Variation in Microbial Community Profiles and Their Energy Metabolism Predictions Under the Influence of Pure and Mixed Fertilizer in Soil Microcosms

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Research Article

Keywords: NPK fertilizers, paddy soil, microcosm, high throughput sequencing, PICRUSt, methane metabolism

Posted Date: March 19th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-319529/v1

Variation in microbial community profiles and their energy metabolism predictions under the influence of pure and mixed fertilizer in soil microcosms

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Abstract

The impact of environmental perturbations (e.g., nitrogen (N), phosphorus (P), potassium (K) and rice straw (Rs)) on the dynamics of soil bacterial and archaeal community are multifactor dependent and seeks more investigation concerning underlyig mechanisms. Current study was designed to establish the effect of pure and mixed fertilizers on microbial community profiles in paddy soil. A short-term microcosm based experiment was established in which each microcosm is amended with N as $C(H_2N)_2O$, P and K as KH_2PO_4, K as KCl and Rs with concentrations equivalent to 160 kg N ha^{-1}, 60 kg P ha^{-1}, 130 kg K ha^{-1} and 1% respectively. Soil pH, electrical conductivity (EC), total C (TC), total nirtrogen (TN), organic matter (OM), available K (AK) and extractable P (EP) were evaluated. To understand the microbial community variation in soil and to predict their metabolic functions, a high throughput sequencing (HTS) approach of 16S rRNA gene along with phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) was employed and analyzed. The results showed that microbial richness and diversity were increased under all amnendments compared to control. *Proteobacteria*, *Actinobacteria* and *Firmicutes* were dominant bacterial phyla. In all amendments, regarding relative abundance, *Chloroflexi*, *Bacteroidetes* and *Verrucomicrobia* showed positive while *Actinobacteria*, *Acidobacteria* and *Gemmatimonadetes* showed negative trends when compared with controlled observations. *Thaumarchaeota* and *Euryarchaeota* were dominant archaeal phyla and exhibited increasing and decreasing trends, respectively. The PICRUSt indicated microbial community shift significantly towards amino acid, carbohydrate, energy, and lipid metabolism while less towards glycan biosynthesis, synthesis of secondary metabolites, terpenoids and biodegradation. Regarding metabolism (methane metabolism), most and least responsive treatments were predicted to be KP and controls, respectively. These findings enhanced our understanding regarding soil quality, fertilizer composition and their impact on microbial diversity.

Keywords: NPK fertilizers, paddy soil, microcosm, high throughput sequencing, PICRUSt, methane metabolism

Declarations:

Funding: The current study was non-funded.

Conflicts of interest/Competing interests: We declare no Conflicts of interest/Competing interests.

Availability of data and material (data transparency): The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. The sequencing data was submitted

in the Sequence Read Archive (SRA) of NCBI (National Center for Biotechnology Information) under the BioProject PRJNA627288 with accession numbers SAMN14661259 to SAMN14661276 and can be accessed.

Code availability (software application or custom code): We strongly believe that all data and materials as well as software application comply with field standards.

Authors' contributions: Conceptualization: [Naeem Ali]; Methodology: [Mohsin Gulzar Barq, Muhammad Mubashar Hassan]; Formal analysis and investigation: [Mohsin Gulzar Barq, Muhammad Mubashar Hassan]; Writing - original draft preparation: [Mohsin Gulzar Barq]; Writing - review and editing: [Mohammad Mubashar Hassan, Noshaba Hassan Malik]; Resources: [Richard Dick]; Supervision: [Naeem Ali].

Ethics approval (include appropriate approvals or waivers): No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with an unregulated invertebrate species.

Consent to participate (include appropriate statements): Not Applicable.

Consent for publication (include appropriate statements): Not Applicable.

Introduction

Soil plays a complex and fundamental part in terrestrial ecosystem and microbial ecology by executing its biotic and abiotic processes that evolve over time [1]. The efficiency and sutainability of terrestrial agroecosystem is highly reliant on micobial diversity and physiology that vaires continuously with the nutrients' status [2, 3]. The substantial role of soil microbiome in energy flow, nutrient cycling [4] and soil quality determinant is well documented [3]. The soil quality is often accompained with nutrient status and microbial community dynamics which make them vital parameters to interrogate in soil ecology studies. A minor shift in organic and inorganic content of soil may shift the microbial community dynamics and modify their underlying mechanisms [5–7] and ultimatley lead to varying yield and soil quality [8]. In this context, the relative abundance and role of specific microbes is considerably important [9]. The 16S rRNA based high-throughput sequencing (HTS) has been well established in recent years to study microbial community ecology dynamics in short and long term studies [10] thus making it an excellent method of choice.

The physicochemical and biological behaviour of paddy soil under flooded irrigation is quite different from upland soil [11, 12]. The nutrient budget of paddy soil is dependent on supplementation of organic (e.g rice straw) and inorganic fertilizers e.g NPK respectively [7]. The role of paddy soils is very important concerning methane status and recycling which is one of the green house gases that has 25 times more ultravoilet (UV) retention capability in atmosphere compared to CO_2 and paddy soilds are the major artificial sites of methanogenesis after natural wetlands. From environmental perspective, methanotrophs are more highlighted as consumers compared to methanogens as producers that are participating in biogeochemical cycling of CH_4 in an antagonistic manner [13]. Typically, aerobic soil is the only biological sink for the oxidation of methane by methanotrophic bacteria sequestering about 6.0% in upland dry soils (i.e., forest and grasslands) and 10.0 to 30.0% in wetland soils [14, 15]. Globally, paddy soils contribute 15–20% CH_4 emission (25-100 Tg/year) which increased during rice cultivation seasons [16, 17]. It is predicted to be increased up to 50% (145 Tg/year) by 2025 due to growing demands of rice production and consumption. Thus it will lead to greater input of NPK fertilizers that may affect the energy metabolism of microbial communities turning soil into source of methane emission rather than sink [18–20]. However, the effect of inorganic fertilizers depends on the type, concentration, mode of application of fertilizers [21]. For instance, NPK fertilization and rice straw has been known to induce varying degree variations in soil physicochemical properties and microbial community dynamics [22–24]

Though it is still unclear how microbial community dynamics transform with different sources of carbon (C), N, P and K, since it fluctuates with multiple factors such as soil texture, pH, EC, OM, availability of mineral nutrients and other accompanied microorganisms as well [25]. Considering the variability in type and application rate of fertilizers in paddy soil, it is vital to know the change in soil microbial community structure, function and chemistry that ultimately affect global methane burden. So, a microcosm-based experiment was established to investigate and predict the said question. The specific hypothesis of the study was to evaluate the effect of short-term supplementation of rice straw and NPK based fertilizers on the composition and relative abundance of bacterial and archaeal community in paddy soil. Additionally, PICRUSt derived functional profiles i-e energy metabolism of contributing microbial community can be predicted to estimate methane metabolism and there may be a correlation between dynamics of microbial communities and physicochemical factors.

Materials and Methods

Experimental soil

Soil samples (non-calcareous, silty clay loam, isohyperthermic Udic Hapludalfs) were acquired from a depth of 10-20 cm in early August 2018 from the rice paddy field in Gujranwala, Pakistan (32°19'N, 74°20'E). The area is 226 m above sea level with hot semi-arid climate (BSh) [26]. The annual rainfall varies around 577 mm with average annual temperature of 23.9 °C. The field soil was transported to the experimental provision in a zipper bag to minimize contamination. The samples were air dried, sieved (2 mm) and stored at -20 ºC till further experimentation. Aseptic conditions were maintained wherever necessary. The soil had a pH of 8.05, TC 0.17% and TN 1.40%.

Microcosm Set-up and supplementation

The microcosms were established using 2.2 kg soil slurry in 64 oz polyethylene plastic pots (20cm height and 15cm diameter) and anaerobic conditions were created by flooding the soil with 3cm of water. Each microcosm was planted with a 26-day old nursery of *Oryza sativa* (var. super basmati). Excluding two controls and time zero sample, 15 different combinations were developed in triplicates using N (as urea), P and K (as KH_2PO_4), K (as KCl) [27] and rice straw (Table 1). All microcosms were amended accordingly with 50ml solution of each fertilizer (per 100 ml: 0.23g N as urea, 0.087g P as KH_2PO_4 and 0.185g K as KCl). Additionally, concentrations of carrier ions were calculated as 0.05g K in KH_2PO_4 and 0.08g Chloride (Cl) in KCl. The supplementation was done at day 0, 5 and 30 as basal dressing and two top dressings. The two controls (with plant and without plant) were provided with the same conditions as that

of samples but without any supplementation. These amendments were in accordance with common rice field agriculture and correspond to per ha 160 kg N as urea, 60 kg P as KH_2PO_4, 130 kg K as KCl and 1% rice straw [28]. The constructed microcosms were placed at an average temperature of 20–25 ºC in a green house facility for 45 days and the water level of 3cm was maintained throughout that period. Soil samples were collected during vegetation phase from each microcosm for further analysis.

Soil Physicochemical Properties

The soil moisture content was calculated following gravimetric method [29, 30] and represented as gravimetric water content (GWC). The pH and EC were measured by dipping glass electrode employing 1:1 soil /water (v/v) ratio. TC and TN were measured by combustion at 1800 ºC using Vario Max CN Analyzer. Soil particle distribution was determined by hydrometer method [31] and textural class was assigned as per US textural classification. EP was determined using Mehlich-3 soil phosphorus test [32].

