Original article

Variation in archaeal and bacterial community profiles and their functional metabolic predictions under the influence of pure and mixed fertilizers in paddy soil

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ABSTRACT

Impact of environmental perturbations i.e., nitrogen (N), phosphorus (P), potassium (K), and rice straw (Rs) on the dynamics of soil bacterial and archaeal communities are multifactor dependent and seeks a contemporary approach to study underlying mechanisms. The current study investigates the effect of pure and mixed fertilizers on soil physicochemical properties, the microbial community structure, and their functional metabolic predictions. It involved amendments with distinct combinations of N as C $(H_2N)_2O$, P and K as KH_2PO_4, K as KCl, and Rs in paddy soil microcosms with concentrations common in rice fields agriculture. Soil pH, electrical conductivity (EC), total carbon (TC), total nitrogen (TN), organic matter (OM), available K (AK), and total extractable P (TEP) were evaluated. To comprehend community variation and functional predictions, 16S rRNA-based high throughput sequencing (HTS) and phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) were employed, respectively. Our findings showed enhanced community richness and diversity in all amendments compared to control. Proteobacteria, Actinobacteria, and Firmicutes were dominant bacterial phyla. Regarding relative abundance, Chloroflexi, Bacteroidetes, and Verrucomicrobia showed positive while Actinobacteria, Acidobacteria, and Gemmatimonadetes showed negative trends compared to controls. Thaumarchaeota and Euryarchaeota were dominant archaeal phyla and exhibited increasing and decreasing trends, respectively. The PICRUSt analysis indicated functional prediction more towards amino acid, carbohydrate, energy, and lipid metabolism while less towards others. Concerning energy metabolism, most and least responsive treatments were KP and controls, respectively. These outcomes enhanced our understanding regarding soil quality, fertilizer composition and application, and functional metabolomics of archaea and bacteria.

1. Introduction

The efficiency and sustainability of terrestrial agroecosystem fluctuate with soil types and are highly reliant on microbial ecology that not only varies continuously with nutrients' status of the soil but also executes biotic and abiotic processes in it (Wang et al., 2017; Wu et al., 2011). The paddy soil is notable for its variable organic and inorganic nutrient status and its physiochemical and biological behavior under flooded irrigation is quite different from upland soil (Kamaa et al., 2011; Kamran et al., 2021). The

nutrient budget of paddy soil depends upon the type of supplementation, concentration, and mode of application (Eo and Park, 2016; Singh and Strong, 2016). For instance, nitrogen, phosphorus, and potassium (NPK) fertilization and rice straw (Rs) have been known to induce varying fluctuations in soil physicochemical properties and microbial community dynamics (Fang et al., 2021; Kuppusamy et al., 2018; Pan et al., 2016). It is still unclear how microbial community transforms with different sources of carbon (C), N, P, and K. Since it is multifactorial and fluctuates with soil texture, pH, electrical conductivity (EC), organic matter (OM), availability of mineral nutrients, and other accompanied microorganisms, it needs to be addressed (Qaswar et al., 2020).

Moreover, paddy soil is a well-known source and sink of methane in which archaeal and bacterial communities are major contributors. Globally, paddy soils contribute 15–20% CH_4 emission (25–100 Tg/year) which increases during rice cultivation seasons (Dubey et al., 2014; Zhang et al., 2011) and is predicted to increase to 50% by 2025 due to growing demands. The role of differential supplementation is very important concerning methane recycling since it has 25 times more ultraviolet (UV) retention capability compared to CO_2 (Kuloyo et al., 2020) and paddy soils are the major non-natural sites of methanogenesis after natural wetlands (Xu et al., 2020). Variation in organic and inorganic fertilization result in varying bacterial and archaeal communities that respond differently in terms of methane metabolism. A shift in organic and inorganic content may direct the community dynamics metabolism positively or negatively that may turn soil into a source of methane rather than sink (Dubey, 2005; Eo and Park, 2016; Ramirez et al., 2012; Zhong and Cai, 2007). In this context, the relative abundance and the physiological response of bacterial and archaeal communities under different nutrient statuses are considerably important (Thielemann et al., 2000).

