Impact of nitrogen fertilization on the soil microbiome and nitrous oxide emissions

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Chapter 3

Impact of long-term N, P, K and NPK fertilization on the composition and potential functions of the bacterial community in grassland soil

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Abstract

Soil abiotic and biotic interactions govern important ecosystem processes. However, the mechanisms behind these interactions are complex and the links between specific environmental factors, microbial community structures and functions are not well understood. Here, we applied DNA shotgun metagenomic techniques to investigate the effect of inorganic fertilizers N, P, K and NPK on the bacterial community composition and potential functions in grassland soils in a 54-year experiment. Differences in total and available nutrients were found in the treatment soils; interestingly, Al, As, Mg and Mn contents were variable in N, P, K and NPK treatments. Bacterial community compositions shifted and *Actinobacteria* were overrepresented under the four fertilization treatments compared to the control. Redundancy analysis of the soil parameters and the bacterial community profiles showed that Mg, total N, Cd and Al were linked to community variation. Using correlation analysis, *Acidobacteria*, *Bacteroidetes* and *Verrucomicrobia* were linked similarly to soil parameters, and *Actinobacteria* and *Proteobacteria* were linked separately to different suites of parameters. Surprisingly, we found no fertilizers effect on microbial functional profiles which supports functional redundancy as a mechanism for stabilization of functions during changes in microbial composition. We suggest that functional profiles are more resistant to environmental changes than community compositions in the grassland ecosystem.

Keywords: metagenomics/microbial ecology/microbial function redundancy/environmental factors

3.1 Introduction

Soil harbors a huge variety of organisms, including microorganisms which are central to terrestrial ecosystem processes such as C and N flows[1]. The recent development of culture-independent techniques and high-throughput sequencing technologies allows detailed study of bacterial communities and the factors that cause shifts in bacterial community structure[2-4]. Many studies apply 16S rRNA amplicon sequence profiling to address not only changes in community composition and function[5, 6] but also the relationship between community components and external drivers (to what extent community compositional shifts are influenced by external factors)[7]. Changes in the soil bacterial communities due to disturbances or altered resource availability may influence ecosystem processes by altering bacterial functions, compositions or interactions. However, the influence of specific environmental factors on changes in bacterial community composition and function due to nutrient inputs alone is not clear.

Studies of bacterial structural drivers at the regional scale have shown that bacterial community structure is driven by soil type and general chemical characteristics, including pH. Soil pH is shown as a key environmental factor that influences bacterial community composition and can explain the distributions of bacterial phyla at local scales[8, 9]. However, there is some indication that pH only indirectly drives shifts in community composition. For instance, with pH change, the soil moisture, cations availability and C:N ratios often co-vary as well[10]. Linking bacterial community shifts only to pH does not take into account the individual influence of soil parameters, e.g., NO_3, NH_4 and Ca. One study in Amazon soils that measured a wide range of soil parameters found a relationship between a specific group of *Acidobacteria* and the available Ca, Mg and Mn[11]. Because of the range of abiotic and biotic interactions between bacteria and their chemical environment, it is important to investigate a suite of soil paramenters in studies of the drivers of bacterial community changes, which we have applied here.

Nutrient amendments in managed grasslands are historically used to improve plant productivity. However, this management practice impacts all components of the grassland ecosystem, including soil functioning. The effect of chronic, long-term (more than five years) N fertilization is intensively studied, e.g. for the relationship between nutrients and plant productivities[12]; or for evaluating anthropogenic effects on bacterial and fungal biomass, community structure, composition and activities[8]. Several studies find long-term N fertilization effects on bacterial community compositional shifts and subsequent alteration of ecosystem functions[13-15]. Long-term application of N fertilizer affects the abundance of

specific groups that are associated with N cycling, as shown in studies targeting nitrifiers and denitrifiers[15, 16], ammonium oxidizing Archaea[17, 18] and methanotrophs[19]. In general, the long-term addition of N appears to select for copiotrophic taxa though these taxa may respond to C dynamics associated with the N additions[13]. Whether a similar mechanism affects bacterial community composition in long-term P and K fertilization is unknown.

In this study, we explored the shifts in abiotic and biotic factors between long-term N-, P-, K- and NPK-fertilization. First, we determined the soil chemical parameters in each long-term fertilization regime. Next, we tracked the compositional and functional changes in the bacterial communities across fertilization regimes. Last, we identified links between soil chemical parameters and bacterial community shifts. We hypothesized that soil factors other than pH were linked to bacterial community compositional and functional alterations (H_1). In addition, we hypothesized that each fertilization regime would alter bacterial community composition and function (H_2). To our knowledge, our study is the first to apply shotgun metagenome techniques to the soil bacterial communities in clay-soil grassland with yearly addition of inorganic fertilizers for such duration (54 years). Advantages over the widely used 16S rRNA amplicon method include the ability to examine community functional potential and to exclude PCR biases. In addition, this study is one of the first to include a suite of soil measurements to explore the environmental factors driving simultaneously soil bacterial community composition and functions under long-term nutrient input.

3.2 Material and methods

3.2.1 Site description

The Ossenkampen Grassland Experiment fields were established in Wageningen, The Netherlands (51°58'15"N; 5°38'18"E) on heavy-clay soil in 1958 to track plant species shifts under long-term application of inorganic fertilizers[20]. Fertilizer amendments, including N (ammonium nitrate, 160 kg N ha^{-1} yr^{-1}), P (superphosphate, 22 kg P ha^{-1} yr^{-1}), K (potassium sulfate, 108 kg K ha^{-1} yr^{-1}) and NPK (ammonium nitrate, superphosphate and potassium sulfate, 160 kg N ha^{-1} yr^{-1}, 33 kg P ha^{-1} yr^{-1} and 311 kg K ha^{-1} yr^{-1}) were applied to the fields annually since 1959. The fields were mown twice a year: once in July and once in October. The mown matter was left on the fields.

3.2.2 Sampling regime and soil parameters

Soil samples were collected on September 20, 2011 from the four treatment fields and one control field (five treatment fields). Each treatment field included 5 m × 2.5 m triplicate plots. Twenty-seven soil cores (10 cm depth and 2 cm diameter) were sampled from each plot and then pooled to give one bulk sample for each plot (Supplementary Figure 1). Five bulk soil samples (3 replicates/sample) were collected and homogenized through a 5 mm sieve. Fractions from two of the three soil samples from each field were sent for physical and chemical measurements at the Soil Science Department of Wageningen University. The remaining soil fractions were stored at -80^{o}C for molecular analyses. For the physical and chemical measurements, levels of soil moisture content, soil pH, extractable N, total carbon and nitrogen concentrations, available potassium, phosphate and sulfur, total organic matter, and available trace elements Al, As, Cd, Cr, Cu, Fe, Mg, Zn, Mn, Na, Ni and Pb were determined.

3.2.3 Shotgun metagenome preparation and processing

DNA from soil fractions (0.3 g) was extracted using the Power Soil kit (MolBio, Carlsbad, CA) with 5.5 m·s^{-1} for 10-min bead beating. DNA concentrations were measured using an ND-1000 spectrophotometer (Nanodrop, Wilmington, DE) to ensure at least 500 ng for library preparation.

The libraries were pyrosequenced on the Roche 454 FLX platform with titanium chemistry (Roche, USA) at Macrogen (South Korea). After demultiplexing and barcode removal, the fragments were trimmed to 300 bp with a quality score cutoff of 30. Artificial duplicates were removed (http://microbiomes.msu.edu/replicates/) from further analysis[21].