Microbial DNA extraction, 16S amplicon production and sequencing

Microbial genomic DNA was extracted employing PowerSoil® DNA isolation kit (MoBio, Carslbad, CA) as per Earth Microbiome Project benchmarked protocols [33, 34]. Microbial community composition was assessed as per protocols and primers described [35] that target archaeal and bacterial hypervariable V4 region (515f/806r) of the 16S rRNA gene [36]. Amplicons of 16S rRNA gene were generated following amplification using HotStarTaq Plus Master Mix Kit (Qiagen) employing subsequent conditions: initial denaturation (94 °C for 3 min) followed by 30 cycles, each set at 94 °C for 30 seconds, 53 °C for 40 seconds and 72 °C for 1 min, with a final elongation step at 72 °C for 5 min. The PCR products were analyzed on 2% agarose gel. Multiple samples were pooled in equal proportions based on DNA concentration and molecular weight. The pooled samples were purified by calibrated Ampure XP beads and used to prepare DNA libraries following Illumina TruSeq DNA library preparation protocol. Sequencing was performed at the Molecular Research DNA laboratory (Shallowater, TX, USA) on an MiSeq (Illumina) platform in an overlapping 2 × 300 bp configuration with a minimum throughput of 20,000 reads for each sample.

Processing of raw Illumina sequencing data

Raw amplicon sequences of 16S rRNA were processed and analyzed following described protocols [37, 38]. In brief, sequences were joined (overlapping pairs) and grouped by samples following the barcodes that were removed afterwards. Then, sequences <150 bp or with ambiguous base calls were removed. Remaining sequences were filtered

using the USEARCH clustering algorithm at 4% sequence divergence to remove chimeras and clusters consisting of only one sequence (i.e. singletons) [39]. The sequencing data for all the 18 samples was submitted in the Sequence Read Archive (SRA) of NCBI (National Center for Biotechnology Information) under the BioProject PRJNA627288 with accession numbers SAMN14661259 to SAMN14661276.

Sequence analysis, taxonomic identification, and diversity analysis

All the resulted sequences were analyzed with Quantitative Insights Into Microbial Ecology (QIIME 2 Core 2019) to obtain all 16S rRNA reads from the amplicon with 97% similarity or 3% divergence with the taxonomy of resulting Operational Taxonomic Units (OTUs) [40, 41]. The OTU selection process was performed with USearch (v6.1.544) using QIIME 2. The total number of OTUs analyzed were 14,087 comprising 1,509,246 reads at species level across 18 samples. Finally, all the OTSs were taxonomically categorized using BLASTn against RDPII and NCBI based database (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu). The microbial diversity patterns were analyzed by calculating alpha OTU diversity using the alpha_rarefaction.py script in QIMME 2 [41]. The Shannon, Pielou E and Faith's Phylogenetic Diversity (PD) indices were calculated alongside observed OTUs ('richness') [42]. While for beta diversity pattern, Bray Curtis, Jaccard, Unweighted Unifrac and Weighted Unifrac distance matrices were calculated (data only shown) and reported [43].

Functional diversity of microbial community

Functional capabilities of microbial communities were predicted using sequencing data of 16S rRNA gene by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [44]. The PICRUSt software store the COG information and KEGG Ortholog (KO) information related to the greengene id and predict metagenomes by standardizing OUT abundance. The KO and COG family information were obtained by greengene id related to each OUT and the KO and COG abundance was obtained. The information of KO, EC and Pathway were obtained from KEGG database thus functional categorization at three levels can be obtained according to OTU abundance [45].

Statistical Analysis

The indexes of microbial alpha-diversity were estimated by mothur (version v1.30.1), including Pileou's E, Faith's PD and Shannon [42]. Means and standard errors (SE) were calculated using Microsoft Excel 365. Multivariate analysis of variance (MANOVA) as Post-HOC test (Tukey's HSD) at the significance level of $\alpha=0.05$ ($p<0.05$) was

performed using SPPP (IBM SPSS Statistics for Windows, Version 26.0., Armonk, NY, USA). The HTS data was computed by QIMME 2 [41] and principal component analysis (PCA) was performed in Canoco for Windows (version 4.5) and drawn in Cano Draw [46]. The hierarchical clustering was plotted using Euclidean distance method and Ward's minimum variance as clustering method and in BioVinci (Version: 1.1.5, r20181005). Details of evaluations are provided in results and discussion.

Results

Physicochemical properties of soil

Soil physicochemical properties showed variable effects of fertilization on bacterial and archaeal community composition. The pH varied between 7.68 – 8.28 in four fertilizer regimes and significant variations were found for different combinations. The EC values were significantly increased in all treatments as compared to control and varied between 259-602 dS/cm being higher in UPK, UPRs, PKRs and UPKRs and lower in each control and U. Soil TC varied between 0.146 – 0.177% and found to increase in all treatments except KP and PKRs while TN varied between 1.34 – 1.64% and found to decrease only in U, P, KP, UK and PKRs. Soil OM and AK increased with the use of fertilizers and ranged between 0.72 – 2.79% and 6.4 – 13.2 mg/kg, respectively. Variations in EP were insignificant in any treatment. Differences based on different treatments using physicochemical properties were outlined using PCA in figure 1. The first two axes explained 33.5% and 25.5% of the overall variance. More variation is found in C_0 and C_f on the positive side of PC1 which is influenced by pH, GWC and EP while TC, TN, OM, EC, and AK tend to influence on left side of biplot and influencing most of the samples. The biplot showed strong correlation between TC and TN; EP and GWC and EC, AK, and OM.

Microbial community composition

The sequencing results showed 3,237,072 reads of 16S rDNA which accounts for 92.51% of the total reads for bacteria. They were clustered into 13,918 OTUs from 18 soil samples and assigned 29 bacterial phyla and 902 genera. Overall, 10 major bacterial phyla contributed over 99% of bacterial community structure with *Proteobacteria* (32-37%), *Actinobacteria* (21-26%) and *Firmicutes* (15-19%) being the dominant ones. Other important bacterial phyla were *Chloroflexi* (9-15%), *Bacteroides* (2-6%), *Acidobacteria* (2-3%) and *Gemmatimonadetes* (1-2%). The relative abundance of dominant phyla and genera is shown (Figure 2). *Chloroflexi*, *Bacteroides*, *Planctomycetes* and *Verrucomicrobia* are found to increase in majority of treatment as compared to control. The dominant genera were

bacillus followed by *conexibacter*, *solirubrobacter*, *bellilinea* and *sphingomonas*. *Bellilinea, Pelobacter*, *Clostridium* and *Dehalococcoides* showed increasing trend, while converse was found for *conexibacter,* Solirubrobacter, *Sphingomonas*, *Acidobacterium*, *Thermoleophilum* and *Frankia*.

For archaea, 193,917 valid reads were obtained which contributed 5.54% of overall diversity and clustered into 169 OTUs which are classified into 3 phyla, and 23 genera. Among the 3 archaeal phyla, *Thaumarchaeota* was the most dominant followed by *Euryarchaeota* and *Crenarchaeota* and their relative abundance in all samples varied from 93-97, 1-6 and 0.2-0.9% respectively (Figure 3). The dominant archaeal genera that gave ~95% community coverage include *Nitrososphaera* (69-78%), *Candidatus* (18-24%), *Methanobacterium* (1-3%) and *Methanocella* (1%). Overall, an increasing trend was observed for *Thaumarchaeota* with their lowest abundance in C_0 (93%) and highest in UK (98%) and decreasing trend was found for *Euryarchaeota* with lowest in K (1.8%) and highest in C_0 (6.2%). For archaeal phyla, *Thaumarchaeota* showed increasing, *Euryarchaeota* proved opposite and *Crenarchaeota* showed both trends. For Archaeal genera, *Nitrososphaera* and *Methanosaeta* showed increasing while the rest showed both trends. The variation in archaeal community is also well pronounced in case of methanogens.

Variations in microbial community composition in different treatments are outlined by PCA (Figure 4). PC1 and PC2 accounted for 56.3% of the variance in microbial community which demonstrated separation and clustering in microbial communities in soil with all treatments.

Observed OTUs, Evenness, Diversity and Bray-Curtis dissimilarity indices of microbial communities.

The observed OTUs and α-diversity indexes like Pielou's E, Faith's PD and Shannon are shown (Table 2). Of all the samples, the least OTUs were found in C_0 and C_f which represent lesser microbial activity without any amendment and vice versa. Pielo's E and and Shannon's indexes were greater in all samples as compared to controls without plants i-e C_0 and C_f while the Faith's PD was also lower in these controls as compared to all other samples.

Functional metabolism profiles prediction (Second and Third level)

The PICRUSt analysis demonstrated six primary functional levels, including, metabolism, genetic information processing, environmental information processing, cellular processes, organ systems, and human diseases. The functional profiles of metabolism at second and third level were predicted using PICRUSt and hierarchically clustered as shown in figure 5. Regarding metabolism, the highest gene counts were found for amino acid and carbohydrate metabolism with lowest in sample C_0, C_f and P and highest in UKRs and KP which also indicate the overall trend of

the community. The gene counts for energy metabolism, lipid metabolism, metabolism of cofactors and vitamin and xenobiotic degradation also showed increase as compared to control without plants i-e C_0 and C_f. An approximate two-fold increase was observed for C_{neg}, U, K, Rs, KP, RsK, UPK, UPRs and UPKRs while three-fold increase was observed for UP and PKRs.

At third level, more pronounced differentiation was observed for carbon fixation pathways in prokaryotes, methane metabolism, nitrogen metabolism and oxidative phosphorylation in KP, UKRs, UPRs, UK and RsP than the rest of samples. The trend for variation against different samples was found to be synchronized with second level. Shift in the abundance and composition of functional metabolism can explain a functional category. The heat map demonstrated gene counts for methane metabolism being highest in KP (7.82%), followed by UKRs (7.24%), UPRs (6.78%), UK (6.64%) and U (6.27%), while least in controls i-e C_0 (2.76%) and C_f (3.02%). At level 3, the microbial communities seem to respond considerably higher for oxidative phosphorylation (21.76%), methane metabolism (18.26%), and carbon fixation pathways in prokaryotes (17.79%).