Considering the variability in type and application rate of fertilizers in paddy soil, it is vital to study the assessment of soil physicochemical properties, variation in archaeal and bacterial community structure, and their functional metabolism i.e., energy metabolism and methane metabolism. To investigate and predict the said question, a microcosm-based experiment was established with paddy soil and 26 days old rice nursery in it. It included pure and mixed combinations of common fertilizers with concentrations equivalent to common rice field agriculture. The 16S rRNA-based high throughput sequencing (HTS) has been well established to study microbial community ecology in short and long-term studies (Li et al., 2019a) thus making it an excellent method of choice in our case. Additionally, phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) derived functional profiles i-e energy metabolism of contributing archaeal and bacterial community is predicted to overview the functional metabolic trend of the community (Douglas et al., 2019).

2. Materials and methods

2.1. Experimental soil

Rhizospheric soil (non-calcareous, silty clay loam, isohyperthermic Udic Hapludalfs) from a depth of 10–20 cm was acquired in early August 2018 from a rice paddy field in Gujranwala, Pakistan (32°19′N, 74°20′E). The sampling site was 226 m above sea level with a hot semi-arid climate (BSh) (Mahmood et al., 2019) with an annual rainfall of around 577 mm and an average annual temperature of 23.9 °C. Field soil samples were transported to an experimental provision in zipper bags to minimize contamination. The soil was air-dried, sieved (2 mm), and stored at −20 °C till further experimentation. Aseptic conditions were maintained wher-

ever necessary. The soil had a pH of 8.05, total nitrogen (TN) 0.17%, and total carbon (TC) 1.40%.

2.2. Microcosm Set-up and supplementation

Paddy soil microcosms were established using 2.2 kg experimental soil slurry in 64 oz polyethylene plastic pots (20 cm height and 15 cm diameter) and anaerobic conditions were created by flooding the soil with 3 cm of water. Each microcosm was planted with a 26-day old nursery of *Oryza sativa* (var. Super Basmati). Excluding two controls and a time zero sample, 15 different combinations were developed in triplicate using N (as urea), P and K (as KH_2PO_4), K (as KCl) (Zheng et al., 2013) as inorganic amendments, and rice straw as an organic amendment (Table 1).

Amendments were carried out with 50 ml solution of each fertilizer (per 100 ml: 0.23 g N as urea, 0.087 g P as KH_2PO_4, and 0.185 g K as KCl) with concentrations as per common rice agriculture practices (Shrestha et al., 2010). Additionally, concentrations of carrier ions were calculated as 0.05 g K in KH_2PO_4 and 0.08 g Chloride (Cl) in KCl. Amendments were done on days 0, 5, and 30 as basal dressing and two top dressings. Two controls (with plant and without plant), devoid of any amendment, were kept under the same conditions. Microcosms were placed at an average temperature of 20–25 °C in a greenhouse facility through vegetation stage and a water level of 3 cm was maintained throughout that period. Soil samples were collected during the vegetation phase from each microcosm and stored at −20 °C till further analysis.

2.3. Soil physicochemical properties

The soil moisture content was calculated employing the gravimetric method (Baldoncini et al., 2019; de Paul Obade, 2019) and represented as gravimetric water content (GWC). Soil pH and EC were measured using a dipping glass electrode employing a 1:1 soil /water (v/v) ratio. TC and TN were calculated by combustion at 1800 °C using Vario Max CN Analyzer. Soil particle distribution was determined by the hydrometer method (Bouyoucos, 1962) and the textural class was assigned as per US textural classification. Total extractable phosphorus (TEP) was determined by the Mehlich-3 soil phosphorus test (Mehlich, 1984).

2.4. Microbial DNA extraction and 16S rRNA amplicon sequencing

Microbial genomic DNA was extracted using PowerSoil® DNA isolation kit (MoBio, Carslbad, CA, USA) as per Earth Microbiome Project protocols (Marotz et al., 2017). Community composition

Table 1
Description and supplementation of each microcosm with symbols.

