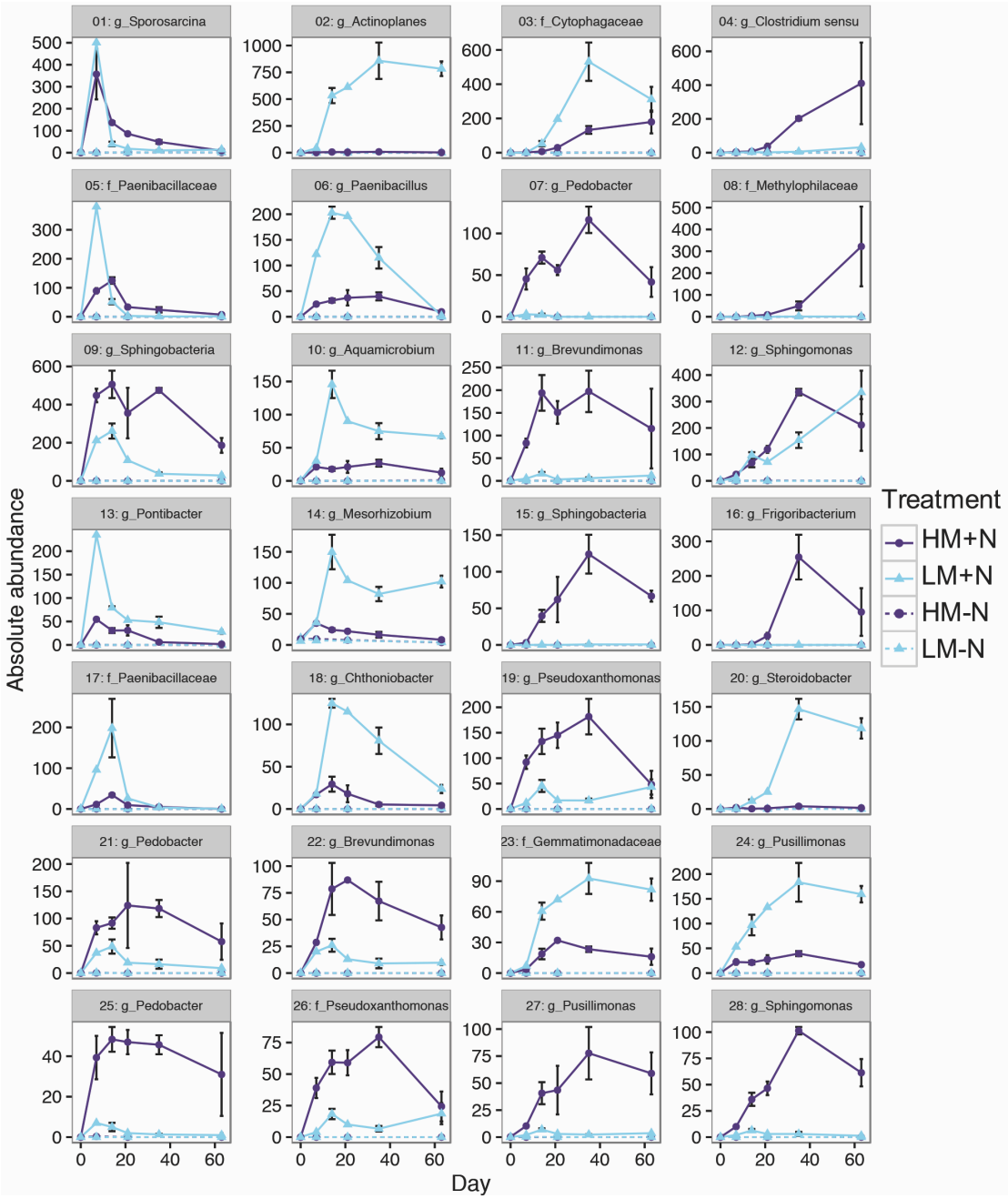
797 moisture [-1.0kPa]). Abbreviations: c: Class; p: Phylum. See supplemental Fig. S8 for

798 relative abundance.

799

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

811 negatively responding OTUs. Shaded areas of branches delineate defined taxonomic
812 groups. See Supplementary file (Table S3) for full classification.
813



814

815 **Fig. 5** Population (16S rDNA) changes (absolute abundance based on 7400 reads per
816 samples) for OTUs identified as positively responsive to urea treatment based on
817 similarity percentages (SIMPER) analysis (representing top 30% cumulative sum).