Correlations

Correlation analysis between physicochemical factors and relative abundance of dominant taxonomic bacterial and archaeal phyla are summarized in Table 3. It strongly indicated an association between pH, OM and AK influencing positively on majority of bacterial phyla. Soil pH and EP negatively affect *Proteobacteria* and *Bacteroidetes* but positively with *Actinobacteria* and *Firmicutes*. pH seems to impact neutrally for archaea except *Crenarchaeota*. The concentration of soil organic matter is positively correlated with all bacterial phyla except *Actinobacteria* and negatively correlated with *Euryarchaeota* and *Crenarchaeota*. Soil EC and AK had a positive impact on *Proteobacteria*, *Bacteroidetes*, *Gemmatimonodetes* and *Thaumarchaeota* and negative impact on *Actinobacteria*, *Chloroflexi*, *Euryarchaeota* and *Crenarchaeota.* Soil TC and TN is positively correlated with *Verrucomicrobia*, *Euryarchaeota* and *Crenarchaeota.* The RDA analysis between soil physicochemical properties and microbial compositions explained 29.1% and 18.5% variance for RD1 and RD2 axes, respectively (Figure 6). *Actinobacteria* ($F= 2.55$, $P = 0.02$) and *Nitrospirae* ($F= 1.71$, $P = 0.05$) were correlated significantly with soil physicochemical properties.

Discussion

As far as organic and inorganic supplementation of paddy soil concerned, the current study characterized a comprehensive investigation of consequent microbial community variations in paddy soil in terms of structure and function. It also focused the quantification, diversification, and metabolic functional prediction at different levels of two key microbial groups i.e., bacteria and archaea. Thus, it gave us better insight on fertilizer usage with respect to methanogens in soil microbiome.

Soil physicochemical properties treated with various fertilizers has been reported to impact bacterial and archaeal community structure [47]. The pH of soil did not vary significantly in our study against different treatments. The flooded conditions in soil have been known to stabilize pH by inhibiting nitrification which is acid producing process [48]. Although pH is known to be a considerate factor in shaping microbial communities [49], some studies has reported otherwise in clay loam [50]. After subsequent inorganic and organic supplementation, significant increase in EC, AK and OM was observed which were strongly correlated to each other and poorly to pH (Figure 1) as previously reported [3]. PCA biplot showed strong correlation between OM and AK; TC and TN; GWC, EP and pH [51] . OM and AK also showed negative correlation with TC and TN while weak correlation with GWC, EP and pH. With respect to different treatments, strong association in microbial composition between KP, PKRs and UK is noted, while the rest of sample showed distinction of varying degree from each other. TC and TN have been showed to positively influence Rs, URs, RsP, UPRs and UPKRs. In particular, the control treatments C_0 and C_f were separated from C_{neg} as well as from other treated samples. No significant variation in TC, TN and EP was observed, which are usually known to increase with straw application and NPK fertilizations, respectively. The possible explanation could be increased CNP efficiency in flooded soil for plant uptake to satisfy their needs. Our results correspond to specific soil used in the study and considerable variation could have occurred due to soil texture, temperature, mineralogy, pH, and OM.

The microbial community succession under the influence of NKP and rice straw are well documented in wetland ecology and rice fields [52]. TC and TN seems to impact negatively or neutrally for majority of bacterial phyla while for archaeal phyla they were positively correlated except *thaumarchaeota*. This exception can be supported by the fact that fungi are more dependent on C and N sources than bacteria and archaea [53]. Total bacterial and archaeal population increased for every test sample as compared to control, however that increase was not sharp in case of bacteria [54] and a moderate increase under flooded conditions has also been reported [55]. Phylum *Proteobacteria* comprised the largest fraction of soil bacterial communities [7, 56] both metabolically and genetically due to

copiotrophic lifestyle of paddy soil [57] and the prevalence of other dominant phyla i-e *Actinobacteria*, *Firmicutes*, *Chloroflexi*, *Bacteroidetes*, *Acidobacteria* etc is also well documented [3, 55, 57, 58] and is in accordance with our results [59]. Bacterial phyla, *Chloroflexi*, *Bacteroidetes*, *Planctomycetes* and *Verrucomicrobia* showed increased in population size as compared to control while *Actinobacteria*, *Acidobacteria* and *Gemmatimonadetes* showed negative trend [10, 60]. Previous studies also report more response of bacterial diversity in the presence of inorganic fertilizer along with rice straw which satisfy our results for all cases except UPKRs [61–63]. One contrary finding in our current study was of *Verrucomicrobia*, which has been reported to decrease with rice straw incorporation and increase during chronic N incorporation [2, 60, 64]. Additionally, RDA analysis showed *Actinobacteria* and *Nitrospirae* being correlated with soil physicochemical properties (Figure 6). It also showed time zero control (C_0) well separated from all treatments with maximum *Actinobacteria* population. Since microbial diversity in soil is always multifactorial dependent, competitive inhibition due to multiple fertilization may justify our results. One such example is of carrier ions (chloride ions in our case from KCl). Chloride ions being a strong oxidant act as a potential biocide and have been studied to obstruct nitrification even at low concentration [65, 66]. This study also suggest that *Bacillus* does not seem to be very responsive genera for almost each combination except UPRs and UPKRs which propose that rice straw in combination U and P may shift the functional dynamics of *Bacillus*. Additionally, rice straw incorporation has been reported extensively to stimulate bacterial communities in paddy soil and our results are in accordance with it [2, 67, 68]. Since multiple bacterial, fungal, and archaeal phyla with various functions were operating, it cannot be concluded which specific factor altered their shift in our study.

The soil archaeal community in rice fields are reported to be more stable unless influenced by temperature or presence of organic matter such as rice straw [55, 69]. Our results suggested archaea (specifically methanogens) being more responsive as compared to bacteria concerning community structure and metabolic functioning due to KCl supplementation. The presence of methanogens such as *Methanosarcinaceae*, *Methanosaetaceae*, *Methanobacteriales*, *Methanomicrobiales*, and *Methanocellales in* rice fields have been well supported [70–72]. There are controversies in literature suggesting N-fertilization can stimulate [73, 74] or inhibit [75, 76] methanogenesis in wetland ecosystems but our results showed mutual cases for the most abundant group i-e *Methanobacteriam*. Most of the test samples showed increasing trend except U, K and UK treatments and reduced methanogenesis due to urea [77] and potassium is documented [78].

Computational methodology to predict functional activities of microbial communities at metabolism level was employed using PICRUSt. The idea was to compare marker genes of HTS with that of KEGG and COG databases [44]. The hierarchical clustering of level 2 KEGG ortholog function prediction at metabolism level showed that the microbial community has responded more towards amino acid (20.66%), carbohydrate (19.85%), energy (10.76%) and lipid metabolism (7.23%) and less towards glycan biosynthesis, synthesis of secondary metabolites, terpenoids and biodegradation (1.8 – 7%). Few studies has predicted the prevalence and abundance of carbon (C), nitrogen (N) and phosphorus (P) cycle related genes [79–82]. At energy metabolism level, methane metabolism, which is confiscated by methanogenesis, was higher since the experimental soil was under flooded conditions. The process is entirely restricted to methanogens which can be either hydrogenotrophic methanogens or acetoclastic methanogens. Previous studies support acetoclastic pathway and the aceticlasic methanogens *Methanosaeta* [57] were also seemed to increase in our test samples.

Conclusions

In the current study, we compared the effects of N, P, K, and rice straw in pure and mixed form on overall microbial community structure and diversity in a planted paddy soil microcosm. Different treatments influenced the physicochemical parameters which were driving factors in microbial community structure. In pure form the highest diversity in found against Rs and least for P as represented by OTUs, Faith's PD and Shannon indices. Also, PCA showed more resemblance of U with C_0 and C_f. In mixed treatments the highest diversity is found in soil supplemented with KP and least in quadruple treatment i-e UPKRs. Overall, archaea were found more responsive against all amendments than bacteria. Compared to each control and single fertilizers, double and triple combinations let to greater diversity. The PICRUSt derived functional profile energy and methane metabolism also indicated KP as most responsive and P, C_0 and C_f as least responsive. It also revealed that mixed fertilization can potentially increase the methane metabolism amongst microbial community. Additionally, the current results imply that a caution must be exercised in flooded agricultural systems regarding the use of KCl to regulate methane emission. The amendments KP, UKRs, UPRs and UK were found most responsive in terms of methane metabolism and oxidative phosphorylation while least response for nitrogen metabolism at the same time.

Acknowledgements

I am thankful for the technical assistance of Muhammad M. Hassan who carried out the sampling and laboratory measurements, Nicola Lorenz who helped me in resources and technical assistance, Noshaba H Malik, who helped me in formal analysis. I am also especially thankful to Richard Dick for resources and supervision and Naeem Ali for supervision and project administration.