3.2.4 Sequence annotation and abundance normalization

The metagenomes were uploaded to the Metagenome Rapid Annotation with Subsystem Technology (MG-RAST; http://metagenomics.anl.gov) web server, version 3.1.2[22]. The sequences were deposited in MG-RAST under ID numbers 4485389.3 to 4485403.3. The metagenomes were compared by BLASTx to the Clusters of Orthologous Groups (COGs) database for the genome size normalization calculation, to the SEED database for functional annotation and the RefSeq microbial database for taxonomic annotation. The sequences with similarity to database entries with the default parameters (E-value of 1x10^{-5}, minimum sequence nucleotide identity of 60% and minimum alignment length of 15 bp)

were counted. The count of metagenome sequences with similarity to the 35 COG marker genes described by Raes *et al.* (2007) [23] [2323] were filtered from the COG tables and used to calculate the effective genome size (EGS) for each metagenome. The annotation abundances were normalized by multiplying each value by the weight of the sample EGS to the average EGS of all sample metagenomes (for detailed method see references[24, 25]. The taxonomic and functional annotation profiles were created by calculating the relative abundances of annotations within each taxonomic or functional category out of the total number of annotations for the sample.

3.2.5 Statistical analyses

Soil parameter values were compared between the control and treatment fields using permutation t-tests (n = 2) and among the five treatments using Analysis of Variance (ANOVA). The t-tests and ANOVA were performed using PAST software version 2.15[26]. The similarity of treatment groups was calculated based on the Euclidean distance matrices between the taxonomic and functional profiles with the Analysis of Similarity (ANOSIM) test. PCA[27] was applied to visualize the samples based on the taxonomic and functional profiles.

Overrepresentation of bacterial phyla or Subsystem Level 1 and 2 categories between the treatment groups and the control group were investigated using the Statistical Analysis of Metagenomic Profiles software (STAMP, version 2.0.0) [28]. Unclassified annotations were not included in the analyses. The two-sided Welch's t-test was applied with Storey's FDR (taxonomic analysis) or Benjamini-Hochberg FDR (functional analysis) multiple test correction and significance was determined based on a 95% confidence interval.

Redundancy analysis (RDA) was conducted to combine the soil parameter and the taxonomic or functional and data. For this analysis, the average of the two values for each soil variable was used as the value for the soil variable of the third replicate. The soil variables were tested for normality and standardized and in two cases were log (Nts) or inverse (Zn) transformed to achieve normality. Pearson correlations were calculated between the soil variables to identify highly correlated variables. The rda function in the "vegan" package in R (version 3.0.2) was applied. The rda function conducts an unweighted linear regression of categories (e.g. relative abundances) against variables (e.g. soil parameters) and performs unweighted singular value decomposition of the influencing variables.

3.3 Results

3.3.1 Soil parameters along the fertilizations

The autocorrelation between soil factors did not result in highly correlated variables across treatments ($p<0.05$), except organic matter correlated with total N (0.92) and with Mg (0.90). Therefore, no variables were removed for further analysis. Seventeen of the 24 analyzed soil parameters differed across the five treatment fields (ANOVA, $p < 0.05$; **Table 1**). The only soil parameter that differed in all of the four treatment fields compared to the control was total extractable N. Several soil parameters differed within the N, P or K and the NPK treatments. Expected differences due to the associated fertilizations were found. For instance, NO_3-NO_2-N was higher in the N and NPK treatments; available K was higher in the K and NPK treatments; and P was higher in NPK treatments. In both the P and NPK treatments, available C was lower than in the control treatment. Al was higher in the N and NPK treatments compared to the control. NH_4-N was lower in the K and NPK treatments compared to the control treatment. Most interestingly, the differences in Mg and Mn values that were found in the N and P comparisons were not encountered in the NPK comparison.

3.3.2 Shotgun metagenome characteristics

The 15 metagenomes comprised a total of 603 million bp contained in 1.6 million reads (**Supplementary Table 1**). Mean lengths of the sequences ranged from 362 to 398 bp and the quality scores were 36 and above. MG-RAST annotation to the M5NR database identified 53% to 57% of the predicted protein coding regions for the 15 sample metagenomes. Rarefaction curve analysis revealed that most of the diversity was sampled in the metagenomes based on number of sequences against number of species from the M5NR comparison (**Supplementary Figure 2**). Alpha diversity of the metagenomes averaged ~640 ± 34 species. The functional diversity, given by the number of functional categories divided by the metagenome size, ranged from 39% to 51%. The samples were dominated by bacteria domain sequences (average relative abundance 94.30%) followed by eukaryota (1.19%) and archaea (1.18%) sequences based on hits to the RefSeq database.

Table 1. Soil physicochemical properties from the non-fertilized (Control) field and the nitrogen (N), phosphorous (P), potassium (K) and nitrogen+phosphorous+potassium (NPK) treatment fields. Significant treatment values are in bold from the control to treatment comparison and the parameters that differed significantly among the five treatments are in bold.

Soil Properties		Values within Treatment Fields				
		Control	**N**	**P**	**K**	**NPK**
Total	**Ct (g/kg)****	59±1.00	62.7±1.33	58.7±0.33	61±0.01	60.7±1.67
	Nt (g/kg)**	5.2±0.03	5.8±0.13	5.1±0.1	5.2±0.01	5.2±0.1
	Nt (mg/kg)**	48±0.01	**68±0.01*****	**46.3±0.01****	**42±0.01****	**51.3±0.01****
	Kt (g/kg)***	10.8±0.24	11.8±0.04	10.5±0.11	12.6±0.06	12.1±0.07
	Pt (g/kg)***	0.7±0.04	0.7±0.01	1.1±0.03	0.07±0.03	1.1±0.04
	OM (%)*	14.7±0.23	15.6±0.4	14.3±0.13	14.7±0.03	14.6±0.33
	pH*	5.27±0.1	4.85±0.06	5.22±0.04	5.05±0.09	5.04±0.05
	moisture (%)	47.2±1.76	**37.5±1.09***	43.3±1.14	41.8±3.04	41.9±1.89
	C:N	11.3±0.12	10.9±0.02	11.5±0.16	11.7±0.01	11.7±0.09
Available (mg/kg)	C	699.7±21.7	716.7±39.3	**652±9.0***	702±3.0	**642±1.7***
	NH_4^+-N**	13.5±1.3	15.0±1.67	11.1±1.57	**7.3±1.17***	**5.5±0.47***
	NO_3^-+NO_2—N	1.6±0.07	**15.3±0.23****	1.9±0.3	1.2±0.13	**14.1±1.67***
	P***	2.3±0.1	2.1±0.07	**3.7±0.23**	2.1±0.1	**3.8±0.03***
	S	11.0±0.53	12.0±0.70	11.9±0.20	10.2±0.90	10.4±0.47
	Al***	8.7±2.23	**20.4±2.73***	15.5±0.53	13.4±1.54	**27.9±1.63****
	As**	0.11±0.01	0.09±0.01	0.08±0.01	0.09±0.01	0.08±0.01
	Cd	0.12±0.01	0.15±0.01	0.13±0.01	0.12±0.01	0.15±0.01
	Cu	0.1±0.00	0.1±0.00	0.1±0.00	0.1±0.00	0.1±0.00
	K***	35±0.01	36.3±2.67	37.3±1.67	**135.7±11.7***	**118.3±11.3***
	Mg**	211.3±9.33	**259.7±16.33****	**179.3±2.67***	222.7±7.33	215.7±1.67
	Mn*	26.3±2.23	**13.7±0.07***	**17.0±2.83***	24.87±4.40	21.7±3.53
	Na	36.7±6.67	32.7±7.33	35.0±4.00	34.7±2.33	31±1.00
	Ni	1.0±0.02	1.2±0.09	1.00±0.04	1.2±0.04	1.0±0.05
	Zn	3.3±0.3	4.3±0.47	2.8.0±0.03	3.5±0.0	4.7±1.53

*p < 0.05, **p < 0.01, ***p < 0.001

3.3.3 Taxonomic comparison of the sampled communities

The taxonomic profiles of the treatment metagenomes were similar to those of the control metagenomes. *Proteobacteria*, *Acidobacteria, Actinobacteria, Firmicutes, Verrucomicrobia* and *Bacterioidetes* were the most abundant phyla in the metagenomes. Fifteen additional bacterial phyla with more than 0.8% of representation in the metagenomes were identified by annotation against the RefSeq database (**Supplementary Table 2**). Principal Component Analysis (PCA) was used to visualize the samples based on relative abundances of annotations within bacterial phyla. Different inorganic fertilizations significantly changed mean relative

abundances of *Actinobacteria, Aquificae Chlorobi*, *Chloroflexi*, *Cyanobacteria, Deferribacteres, Dictyoglomi, Firmicutes, Lentisphaerae, Nitrospirae, Planctomycetes, Proteobacteria, Spirochaetes, Synergistetes* and *Verrucomicrobia.* (ANOVA, corrected $p < 0.05$). The samples were found to form distinct treatment groups by the PCA which explained ~96% of the variance between samples. This was confirmed by the ANOSIM test ($R = 0.835$, $p < 0.0001$; **Supplementary Figure 3a**).