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

was evaluated as per protocols and primers described (McHugh and Schwartz, 2016) that targeted archaeal and bacterial hypervariable V4 region (515f/806r) of 16S rRNA gene (Caporaso et al., 2011). Amplicons were generated using HotStarTaq Plus Master Mix Kit (Qiagen) by following subsequent conditions for amplification: initial denaturation (94 °C for 3 min) followed by 30 cycles, each at 94 °C for 30 s, 53 °C for 40 s, and 72 °C for 1 min, with a final elongation step at 72 °C for 5 mins. 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 a MiSeq (Illumina) platform in an overlapping 2×300 bp configuration with a minimum throughput of 20,000 reads for each sample.

2.5. Processing of Illumina sequencing data

Raw amplicon sequences of 16S rRNA were processed and analyzed following described protocols (Dowd et al., 2008; Handl et al., 2011). In brief, sequences were joined (overlapping pairs) and grouped by sample barcode that was removed afterward. Sequences having < 150 bp or ambiguous base calls were removed. The 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) (Edgar et al., 2011). The sequencing data was submitted in the Sequence Read Archive (SRA) of NCBI (National Center for Biotechnology Information) under the BioProject PRJNA627288.

2.6. Sequence analysis, taxonomic identification, and diversity analysis

All the resulted sequences were analyzed with Quantitative Insights Into Microbial Ecology (QIIME 2) to obtain 16S rRNA reads from amplicon with 97% similarity with the taxonomy of resulting Operational Taxonomic Units (OTUs) (Bolyen et al., 2019; Jiang et al., 2019). The OTU selection process was performed with USearch (v 6.1.544) using QIIME 2. In total 14,087 OTUs were analyzed, comprising 1,509,246 reads at the species level. Finally, all OTUs were taxonomically categorized using BLASTn against RDPII and NCBI databases (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 (Bolyen et al., 2019). The Shannon, Pielou E, and Faith's Phylogenetic Diversity (PD) indices were calculated alongside observed OTUs ('richness') (Schloss et al., 2009).

2.7. Functional diversity of the archaeal and bacterial community

Functional capabilities of archaeal and bacterial communities were predicted using sequencing data of 16S rRNA gene by PICRUSt (Langille et al., 2013). The software stores Clusters of Orthologous Groups of proteins (COG) and KEGG Ortholog (KO) information related to the greengene id and predicts metagenomes by standardizing OTU abundance. The KO and COG family information was obtained by greengene id related to each OTU and the KO and COG abundance was obtained. The information of COG and KO pathways obtained from the KEGG database was used to predict functional categorization at three levels according to OTU abundance (Malik et al., 2018).

2.8. Statistical analysis

The indices of microbial alpha diversity were estimated by mothur (version v1.30.1) and included Pileou's E, Faith's PD, and

Shannon (Schloss et al., 2009). 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 while principal component analysis (PCA) and redundancy analysis (RDA) was performed in Canoco for Windows (version 4.5) and drawn in Cano Draw (ter Braak and Šmilauer, 2002). The hierarchical clustering was plotted using Euclidean distance and Ward's minimum variance as clustering method in R (Version: 4.0.5, Package: pheatmap, dplyr and, ggplot2). Details of evaluations are provided in the results and discussion section.

3. Results

3.1. Physicochemical properties of soil

Soil pH varied between 7.68 and 8.28 in four fertilizer regimes and it varied significantly with different combinations of amendments (Table 2). Soil EC significantly increased in all treatments compared to control and varied between 275 and 645 dS/cm. It was observed higher in UPK, UPRs, PKRs, and UPKRs and lower in controls and U. TN and TC varied insignificantly within 0.15–0.18% and 1.35–1.65% respectively. Soil OM and available potassium (AK) increased with the use of fertilizers and ranged within 0.71–2.77% and 6.4–13.5 mg/kg, respectively while variations in TEP were insignificant in all treatments. Physicochemical-based variations within different treatments are outlined using PCA in Fig. 1. The first two axes explained 32.9% and 26.2% of the overall variance. More variation was found in C_0 and C_f on the positive side of PC1 which is influenced by pH and GWC while TC, TN, OM, EC, TEP, and AK tend to dominate the negative side of the plot and influenced most of the samples. The biplot showed a strong correlation between TC and TN; TEP, OM, EC, and AK.