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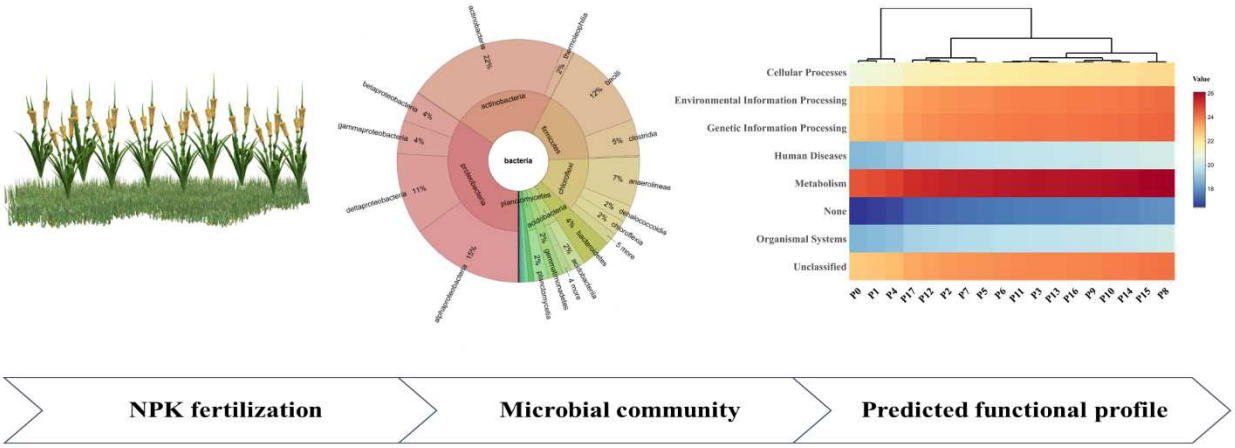
Graphical Abstract



569 **Tables**

570 *Table 1: Description and supplementation of each microcosm setup with symbols used in this context.*

Pot ID	***Treatment***	***Symbol***
0	Non-supplemented, Non-flooded	C_0
1	Non-supplemented, Flooded	C_f
2	Non-supplemented, Flooded, Planted	C_{neg}
3	$CO(NH_2)_2$, Planted	U
4	KH_2PO_4, Planted	P
5	KCl, Planted	K
6	Rice Straw, Planted	Rs
7	$CO(NH_2)_2$ +KH_2PO_4, Planted	UP
8	KCl + KH_2PO_4, Planted	KP
9	Rice Straw + KH_2PO_4, Planted	RsP
10	$CO(NH_2)_2$ + KCl, Planted	UK
11	Rice Straw + KCl, Planted	RsK
12	$CO(NH_2)_2$ + Rice Straw, Planted	URs
13	$CO(NH_2)_2$ + KH_2PO_4 + KCl, Planted	UPK
14	$CO(NH_2)_2$ + KH_2PO_4 + Rice Straw, Planted	UPRs
15	$CO(NH_2)_2$ + KCl + Rice Straw, Planted	UKRs
16	KH_2PO_4 + KCl + Rice Straw, Planted	PKR
17	$CO(NH_2)_2$ + KH_2PO_4 + KCl + Rice Straw, Planted	UPKRs

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Table 2: Observed OTUs and Alpha diversity indices for all treatments.

Treatment	*Observed OTUs*	*Pielou's E*	*Faith's PD*	*Shannon's Index*
C_0	1119	0.92	85.31	9.35
C_f	1306	0.92	99.69	9.55
C_{neg}	1852	0.92	128.15	9.96
U	1981	0.92	132.56	10.02
P	1391	0.92	109.00	9.56
K	1733	0.90	117.06	9.74
Rs	2043	0.91	134.86	10.01
UP	1844	0.91	129.45	9.91
KP	2421	0.91	155.74	10.25
RsP	2124	0.92	137.31	10.15
UK	2102	0.91	133.98	10.08
RsK	2067	0.91	138.41	10.06
URs	1782	0.92	123.34	9.90
UPK	1982	0.91	129.11	9.99
UPRs	2260	0.92	142.80	10.21
UKRs	2256	0.91	141.06	10.14
PKRs	2125	0.92	137.16	10.14
UPKRs	1630	0.91	119.06	9.72

Table 3: Correlation between physicochemical variables with bacterial and archaeal phyla (indicates archaeal phyla)*

	pH	***EC***	***Total C***	***Total N***	***OM***	***AK***	***EP***
Proteobacteria	-0.30	0.23	0.13	-0.01	0.23	0.24	-0.28
Actinobacteria	0.11	-0.24	0.01	-0.17	-0.62	-0.56	0.48
Firmicutes	0.44	-0.07	-0.16	-0.14	0.09	-0.04	0.09
Chloroflexi	-0.04	-0.14	0.01	0.22	0.09	-0.08	-0.10
Bacteroidetes	-0.35	0.27	0.09	0.10	0.30	0.40	-0.13
Acidobacteria	0.48	-0.19	-0.26	-0.33	-0.13	0.11	-0.32
Planctomycetes	0.18	-0.01	-0.20	-0.08	0.21	0.04	0.00
Gemmatimonadetes	0.13	0.28	-0.17	-0.22	0.34	0.56	-0.09
Verrucomicrobia	-0.27	0.25	0.42	0.53	0.34	0.15	0.26
Nitrospirae	0.57	-0.17	-0.37	-0.30	-0.10	-0.04	-0.19
Thaumarchaeota*	-0.06	0.24	-0.28	-0.20	0.31	0.44	-0.08
Euryarchaeota*	0.02	-0.21	0.28	0.18	-0.30	-0.44	0.12
Crenarchaeota*	0.32	-0.40	0.14	0.33	-0.25	-0.25	-0.21

615 **Figures**

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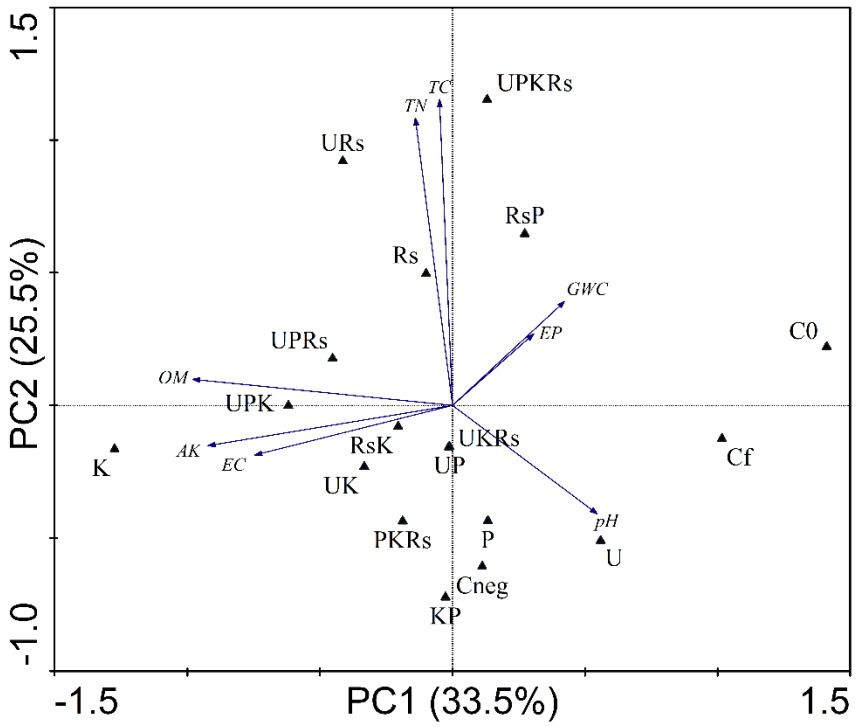

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618 *Fig 1: Principal component analysis (PCA) showing correlation biplot between explanatory soil variables (black*

619 *triangles) and loadings (blue lines). The symbols denote samples and are explained in Table 1.*

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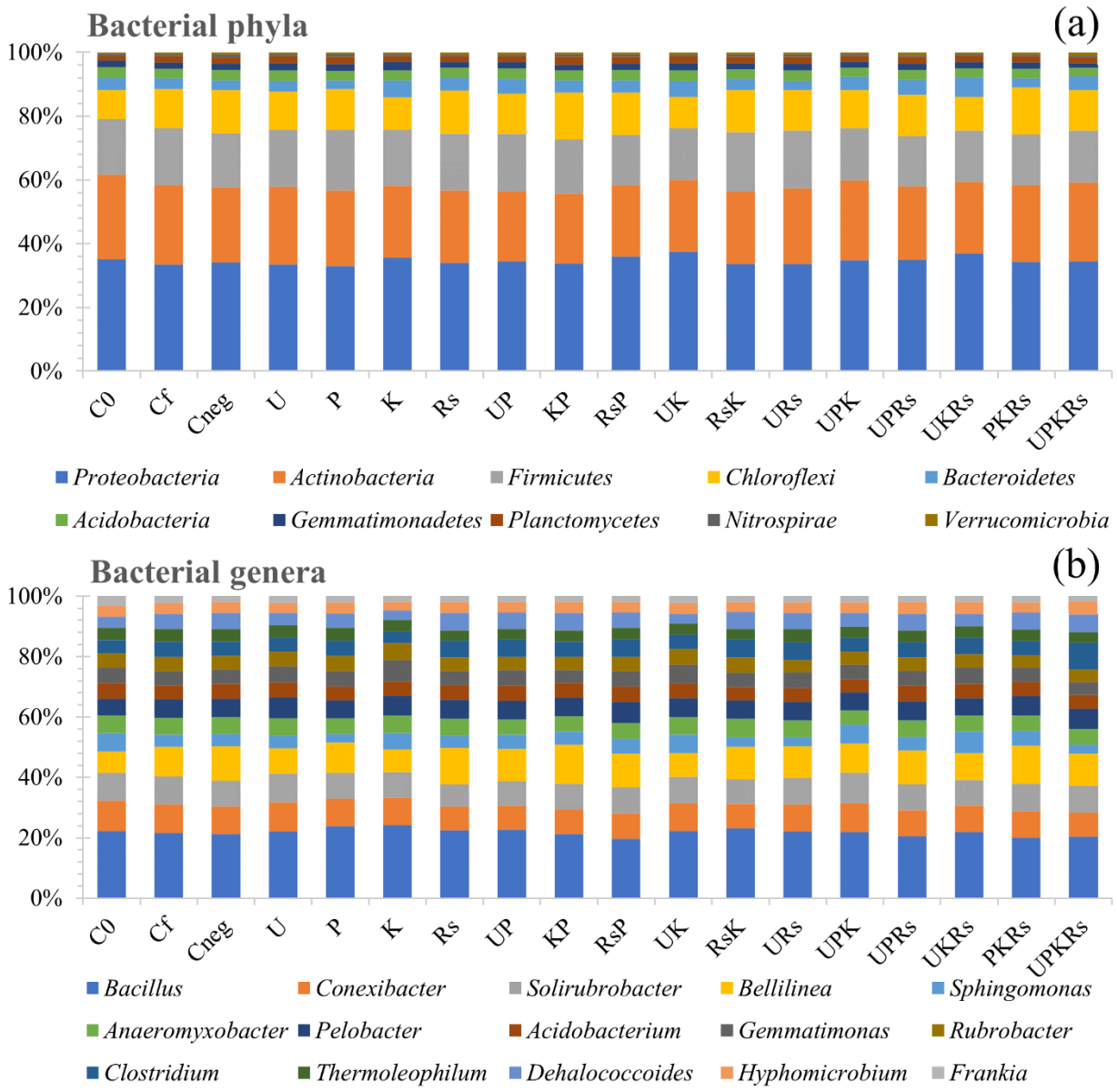


Fig 2: Relative abundance of major bacterial phyla that accounts for ≈ 99% of bacterial community (a) and genera (b) in all treatments.