Two-group comparisons revealed over- and under-represented phyla between the control and treatment groups, with the exception of the P treatment (**Figure 1**). The N, K and NPK treatments showed an overrepresentation in *Actinobacteria* compared to the control (corrected $p < 0.03$). In addition, the N treatment showed an underrepresentation in *Aquificae*, *Chlorobi*, *Cyanobacteria*, *Firmicutes*, *Nitrospirae*, *Planctomycetes*, and *Verrucomicrobia* (corrected $p < 0.03$), the K treatment an underrepresentation in *Firmicutes* and *Verrucomicrobia* (corrected $p < 0.04$) and the NPK treatment an underrepresentation in *Planctomycetes* (corrected $p < 0.05$).

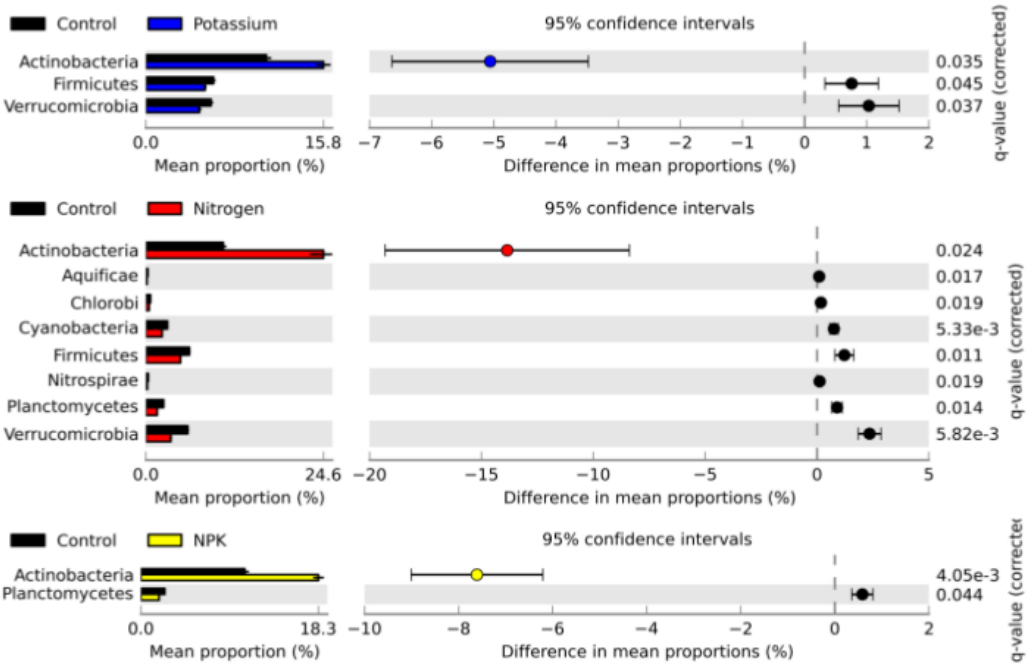


Figure 1. The bacterial phyla that were significantly overrepresented in comparisons between the treatments (Potassium, Nitrogen and NPK) and the control are shown (Welch's t-test, $p < 0.05$). Control=black, Potassium=blue, Nitrogen=red, NPK=yellow. In the Control to Phosphorus treatments no overrepresented phyla were found.

3.3.4 Functional comparison of the sampled communities

Functional profiles of the metagenomes were created from relative abundances of annotations to hierarchical subsystems through MG-RAST. The 15 sample metagenomes exhibited similar functional profiles across the 28 Subsystem Level 1 categories. The top 10 subsystems represented in the metagenomes were Carbohydrates (average 15.6%), Clustering-based subsystems (14.0%), Amino Acids and Derivatives (9.9%), Miscellaneous (7.6%), Protein Metabolism (6.0%), Cofactors, etc. (6.0%), RNA Metabolism (5.0%), Cell Wall and Capsule (4.0%), Fatty Acids, etc. (4.1%) and Virulence, etc. (3.3%). Principal Component Analysis (PCA) was used to visualize the samples based on relative abundances of annotations within Subsystems Level 1 and 2 categories. In the Level 1 comparison, the samples did not form distinct treatment groups in the PCA (~60% of the variance) which was reflected in the ANOSIM test ($R = 0.2889$, $p < 0.0246$; **Supplementary Figure 4**). In the Level 2 comparison, the treatment effects were more distinctly displayed in the PCA (~48% of variance) and this was reflected in the ANOSIM test result ($R = 0.6193$, $p < 0.0003$; **Supplementary Figure 3b**). Of the Level 2 categories, no category differed between treatments based on the multiple group comparison. Two-group comparisons revealed no over- or under-represented functional categories between the control and treatment groups based on Subsystems Level 1 and 2.

3.3.5 Linking environmental variables with bacterial taxa and functional categories

The links between the measured soil parameters and the relative abundances within bacterial phyla or functional Level 2 categories were investigated through RDA. Mn, As, Mg, Cd, Al, Pt, K, Kt, and Nt were found to be related to the samples based on the taxonomic (RDA, adjusted R-squared = 0.82) and functional Level 2 (RDA, adjusted R-squared = 0.34) profiles (**Figure 2a and Figure 2b**).

In order to examine the correlation between environmental factors and bacterial taxa, Spearman correlations were calculated between the relative abundance values of different bacterial taxa and the values for soil parameters for each control versus treatment comparison (**Table 2**). Of the soil parameters that were significantly different in the control to treatment comparisons, Al, K, Nt, $N\text{-}NH_4$, $NO_3\text{-}NO_2\text{-}N$, P and Mg were strongly correlated ($r > |0.70|$) with the relative abundances of the six major bacterial phyla. Three groups could be made of the bacterial phyla with similar correlations. First, the *Acidobacteria, Bacteroidetes* and *Verrucomicrobia* were correlated to similar soil parameters in at least two

comparisons: negatively correlated to Kt, Al, K and Ni and positively correlated with N-NH_4, NO_3-NO_2-N and Nt. The *Actinobacteria* were positively correlated to Kt, Al, K, and Ni and negatively correlated to N-NH_4 and Nt in at least two comparisons. Last, the *Firmicutes* and *Proteobacteria* were positively correlated with P, N-NH_4, NO_3-NO_2-N, Nt, Ct, OM and moisture in at least one comparison (**Supplementary Table 3**).

For investigating the correlation between soil factors and functional categories, Spearman correlations were also calculated between the relative abundance values within functional categories and the values of soil parameters. The relative abundances of certain functional groups were correlated with specific environmental factors. For example, available Cd, Al and Ni together with total K concentrations were highly positively correlated with stress responses related genes abundance (**Table 3**), which had a negative correlation with soil pH and moisture content. Available Cd, Ni and total C were negatively correlated with virulence related gene abundances whereas soil pH and moisture content were positively correlated with this function category. Total extractable N had positive correlation with cell division and cell cycle and DNA metabolism related functional profiles abundance.