3.2. Microbial community composition

Sequencing showed 3,237,072 reads of 16S rDNA that accounted for 92.51% bacterial reads. They were clustered into 13,918 OTUs and assigned 29 bacterial phyla and 902 genera. Overall, 10 bacterial phyla contributed over 99% of the bacterial community with Proteobacteria (32–37%), Actinobacteria (21–26%), and Firmicutes (15–19%) were the dominant ones. Other important phyla were Chloroflexi (9–15%), Bacteroides (2–6%), Acidobacteria (2–3%) and Gemmatimonadetes (1–2%). The relative abundance of major phyla and genera is shown (Fig. 2). Chloroflexi, Bacteroides, Planctomycetes, and Verrucomicrobia increased in most of the treatments compared to control. The dominant genera were Bacillus followed by Conexibacter, Solirubrobacter, Bellilinea, and Sphingomonas. Bellilinea, Pelobacter, Clostridium, and Dehalococcoides showed an increasing trend, while the converse was found for Conexibacter, Solirubrobacter, Sphingomonas, Acidobacterium, Thermoleophilum, and Frankia.

For archaea, 193,917 valid reads were obtained that contributed 5.54% of overall diversity and clustered into 169 OTUs that were classified into 3 phyla, and 23 genera. Thaumarchaeota was the most dominant followed by Euryarchaeota and Crenarchaeota and their relative abundance varied from 93 to 97, 1–6, and 0.2–0.9% respectively (Fig. 3). The dominant archaeal genera gave $\sim 99\%$ community coverage and included Nitrososphaera (69–78%), Candidatus (18–24%), Methanobacterium (1–3%), and Methanocella (1%). Overall, an increasing trend was observed for Thaumarchaeota with the lowest abundance in C_0 (93%) and highest in UK (98%), and decreased trend was found for Euryarchaeota

Table 2
Physico-chemical properties of soil against different supplements. Values represent means (n = 3) with SE. Different lowercase letters represent a significant difference at p < 0.05. EC = electrical conductivity, TC = total carbon, TN = total nitrogen, OM = organic matter, AK = available potassium, TEP = total extractable phosphorus, C_0 = non-supplemented and non-flooded control, C_f = non-supplemented and flooded control, C_{neg} = non-supplemented, flooded control + plant, U = urea + plant, P = KH_2PO_4 + plant, K = KCl + plant, Rs = rice straw + plant, UP = urea + KH_2PO_4 + plant, KP = KCl + KH_2PO_4 + plant, RsP = rice straw + KH_2PO_4 + plant, UK = urea + KCl + plant, RsK = rice straw + KCl + plant, URs = urea + rice straw + plant, UPK = urea + KH_2PO_4 + KCl + plant, UPRs = urea + KH_2PO_4 + rice straw + plant, UKRs = urea + KCl + rice straw + plant, PKRs = KH_2PO_4 + KCl + rice straw + plant, UPKRs = urea + KH_2PO_4 + KCl + rice straw + plant.