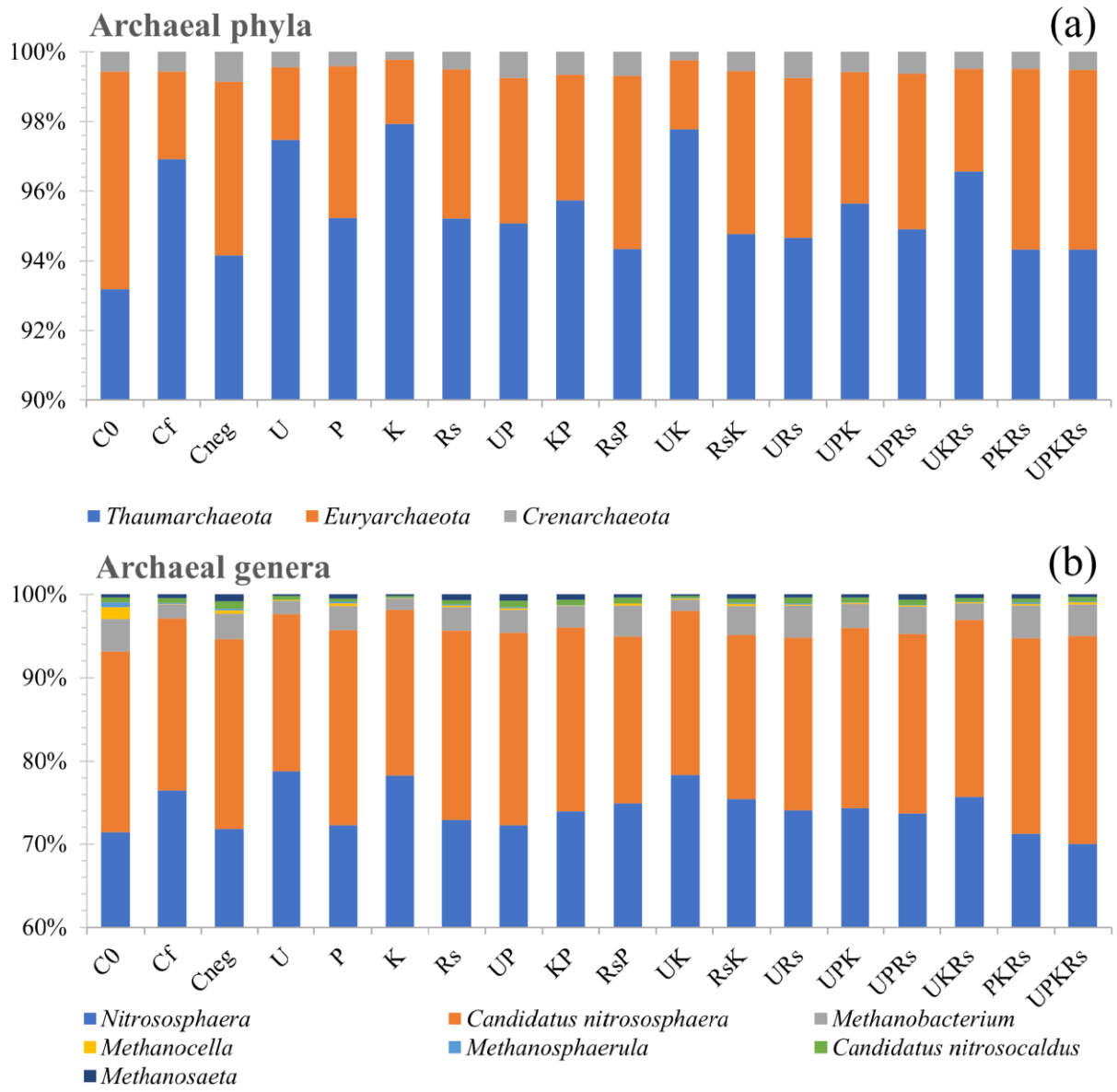
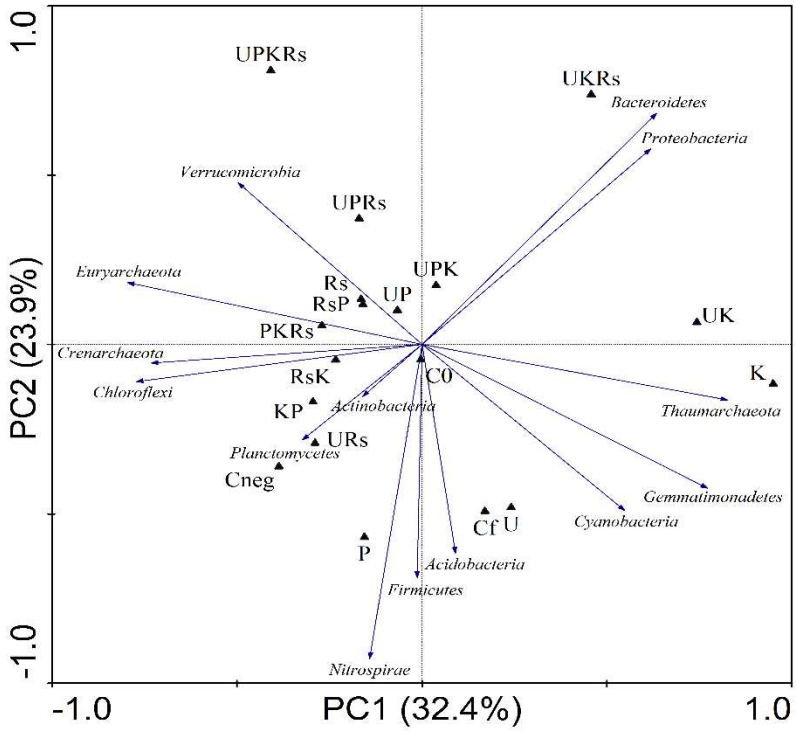


Fig 3: Relative abundance of archaeal phyla and genera in all samples.



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632 *Fig 4: Principal component analysis (PCA) showing correlation biplot between explanatory soil variables (black*

633 *triangles) and loadings as bacterial and archaeal phyla (blue lines). The symbols are explained in Table 1.*