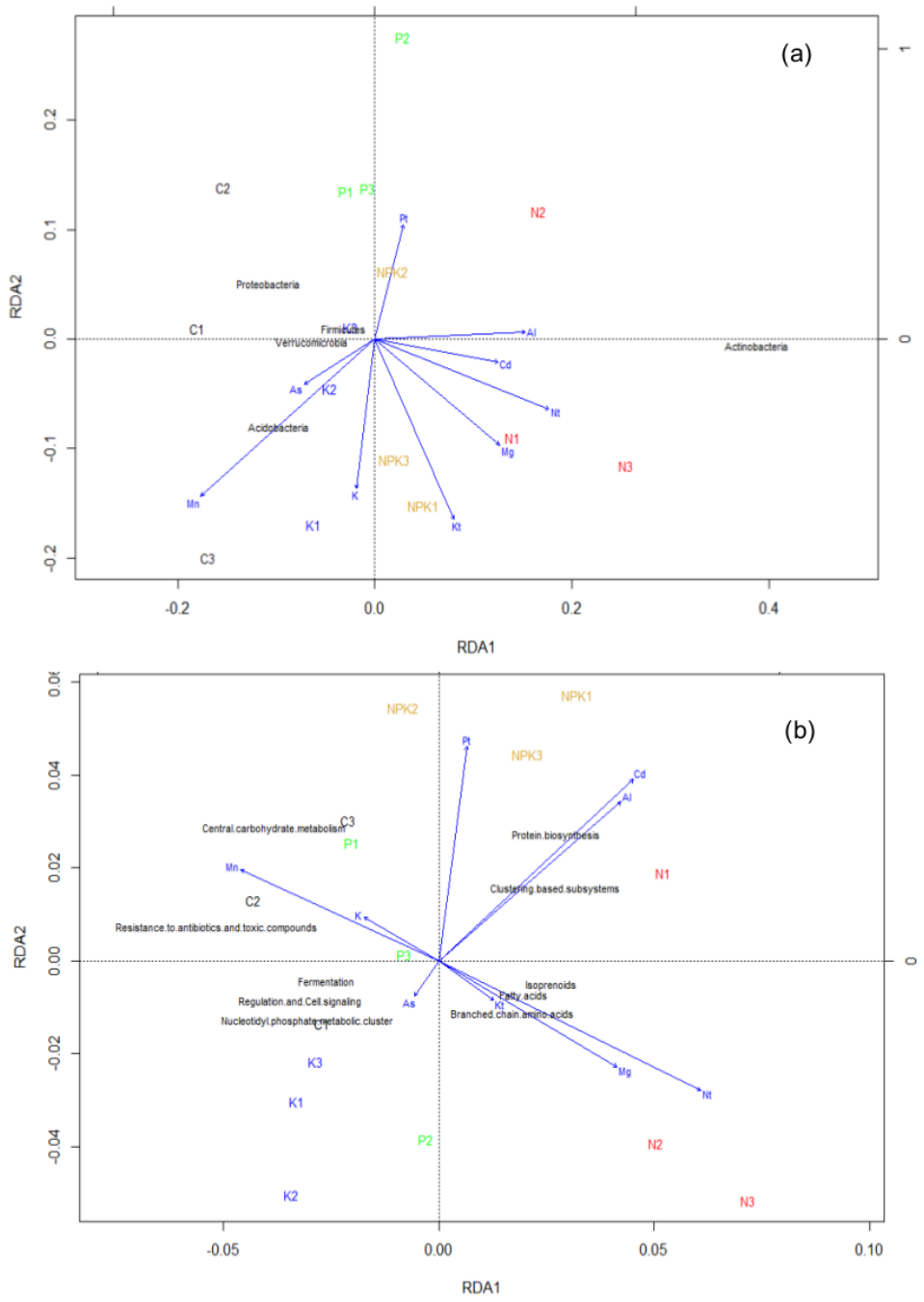
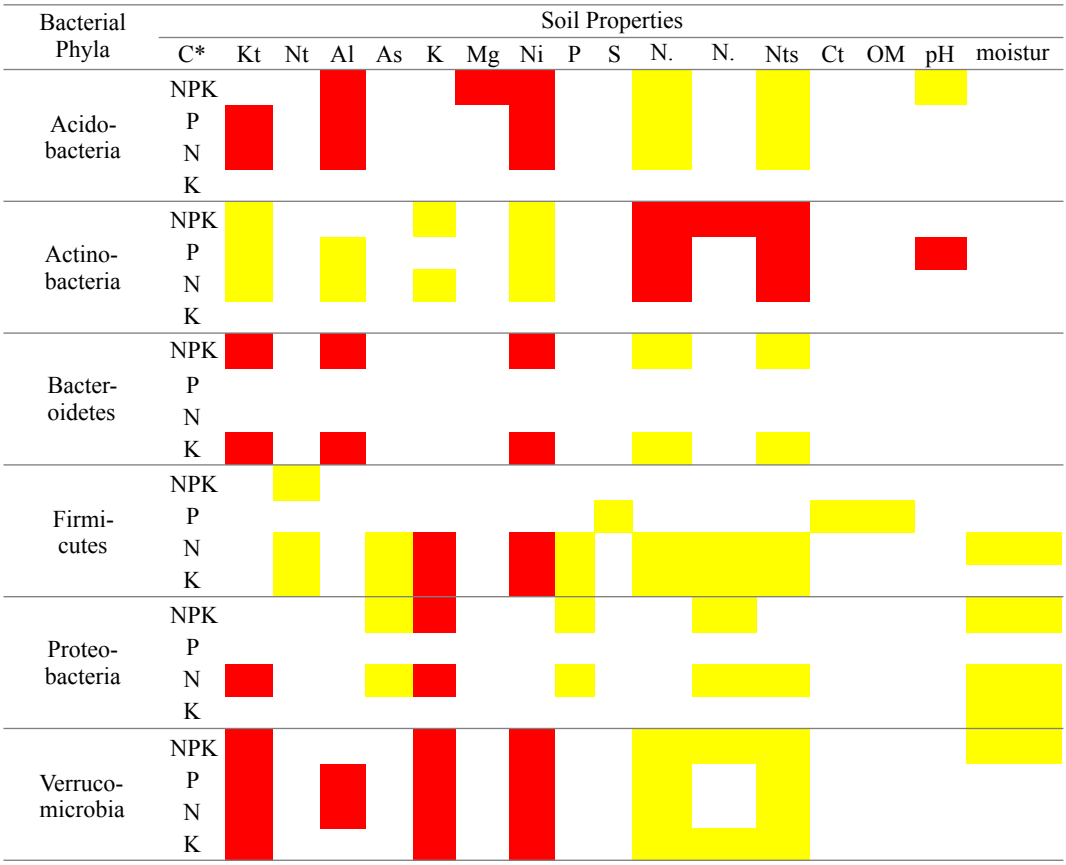


Figure 2. Redundancy analysis (RDA) of the samples based on (a) relative abundances within RefSeq Bacterial phyla or (b) relative abundances within Subsystem Level 2 categories and values of environmental variables. C=Control, N=N fertilized samples, P=P fertilized samples, K=K fertilized samples, NPK=NPK fertilized samples. 1, 2, 3 = replicates. Only the environmental variables (blue lines with arrow heads) that explained a significant amount of the variations (Monte Carlo test, p value < 0.05) are included in the figure.

Table 2. Spearman correlation values between the relative abundances of annotations within the six major bacterial phyla and soil parameters. Only the correlations values greater than |0.7| are shown. Positive correlations=yellow and negative correlations=red.



C* = control to treatment comparison; soil parameters

Table 3. Spearman correlation between soil parameters and functional profiles based on subsystems hierarchy 1 (MG-RAST). Only the correlations values greater than |0.7| are shown.