| Treatment	pH	EC	TN	TC	OM	AK	TEP
		dS/cm	%	%	%	mg/kg	mg/kg
C_0	8.05 ± 0.08a	275.33 ± 5.78a	0.17 ± 0.00a	1.43 ± 0.03a	0.71 ± 0.01a	6.47 ± 0.12a	0.31 ± 0.02a
C_f	8.08 ± 0.02ab	283.67 ± 7.22a	0.16 ± 0.01a	1.48 ± 0.04a	0.80 ± 0.08a	6.57 ± 0.32a	0.32 ± 0.01a
C_{neg}	8.28 ± 0.01c	446.33 ± 6.94b	0.16 ± 0.01a	1.44 ± 0.08a	1.55 ± 0.03b	9.53 ± 0.19bc	0.31 ± 0.01a
U	8.20 ± 0.02bc	342.33 ± 7.69c	0.16 ± 0.01a	1.38 ± 0.05a	1.17 ± 0.05c	9.53 ± 0.18cf	0.31 ± 0.01a
P	8.06 ± 0.02ab	522.33 ± 5.61dgh	0.16 ± 0.00a	1.38 ± 0.03a	1.75 ± 0.07d	8.57 ± 0.20d	0.34 ± 0.02a
K	7.84 ± 0.02de	645.67 ± 11.46e	0.16 ± 0.01a	1.46 ± 0.01a	2.77 ± 0.08e	13.53 ± 0.24e	0.31 ± 0.02a
Rs	7.82 ± 0.02def	300.33 ± 9.26ac	0.17 ± 0.01a	1.58 ± 0.07a	1.54 ± 0.05f	9.77 ± 0.09cf	0.31 ± 0.02a
UP	8.02 ± 0.02a	504.67 ± 7.62dh	0.16 ± 0.01a	1.50 ± 0.12a	1.64 ± 0.07b	8.63 ± 0.27d	0.36 ± 0.02a
KP	7.83 ± 0.01de	338.33 ± 7.80c	0.15 ± 0.01a	1.35 ± 0.11a	1.79 ± 0.07 g	9.47 ± 0.26cf	0.34 ± 0.02a
RsP	8.04 ± 0.02a	340.67 ± 8.09c	0.18 ± 0.01a	1.61 ± 0.07a	1.41 ± 0.03 h	8.53 ± 0.29bd	0.36 ± 0.01a
UK	7.82 ± 0.01def	602.33 ± 9.94f	0.16 ± 0.00a	1.38 ± 0.01a	1.75 ± 0.08i	9.53 ± 0.22cf	0.32 ± 0.02a
RsK	8.08 ± 0.02ab	549.67 ± 5.55 g	0.17 ± 0.01a	1.45 ± 0.03a	1.95 ± 0.03j	9.17 ± 0.24cf	0.33 ± 0.01a
URs	7.85 ± 0.02de	342.67 ± 7.54c	0.18 ± 0.00a	1.65 ± 0.10a	2.55 ± 0.10 k	9.83 ± 0.37 fg	0.31 ± 0.02a
UPK	7.75 ± 0.02def	544.33 ± 7.88dgh	0.17 ± 0.01a	1.47 ± 0.12a	2.04 ± 0.03j	9.83 ± 0.37cfg	0.38 ± 0.02a
UPRs	7.85 ± 0.02de	523.33 ± 7.31dgh	0.17 ± 0.01a	1.53 ± 0.14a	1.73 ± 0.03i	10.43 ± 0.20 g	0.34 ± 0.03a
UKRs	7.88 ± 0.02d	346.33 ± 9.84c	0.16 ± 0.00a	1.45 ± 0.05a	1.70 ± 0.07i	9.47 ± 0.18bc	0.30 ± 0.02a
PKRs	7.68 ± 0.01f	482.67 ± 10.71b	0.15 ± 0.01a	1.35 ± 0.08a	1.87 ± 0.05d	8.53 ± 0.27d	0.37 ± 0.02a
UPKRs	7.72 ± 0.03ef	504.33 ± 7.84dh	0.18 ± 0.01a	1.64 ± 0.12a	1.76 ± 0.01i	7.57 ± 0.29 h	0.36 ± 0.03a
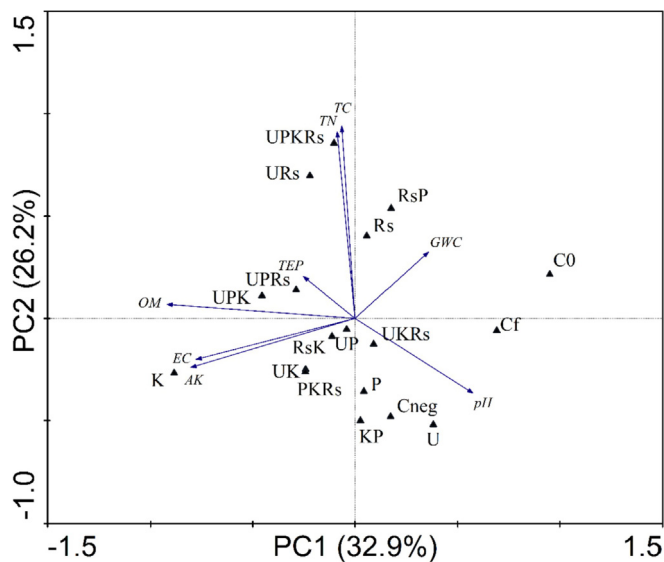
Fig 1. Principal component analysis (PCA) showing correlation biplot of first two PCs between explanatory soil treatments and loadings (blue lines). Total carbon (TC), total nitrogen (TN), gravimetric water content (GWC), electrical conductivity (EC), organic matter (OM), available potassium (AK), and total extractable phosphorus (TEP).