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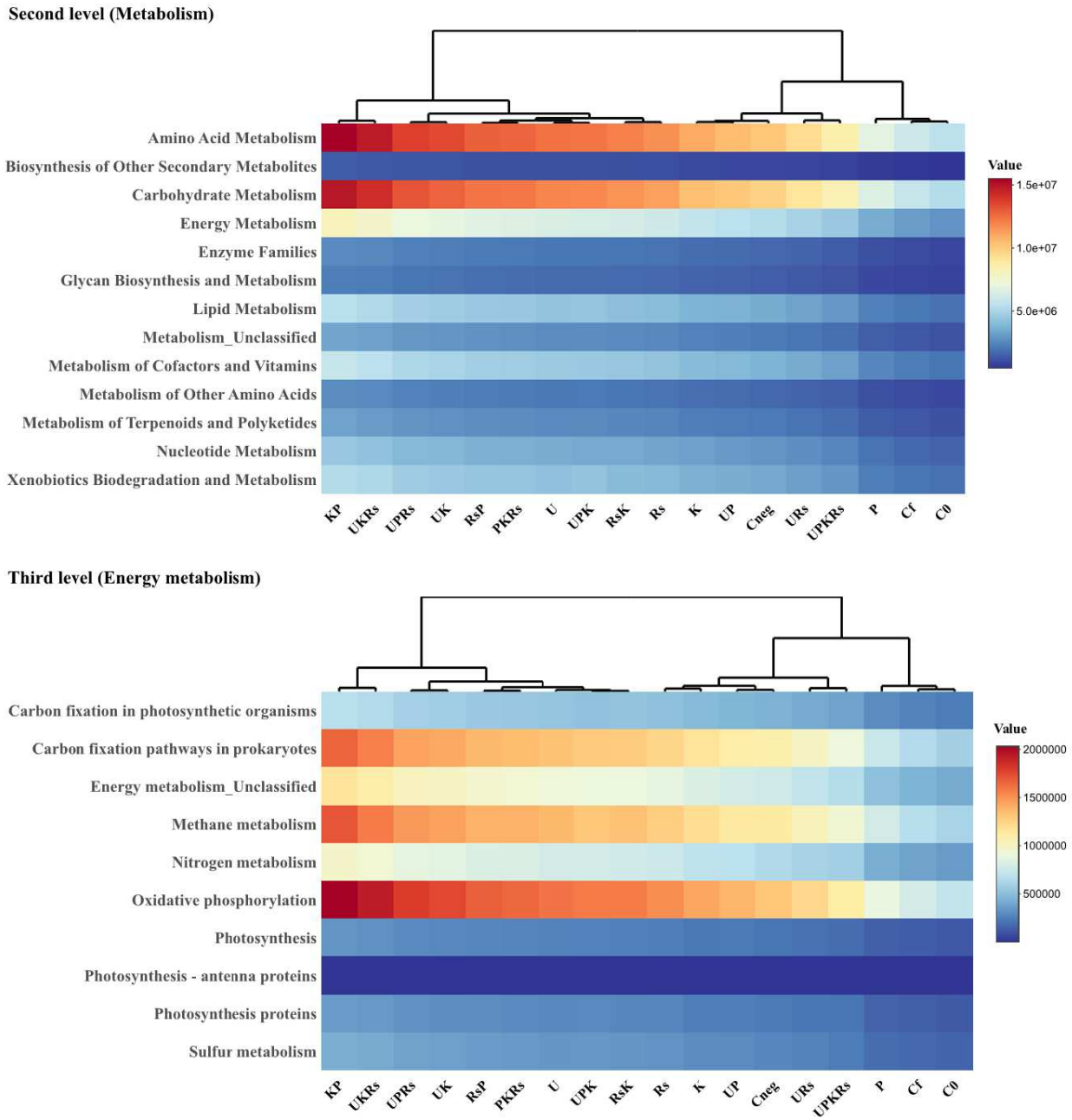
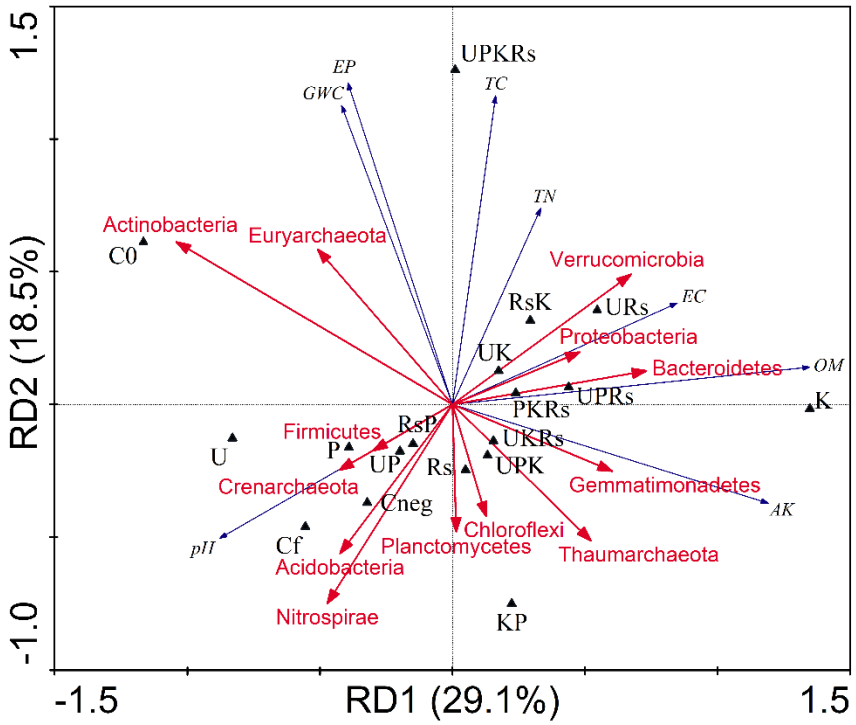


Fig 5: Heat map demonstrating PICRUSt derived hierarchical clustering of predicted functional profiles at second level (metabolism) and third level (Energy metabolism).



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642 *Fig 1: Redundancy analysis (RDA) of soil microbial community as explained by soil physicochemical parameters.*

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Supplementary Information (SI)

Table 1: Soil physico-chemical properties of soil against different supplements. Different lowercase represents significant difference.

Treatment	*pH*	*EC* dS/cm	*TC* %	*TN* %	*OM* %	*AK* mg/kg	*EP* mg/kg
C_0	8.05d	275a	0.17a	1.40a	0.73a	6.50a	0.31a
C_f	8.08de	283a	0.16a	1.48a	0.72a	6.40a	0.30a
C_{neg}	8.28g	446d	0.16a	1.44a	1.58d	9.40de	0.29a
U	8.20fg	342bc	0.16a	1.38a	1.15b	9.60ef	0.32a
P	8.06de	522efg	0.16a	1.38a	1.80g	8.40c	0.31a
K	7.84bc	645i	0.16a	1.46a	2.79k	13.20i	0.30a
Rs	7.82abc	300ab	0.17a	1.58a	1.64e	9.60ef	0.28a
UP	8.02d	504ef	0.16a	1.50a	1.57d	8.70c	0.29a
KP	7.83bc	338bc	0.15a	1.35a	1.84h	9.50ef	0.29a
RsP	8.04d	340bc	0.18a	1.61a	1.36c	8.80cd	0.30a
UK	7.82abc	602h	0.16a	1.38a	1.78f	9.60ef	0.31a
RsK	8.08de	549g	0.17a	1.45a	1.98i	9.50ef	0.30a
URs	7.85bc	342bc	0.18a	1.65a	2.45j	10.10gh	0.29a
UPK	7.75abc	544fg	0.17a	1.47a	1.99i	10.00efg	0.28a
UPRs	7.85bc	523efg	0.17a	1.53a	1.75f	10.60h	0.29a
UKRs	7.88c	346c	0.16a	1.45a	1.77f	9.40de	0.28a
PKRs	7.68a	482de	0.15a	1.35a	1.82g	8.50c	0.30a
UPKRs	7.72ab	504ef	0.18a	1.64a	1.76f	7.60b	0.33a

Figures

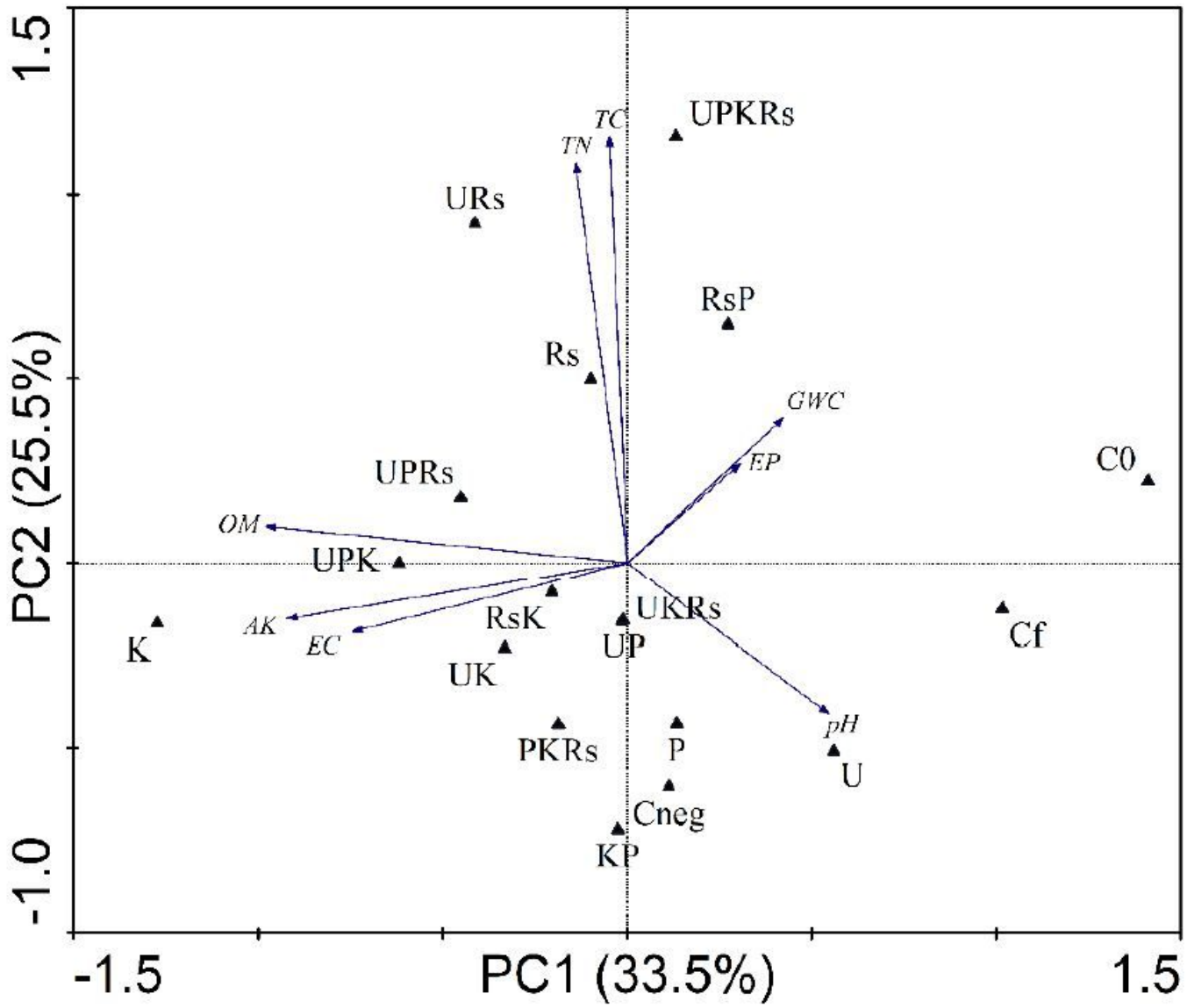

Figure 1

Principal component analysis (PCA) showing correlation biplot between explanatory soil variables (black triangles) and loadings (blue lines). The symbols denote samples and are explained in Table 1.