818 Treatments = +/- N [+/-1000 µg N/g dry soil] under two moisture conditions (LM =
819 low moisture [-10kPa]; HM = high moisture [-1.0kPa]).
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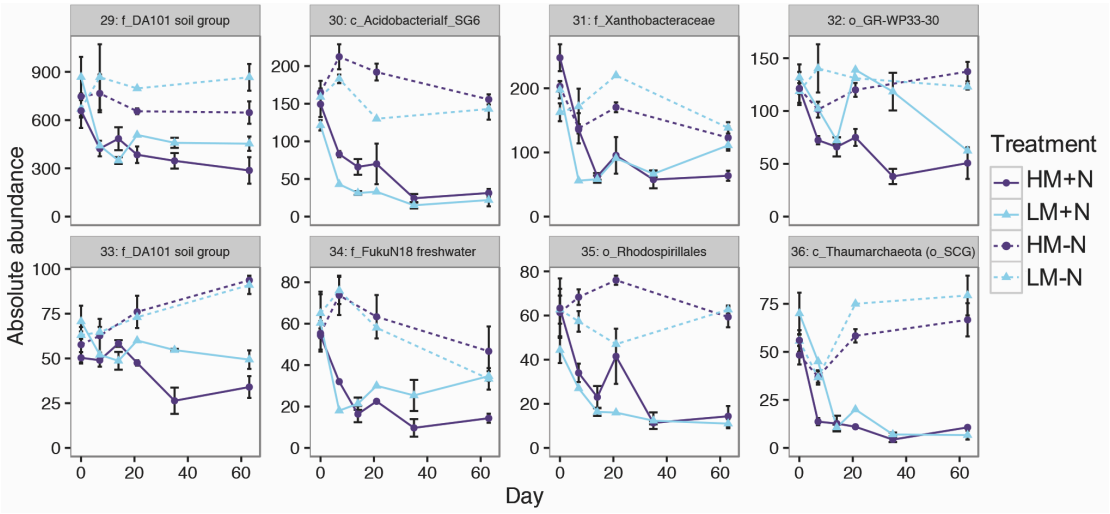

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825 **Fig. 6** Population (16S rDNA) changes (absolute abundance based on 7,400 reads per
826 samples) for OTUs identified as negatively responsive to urea treatment based on
827 similarity percentages (SIMPER) analysis (representing top 30% cumulative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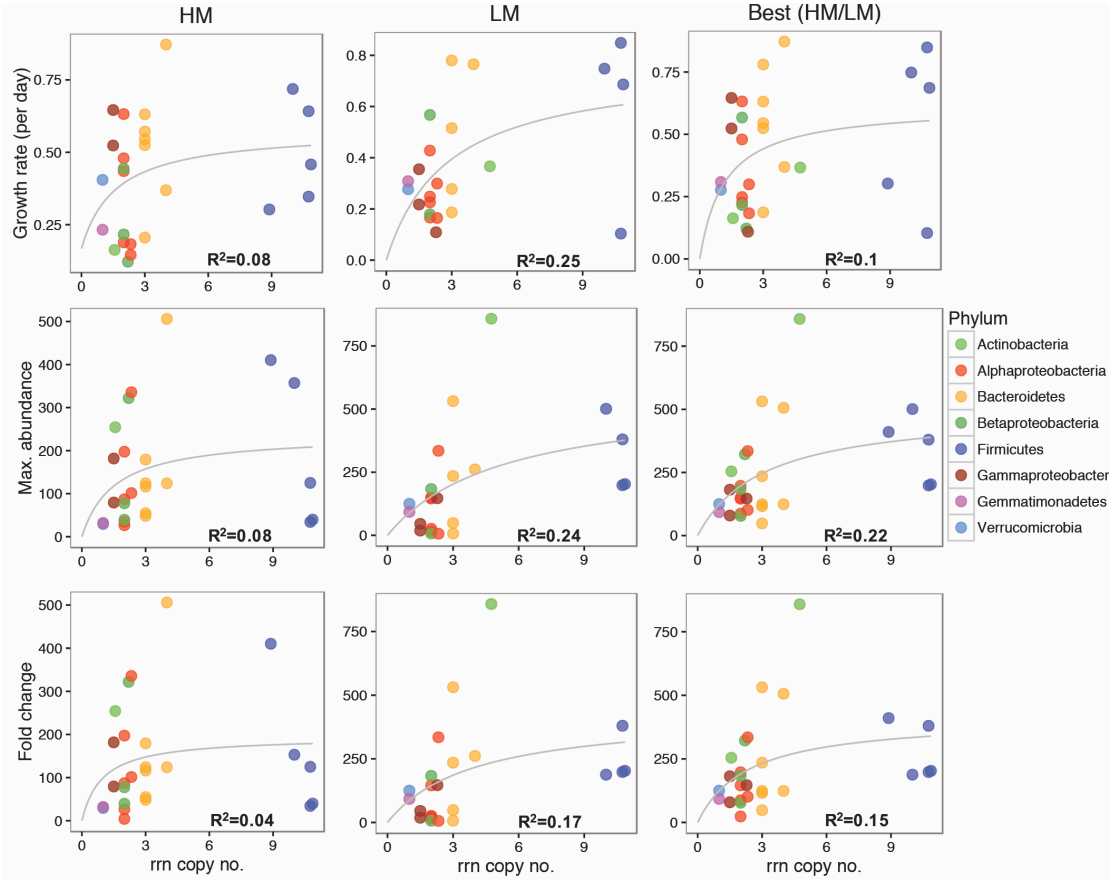

Fig. 7 Relationship between predicted ribosomal RNA operon (rrn) copy numbers and growth rate (per day), maximum observed population change, or fold change in response to N treatment under both high moisture (HM) content, low moisture (LM) content and best growth either in HM or in LM (based on maximum 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.