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

with the lowest in K (1.8%) and highest in C_0 (6.2%). For archaeal phyla, Thaumarchaeota showed increasing, Euryarchaeota showed opposite and Crenarchaeota showed both trends. For archaeal genera, Nitrososphaera and Methanosaeta showed increasing while the rest showed both trends. The variation in the archaeal community is also well pronounced in the case of methanogens. Variations in microbial community composition in different treatments are outlined by PCA, using all the identified genera of archaea and bacteria (Fig. 4). PC1 and PC2 accounted for 29.6% and 7.8% variance in the microbial community which demonstrated separation and clustering in microbial communities in soil with all treatments. It showed a clear distinction of C_0, C_f, and P with other treatments. Moreover, RDA analysis between soil physicochemical properties
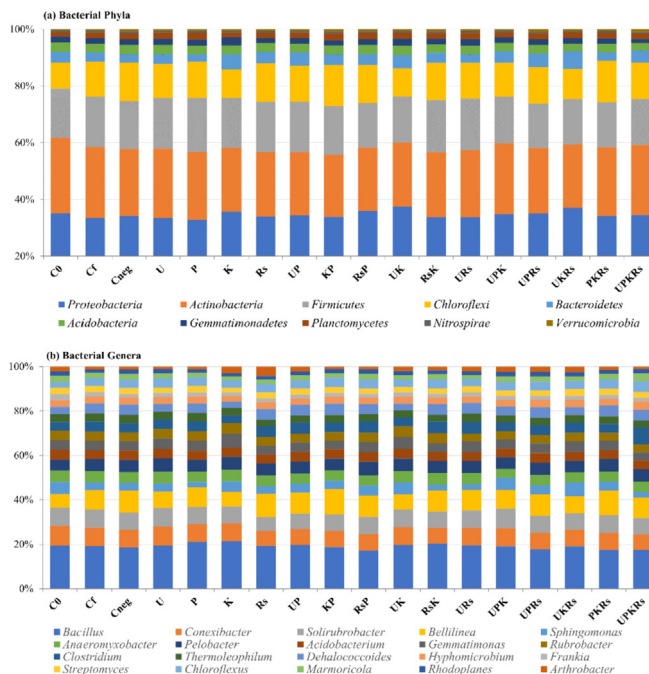
and microbial composition explained 23.3% and 14.3% variance for RD1 and RD2 axes, respectively (Fig. 5). The biplot showed strong correlation of gravimetric water content (GWC) (P = 0.02, F = 2.10), pH (P = 0.05, F = 1.15), TC and TN with the first axis. EC, OM, AK, and TEP did not show a correlation with the first axis.