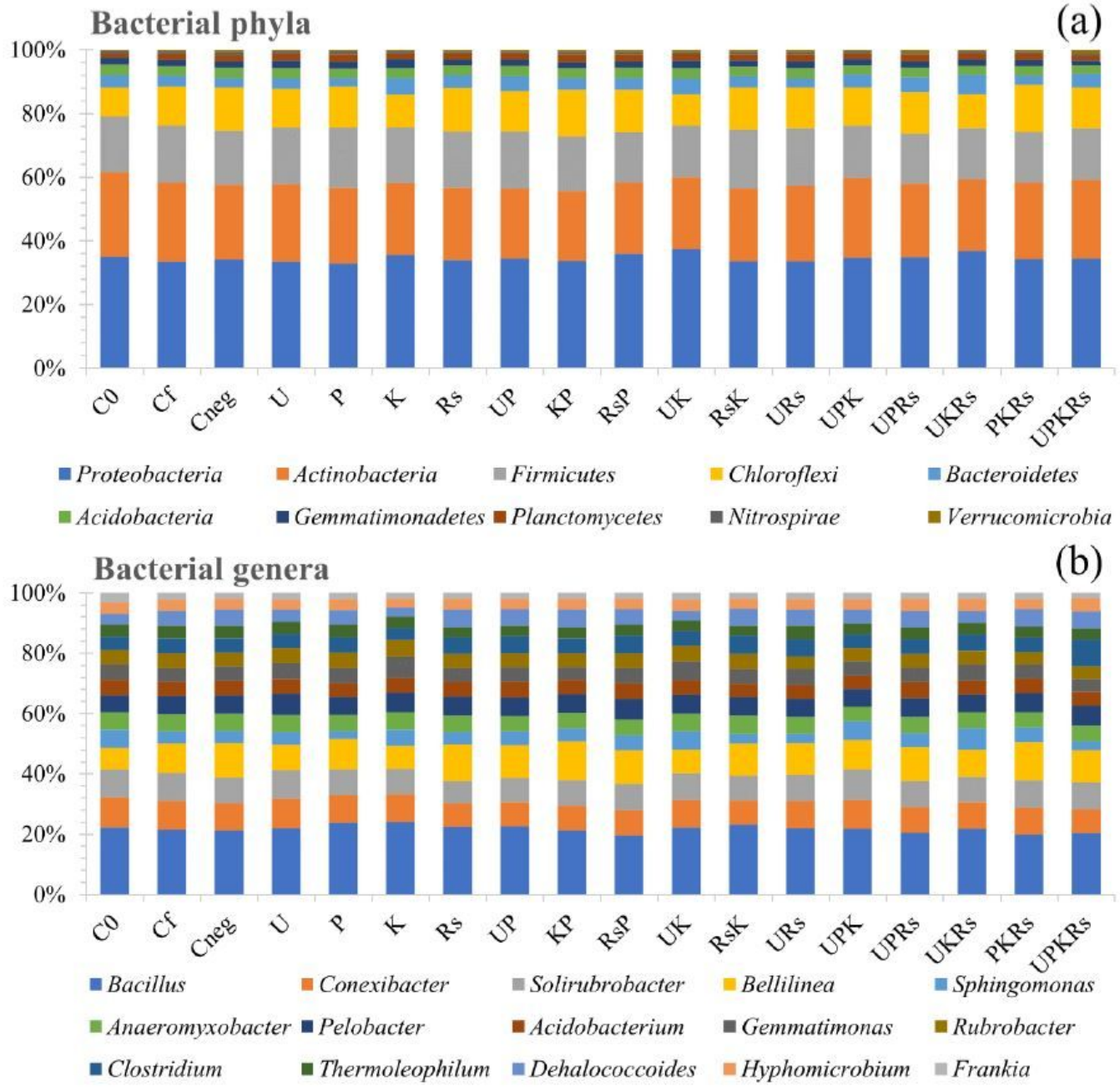

Figure 2

Relative abundance of major bacterial phyla that accounts for ≈ 99% of bacterial community (a) and genera (b) in all treatments.

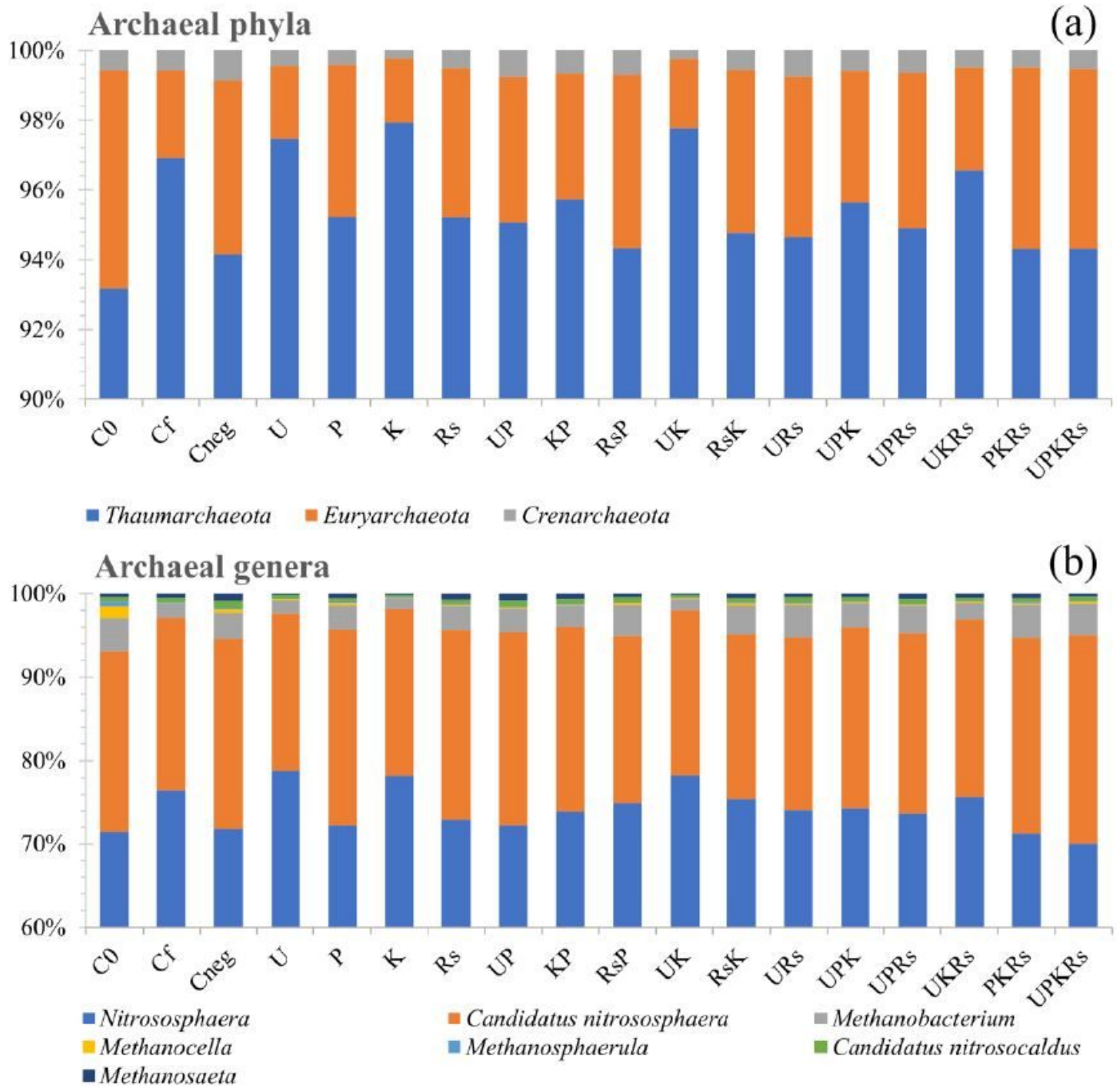

Figure 3

Relative abundance of archaeal phyla and genera in all samples.