Function category	Soil Properties													
	pH	Moisture	C:N	Ct[a]	Nt[a]	Kt[a]	Pt[a]	(NO_3+NO2)-N[b]	Al[b]	Cd[b]	Mn[b]	Na[b]	Ni[b]	Pb[b]
Amino Acids and Derivatives														
Cell Division and Cell Cycle					0.7									
Cofactors, Vitamins, Prosthetic Groups, Pigments		-0.7		0.7						0.7	-0.8	0.7	0.7	0.7
DNA Metabolism					0.7			0.8	0.7	0.8				0.8
Motility and Chemotaxis														
Protein Metabolism														
Regulation and Cell signaling														-0.7
Respiration			0.7				0.7							
Secondary Metabolism														
Stress Response	-0.7	-0.7				0.7			0.8	0.8			0.7	
Virulence, Disease and Defense	0.8	0.8		-0.7						-0.8			-0.8	

a = g/kg; b = mg/kg

3.4 Discussion

Here we explored the correlations between abiotic and biotic factors in a long-term N-, P-, K- and NPK-fertilized grassland. We hypothesized that each inorganic fertilization treatment would alter bacterial community composition and functions (H_1). Bacterial community compositional and functional alterations were hypothesized to be linked to soil parameters other than pH (H_2). Our results indicated that soil chemical profiles and bacterial community composition but not functions shifted with inorganic fertilization. Several soil parameters were correlated to compositional and functional shifts in the bacterial community.

Chronic deposition of N, P and K fertilizers resulted in distinct soil profiles, i.e. with saturated levels of extractable N and NO_3-NO_2-N, P and K in the respective treatments (deduced by similar levels of these soil parameters in the NPK treatment). Thus, the treatments can be conceptualized as resource manipulations in which bacterial community assembly was driven by the four different treatment "habitats." Hereafter we will refer to each treatment as saturated with the associated fertilizer, e.g. N-saturated for the N fertilizer treatment, to facilitate our discussion on the drivers of compositional shifts that were observed within the habitats.

Microbial mining hypothesis links phyla and soil properties in N- and K-saturated fields

N-saturations of 160 kg N ha^{-1} yr^{-1} resulted in the largest differences in bacterial community compositions compared to the control field. Shen, Zhang [29] also showed significant changes in the microbial community composition in a 20-year inorganic N-fertilized field (135 kg N ha^{-1} yr^{-1}) in China. Fierer, Lauber [14] detected significant differences in microbial community profiles only in fields with the highest inorganic N-input of 136 to 145 kg N ha^{-1} yr^{-1}, which is in the range of our N treatment, and not in fields that received intermediate N input of 17 to 101 kg N ha^{-1} yr^{-1}. However, in that study, the duration of fertilization of the fields was 27 and 8 years while ours was 54 years. These studies and ours results confirm that N-saturation has variable effects on microbial community composition that depend on the duration of the experiment [13]. Total extractable N availability was significantly higher in our N-saturated soil compared to the control treatment while NH_4^+ levels were the same. Coupled with the taxonomic differences observed in the N- and K-saturated communities, the microbial mining theory offers a possible explanation. N stored in organic matter is the main source of N for microbial growth and maintenance [30] and microbes usually mineralize organic N into ammonium-N in order to access this nitrogen. In N-amended soil,

there is no need for microbes to mine N and to compete with other microbes to provide N sources. Instead, bacteria that utilize high levels of available N may be favored. We observed this prediction as a shift to copiotrophic taxa under N and K saturation. The copiotroph-oligotroph tradeoff [31] seems to explain our result of increased abundances of *Actinobacteria* and decreased abundances of *Acidobacteria*/*Verrucomicrobia/Firmicutes* in the N and K saturated fields. *Actinobacteria* are regarded as copiotrophs, whereas *Acidobacteria, Firmicutes* and *Verrucomicrobia* are regarded as oligotrophs. In support, studies have shown that N addition may decrease the decomposition of recalcitrant carbon [32], which may affect members of the phylum *Actinobacteria* since they are important decomposers and play a vital role in the carbon cycle [33]. The generally oligotrophic phyla *Verrucomicrobia* may highly depend on C availability due to a slow-growing life strategy [34, 35].

P mobility in N- and P-saturated fields may explain the chemical profiles

Our finding of no compositional differences in the P-saturated field compared to that of the control field was unexpected. In contrast, in a long-term (42-year) phosphorus fertilization field, the phosphorus addition resulted in a decrease of the relative abundance of *Acidobactera* and *Pseudomonas* in pasture soils [36]. Long-term (8-year) P-amendment also shifted the bacterial community in an alfalfa field [37]. In the P-saturated fields we observed decreased levels of available Mg and Mn compared to the control field. Because no compositional differences in the bacterial community between control and the P-saturated fields were found, a hypothesis based on P mobility may explain the differences in the chemical profiles that were observed. In acidic soils, phosphorous conversion to insoluble forms may occur by precipitation of phosphate ions with Ca, Mg, Al and Fe (precipitation); alternatively, phosphate might react to form plant-available soluble forms by reacting with Mg or Mn (reactive processes). We hypothesize that both precipitation and reactive processes are on-going in the P-saturated fields due to the excess availability of P. In support of this hypothesis, Mg and Mn levels were decreased in the P-saturated but not the NPK-saturated fields compared to the control fields; strikingly, available C levels were decreased in both fields. Our results suggest that in the P-saturated fields, phosphate reactions involving Mn and Mg have removed excess P while in the NPK-saturated fields the additions of N and K fertilizers diverted the P to alternate reactions.

Under fertilization, total community functions did not shift

Surprisingly, we found no fertilizer effect on the Level 1 and Level 2 functional profiles. This supports functional redundancy as a mechanism for stabilization of functions during changes in microbial composition [7, 38]. Functional redundancy refers to the hypothesis that each member of the community has the same functional capability, such that if the composition of the community changes, the metabolic output does not. This hypothesis suggests that functional profiles are more resistant to environmental changes than community compositions. Our result is in contrast to the observations of Fierer *et al.* [14]. They found distinct different functional profiles in the soils with the highest N-saturation at the highest hierarchical level of annotations. However, our observation was supported by a meta-analysis according to Allison and Martiny [39], who showed that microbial community composition is sensitive to environmental changes. Therefore, it is still difficult to make solid conclusions whether we could predict functional redundancy using whole community data. Further studies are needed to compose mock communities to test whether the link between community functions and metagenomic characteristics generally exists or whether the link only can be observed within certain taxa.

Abiotic factors and soil bacterial group correlations

In many long-term fertilization studies, community composition or functional changes are most often correlated with soil pH and C/N ratio [29, 40]. In addition to pH and C and N links, we report strong correlations of bacterial group abundances to several other measured parameters as well as functional categories. For example, we found that *Acidobacteria*, *Actinobacteria* and *Verrucomicrobia* were correlated with Kt, K, Al, Ni, N-NH_4 and Nt in at least 2 comparisons. Navarrete *et al.* Navarrete, Kuramae [11] has reported a correlation between Al with *Acidobacteria* in the Amazon area. *Verrucomicrobia* are positively correlated with soil moisture [41] and negatively correlated with elevated CO_2. Faoro, Alves [42] demonstrated the importance of Ca^{2+}/Mg^{2+} ratio, Al^{3+} and phosphorus content in shaping soil microbial community composition in the Southern Brazilian Atlantic Forest. The studies suggest that besides pH and C/N ratios other abiotic parameters may also influence soil microbial community structures. Cd, Al and Ni are regarded as toxic (stress) compounds to microbes [43]. In order to survive, the microbes need to have the ability to detoxify, which can explain why in our study available Cd, Al, Ni and total K concentrations had a high positive correlation with stress response-related gene relative abundances. The positive correlations

between cell division and DNA metabolism genes and total extractable N suggest that the increased N nutrient level may lead to enhanced microbial reproduction [44].

Conclusions

Of the numerous studies investigating the influence of long-term fertilizers on microbial community compositions, many generalize the different soil sources e.g., grassland, pasture or forest, and use 16S rRNA amplicon profiling. Here we focused on the effects of only inorganic fertilization on long-term bacterial community changes in clay soil grassland. We applied shotgun sequencing which allowed us to circumvent PCR biases as occurs 16S rRNA amplicon studies and also to evaluate the effects of fertilization not only on bacterial community composition but also on potential functions. During our analysis, we took a coarse-grained approach by examining the changes in the bacterial communities at the domain, phyla or Subsystems category levels. This was done to overcome database biases and spurious annotations from a finer resolution. Last, we demonstrated the importance of measuring a suite of soil parameters in future studies that aim to find links between abiotic and biotic ecosystem components. Whether our findings of the correlations between different soil factors (especially Fe, Al, Mg, Mn) and specific bacterial groups and functional categories have biological meanings still needs to be verified in both laboratory and field conditions.

3.5 Declarations

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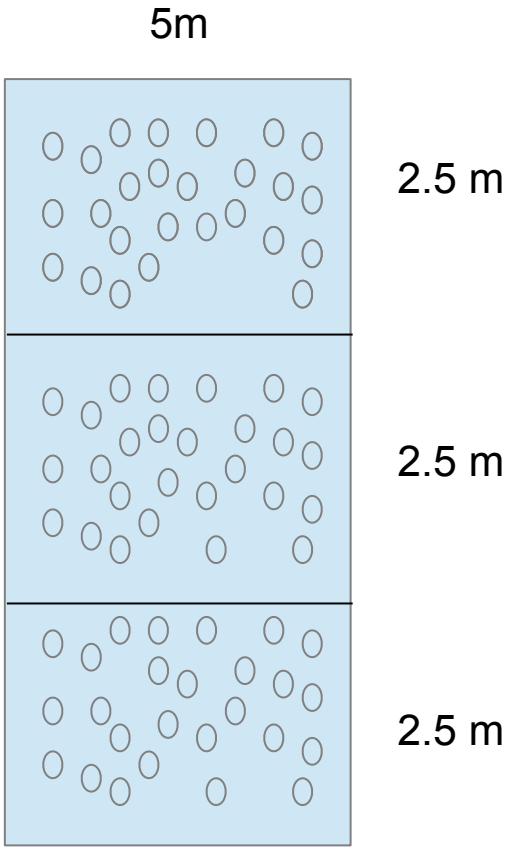
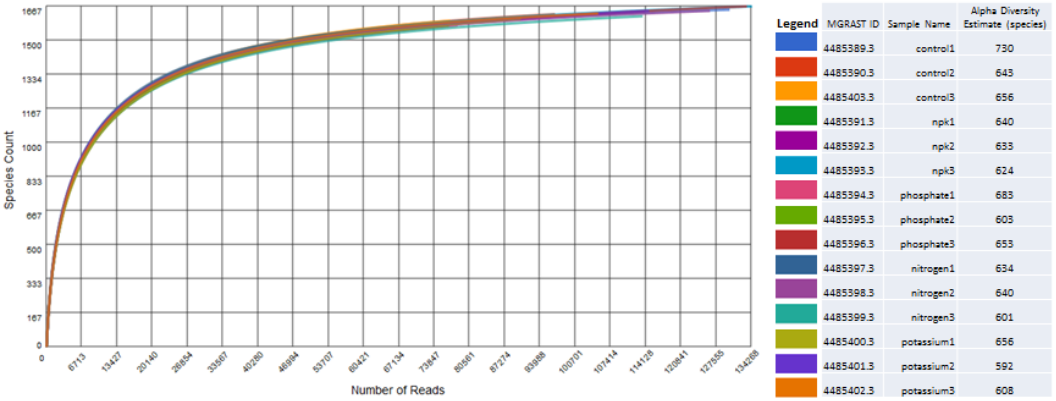
3.6 Supplementary Material

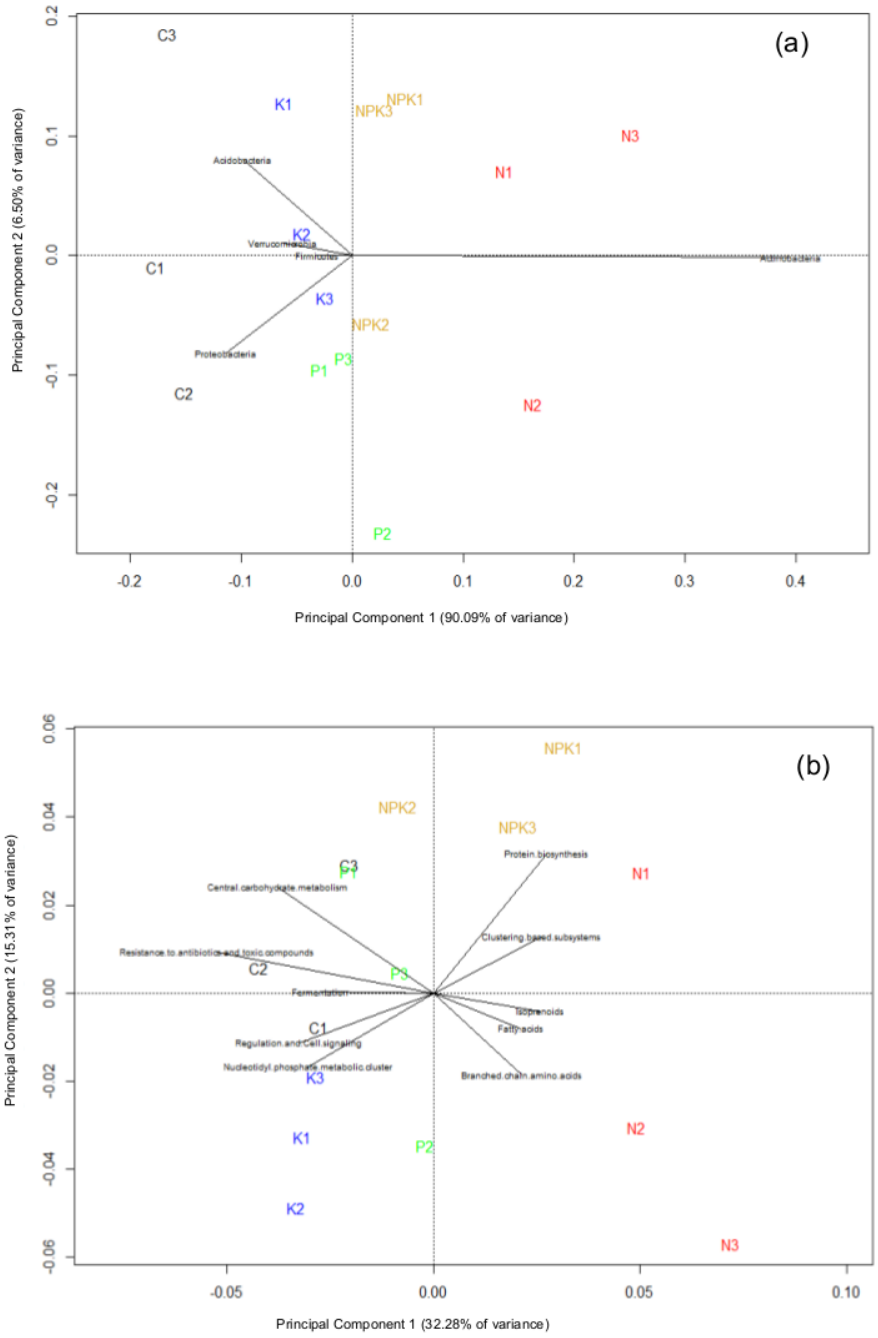


Supplementary Figure 1. One field is divided to 3 plots, 27 soil cores from each plot were sampled and pooled as one replicate for the field, and each field has 3 replicates.