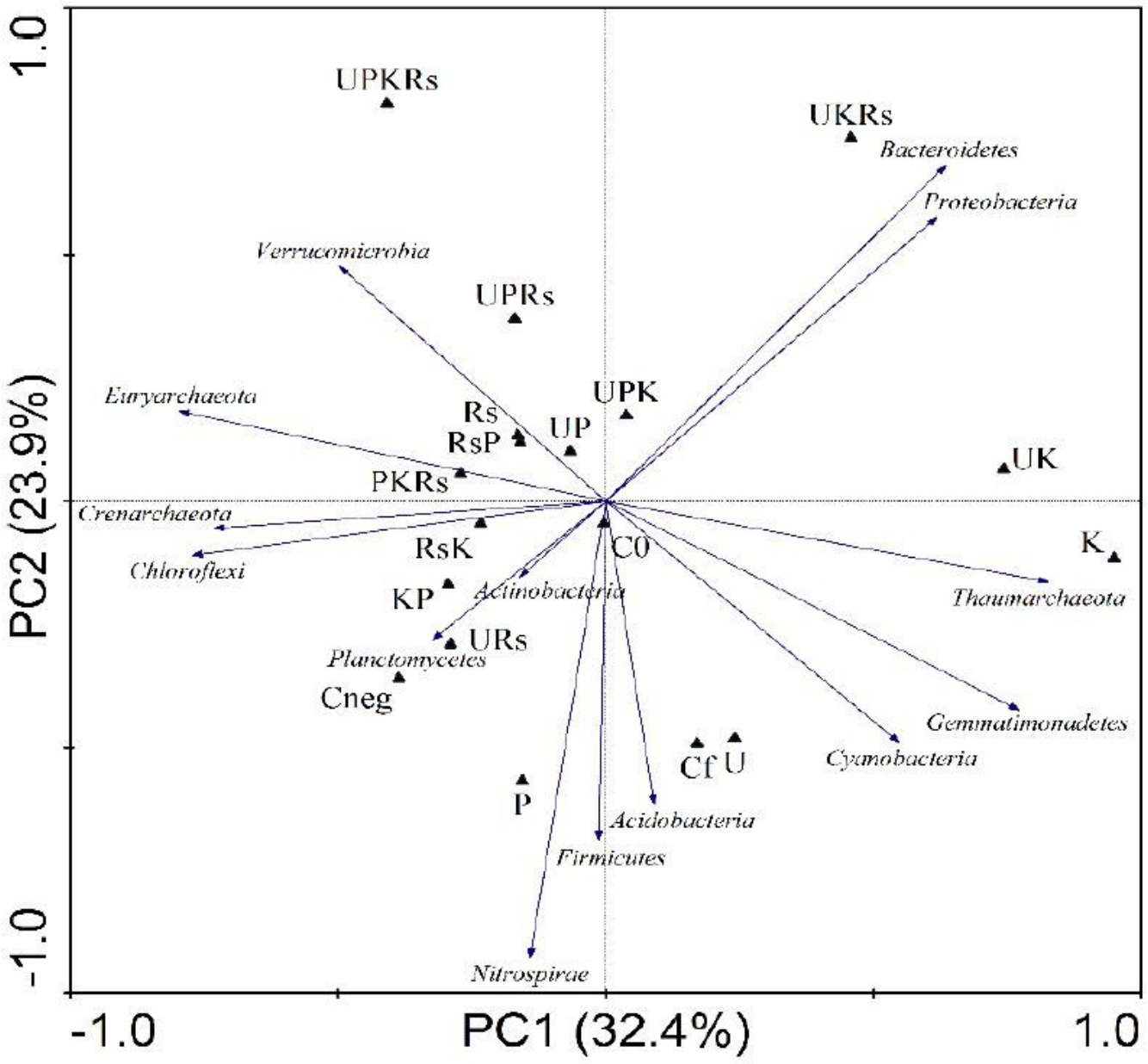

Figure 4

Principal component analysis (PCA) showing correlation biplot between explanatory soil variables (black triangles) and loadings as bacterial and archaeal phyla (blue lines). The symbols are explained in Table 1.

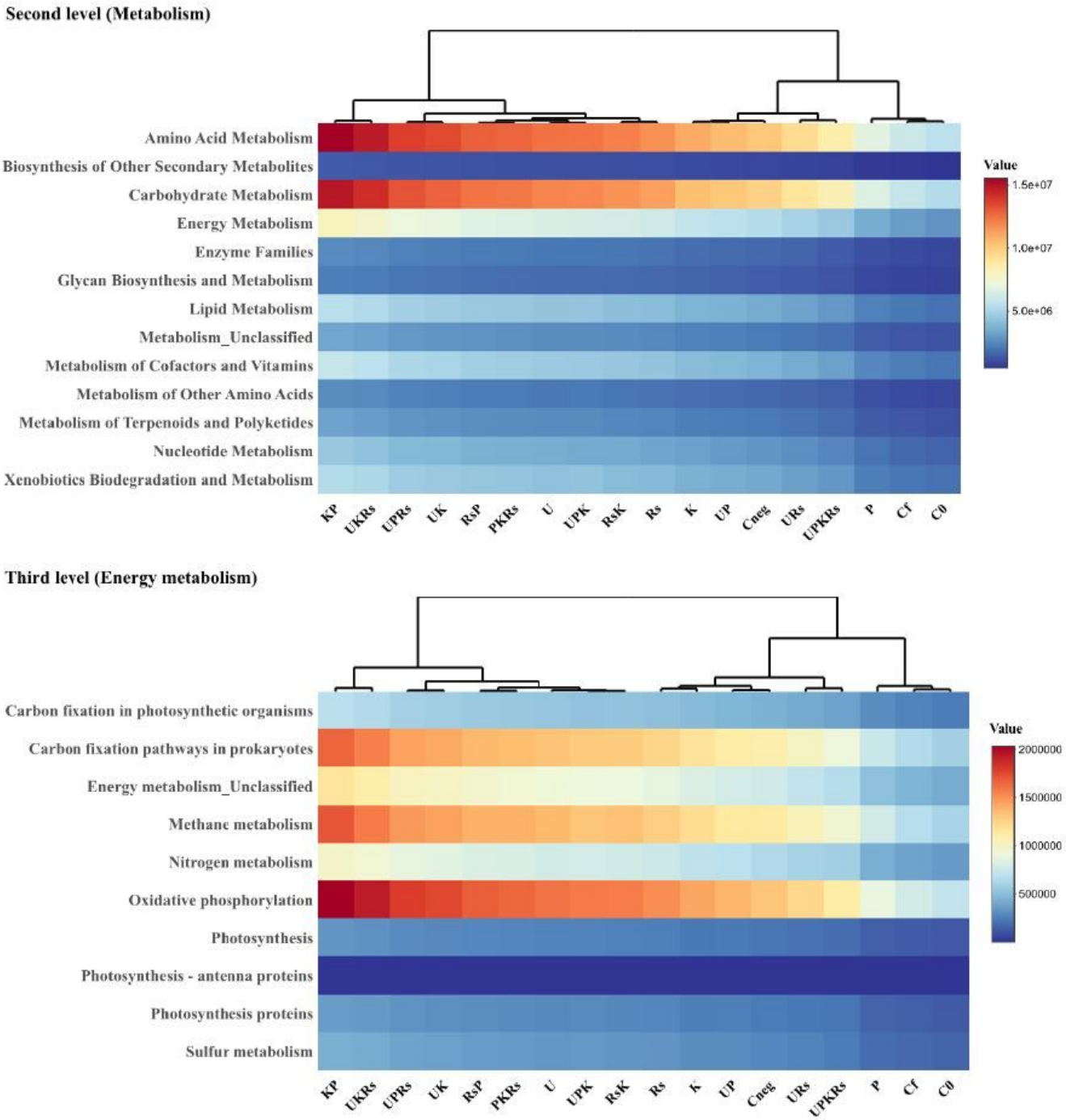

Figure 5

Heat map demonstrating PICRUSt derived hierarchical clustering of predicted functional profiles at second level (metabolism) and third level (Energy metabolism).

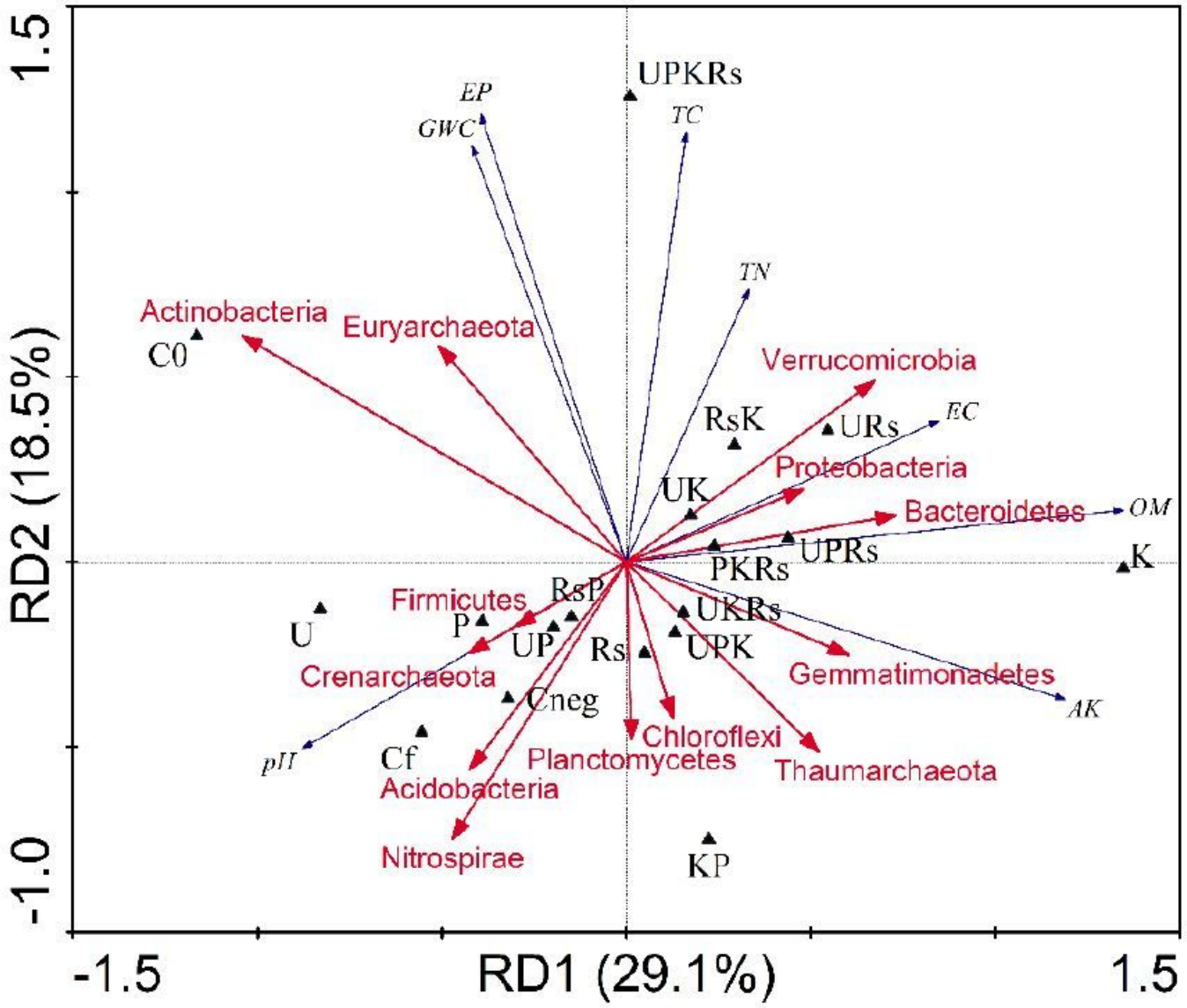

Figure 6

Redundancy analysis (RDA) of soil microbial community as explained by soil physicochemical parameters.

Supplementary Files

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- graphicsabstract.jpg