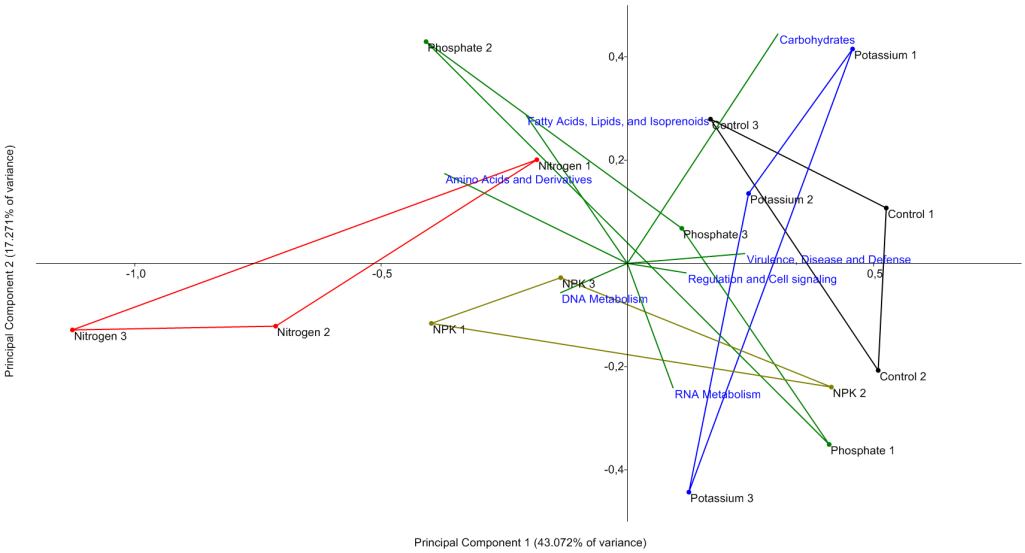


Legend	MGRAST ID	Sample Name	Alpha Diversity Estimate (species)
	4485389.3	control1	730
	4485390.3	control2	643
	4485403.3	control3	656
	4485391.3	npk1	640
	4485392.3	npk2	633
	4485393.3	npk3	624
	4485394.3	phosphate1	683
	4485395.3	phosphate2	603
	4485396.3	phosphate3	653
	4485397.3	nitrogen1	634
	4485398.3	nitrogen2	640
	4485399.3	nitrogen3	601
	4485400.3	potassium1	656
	4485401.3	potassium2	592
	4485402.3	potassium3	608

Supplementary Figure 2. Rarefaction curve analysis of the 15 sample metagenomes and estimated alpha diversity of the communities. Figure taken from MG-RAST based on subsamples plotted against M5NR species annotations.



Supplementary Figure 3. Principal Component Analysis (PCA) of the samples based on relative abundances of metagenome sequences annotated within (a) RefSeq bacterial phyla and (b) Subsystem Level 2 categories. Only the phyla that contributed to more than |0.1| units of loading and the Subsystems categories that contributed to more than |0.2| units of loading are included in the plots. C=Control, N=N fertilized samples, P=P fertilized samples, K=K fertilized samples, NPK=NPK fertilized samples. 1, 2, 3 = replicates.



Supplementary Figure 4. Principal Component Analysis (PCA) of the samples based on relative abundances of metagenome sequences annotated within Subsystem Level 1 categories. Only the phyla or Subsystem categories that contributed to more than |0.1| units of loading are included in the plots. Control=black, Phosphorus=green, Potassium=blue, Nitrogen=red, NPK=yellow; 1, 2, 3 = replicates.

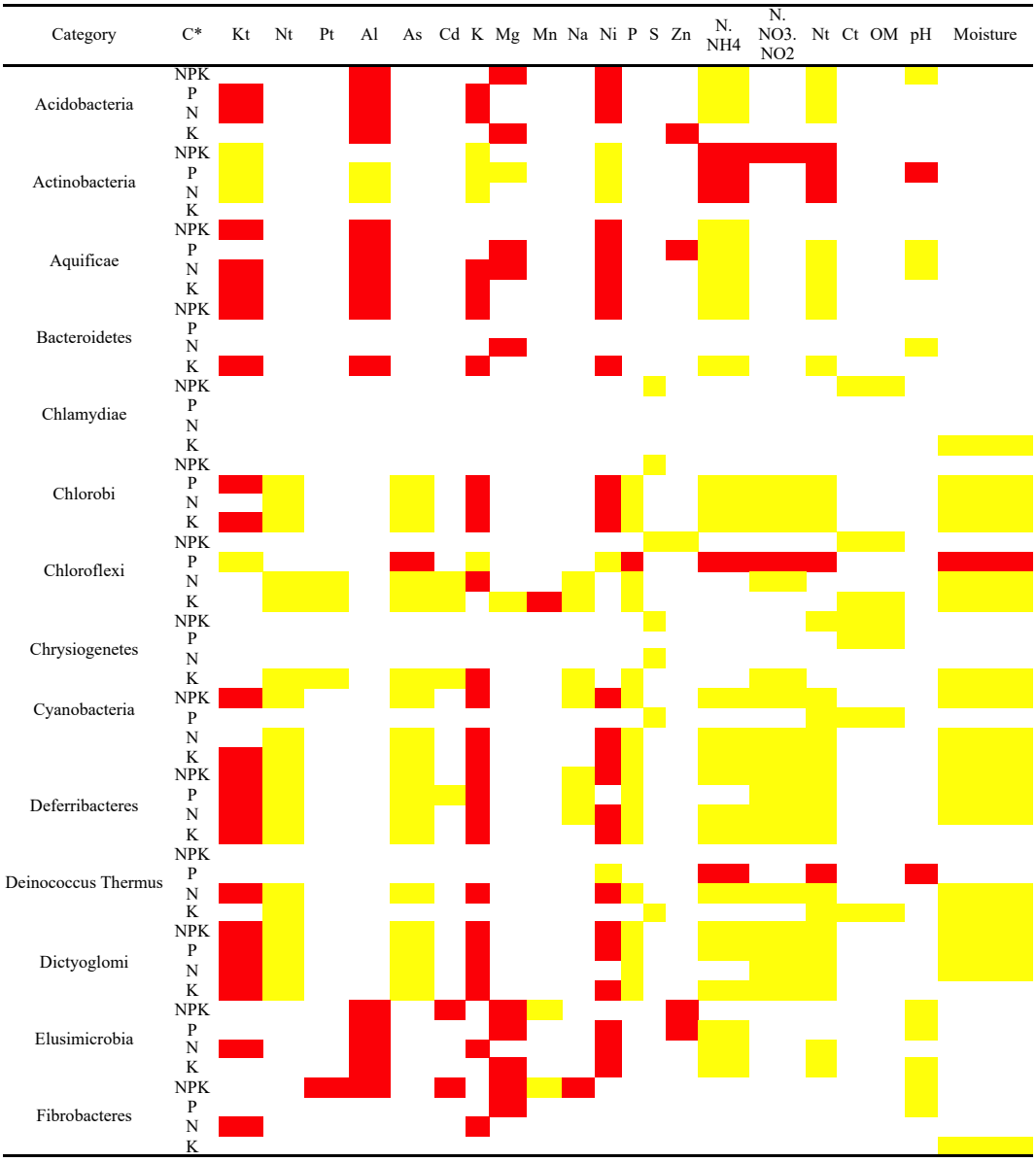
Supplementary Table 1. Sequencing information for the fifteen sample metagenomes.

Sample Name	MGRAST ID	# Sequences	# bp	Mean Sequence Length (bp)
control1	4485389.3	105010	40423549	384 ± 131
control2	4485390.3	114709	44905782	391 ± 127
control3	4485403.3	126331	49728531	393 ± 120
npk1	4485391.3	134389	52721323	392 ± 123
npk2	4485392.3	113883	42073456	369 ± 125
npk3	4485393.3	96793	38425397	396 ± 120
phosphate1	4485394.3	84053	33521299	398 ± 119
phosphate2	4485395.3	76677	29433082	383 ± 121
phosphate3	4485396.3	83399	32356773	387 ± 119
nitrogen1	4485397.3	76859	28573338	371 ± 124
nitrogen2	4485398.3	78248	28396437	362 ± 126
nitrogen3	4485399.3	90340	34738730	384 ± 123
potassium1	4485400.3	130016	51665672	397 ± 123
potassium2	4485401.3	133309	52226427	391 ± 120
potassium3	4485402.3	113565	43656772	384 ± 125

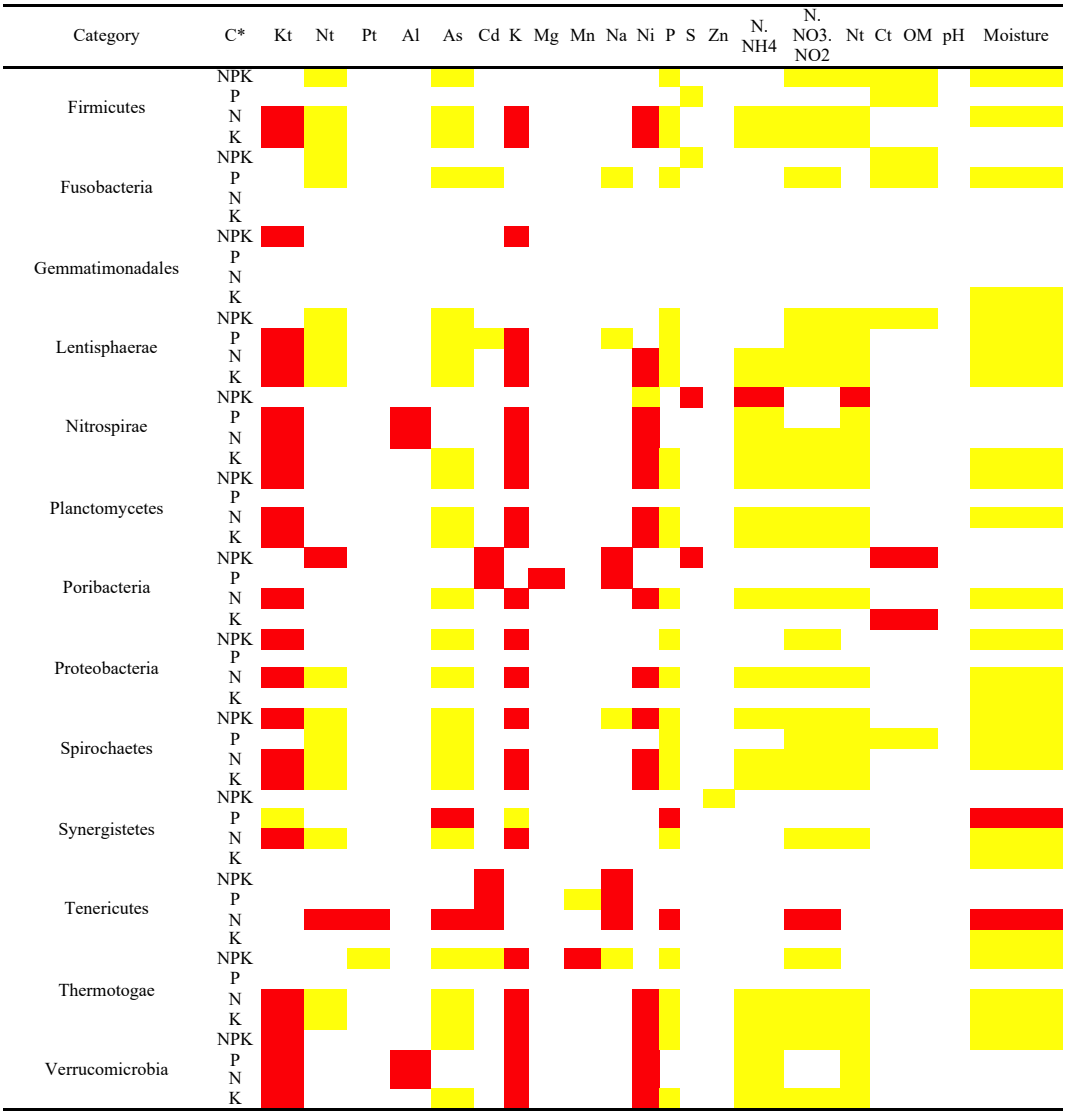
Supplementary Table 2. Relative abundances of metagenome read annotations within taxonomic categories. Values above a cutoff of 0.08% are presented and those in bold were significantly different between treatments (ANOVA, corrected $p < 0.05$).

		Average Relative Abundance of Reads (%)				
Domain	Phylum	Control	Nitrogen	Phosphorous	Potassium	NPK
Archaea	Crenarchaeota	0,14	0,12	0,12	0,12	0,17
	Euryarchaeota	0,90	0,64	0,82	0,72	0,79
Bacteria	Acidobacteria	13,78	10,59	10,44	13,36	12,20
	Actinobacteria	**10,35**	**23,70**	**16,18**	**15,19**	**17,78**
	Aquificae	**0,26**	**0,16**	**0,22**	**0,19**	**0,23**
	Bacteroidetes	4,28	3,82	4,05	3,50	3,57
	Chlamydiae	0,15	0,14	0,14	0,15	0,14
	Chlorobi	**0,61**	**0,44**	**0,54**	**0,53**	**0,58**
	Chloroflexi	**2,19**	**1,93**	**2,39**	**2,09**	**2,25**
	Cyanobacteria	**2,90**	**2,16**	**2,71**	**2,56**	**2,54**
	Deferribacteres	**0,13**	**0,08**	**0,09**	**0,08**	**0,10**
	Deinococcus-Thermus	0,58	0,54	0,60	0,53	0,60
	Firmicutes	**5,81**	**4,62**	**5,63**	**5,06**	**5,51**
	Fusobacteria	0,10	0,10	0,08	0,12	0,09
	Gemmatimonadetes	0,35	0,31	0,35	0,33	0,34
	Lentisphaerae	**0,16**	**0,11**	**0,12**	**0,11**	**0,14**
	Nitrospirae	**0,32**	**0,20**	**0,26**	**0,24**	**0,36**
	Planctomycetes	**2,38**	**1,51**	**2,37**	**2,11**	**1,82**
	Proteobacteria	**45,90**	**42,19**	**44,49**	**44,88**	**43,46**
	Spirochaetes	**0,26**	**0,17**	**0,25**	**0,21**	**0,22**
	Synergistetes	**0,14**	**0,10**	**0,15**	**0,13**	**0,15**
	Thermotogae	0,21	0,13	0,20	0,17	0,19
	Verrucomicrobia	**5,60**	**3,32**	**4,36**	**4,58**	**4,58**
Eukaryota	Arthropoda	0,12	0,17	0,16	0,09	0,11
	Ascomycota	0,79	1,23	1,10	1,30	0,55
	Basidiomycota	0,21	0,15	0,44	0,32	0,15
	Chordata	0,21	0,21	0,30	0,24	0,25
	Streptophyta	0,34	0,43	0,65	0,40	0,41

Supplementary Table 3. Spearman correlation values between the relative abundances of annotations within bacterial phyla and soil parameters using data from all five sample treatments. Only the phyla and parameters with correlations values greater than |0.6| are shown. Positive correlations=yellow and negative correlations=red. Continued on next page.



Supplementary Table 3 con't.



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