3.3. Observed OTUs and alpha diversity

Least OTUs were reported in C_0 and C_f that represent lesser microbial diversity without any amendment and vice versa. Pielou's E and Shannon's indices were greater in all samples compared
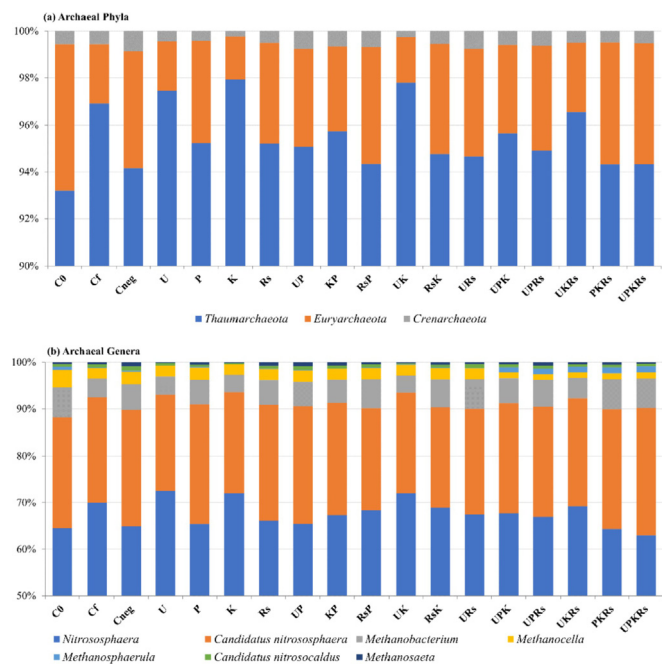
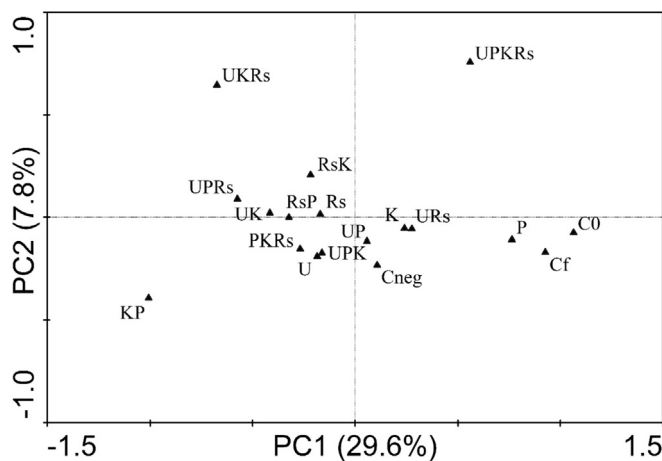**Fig 3.** Relative abundance of archaeal phyla and genera in all samples.

Fig 5. Biplot of redundancy analysis (RDA) showing community succession in all samples and their relation to environmental variables i.e., pH, Total carbon (TC), total nitrogen (TN), gravimetric water content (GWC), electrical conductivity (EC), organic matter (OM), available potassium (AK) and total extractable phosphorus (TEP).

Fig 4. Principal component analysis (PCA) showing a plot of the first two PCs for all amplified 16S rRNA gene fragments from archaeal and bacterial communities. The symbols for each treatment are explained in Table 1.

Table 3

Observed OTUs and Faith's PD index for all treatments. OTU = Operational Taxonomic Units, PD = phylogenetic diversity, C_0 = non-supplemented and non-flooded control, C_f = non-supplemented and flooded control, C_{neg} = non-supplemented, flooded control + plant, U = urea + plant, P = KH_2PO_4 + plant, K = KCl + plant, Rs = rice straw + plant, UP = urea + KH_2PO_4 + plant, KP = KCl + KH_2PO_4 + plant, RsP = rice straw + KH_2PO_4 + plant, UK = urea + KCl + plant, RsK = rice straw + KCl + plant, URs = urea + rice straw + plant, UPK = urea + KH_2PO_4 + KCl + plant, UPRs = urea + KH_2PO_4 + rice straw + plant, UKRs = urea + KCl + rice straw + plant, PKRs = KH_2PO_4 + KCl + rice straw + plant, UPKRs = urea + KH_2PO_4 + KCl + rice straw + plant.

Treatment	Observed OTUs	Faith's PD
C_0	1119	85.31
C_f	1306	99.69
C_{neg}	1852	128.15
U	1981	132.56
P	1391	109.00
K	1733	117.06
Rs	2043	134.86
UP	1844	129.45
KP	2421	155.74
RsP	2124	137.31
UK	2102	133.98
RsK	2067	138.41
URs	1782	123.34
UPK	1982	129.11
UPRs	2260	142.80
UKRs	2256	141.06
PKRs	2125	137.16
UPKRs	1630	119.06

to controls without plants i-e C_0 and C_f while Faith's PD was also lower in those controls as compared to all samples. Observed OTUs and α-diversity index i-e., Faith's PD for each sample is shown (Table 3). Other α-diversity indices i.e., Pielou's E and Shannon's are shown in the supplementary material (S1).

3.4. Functional metabolism profiles prediction (Second and third level)

The functional profiles of metabolism at the second and third levels were predicted using PICRUSt and hierarchically clustered as a heat map for comparison as shown in Fig. 6. Regarding metabolism, the functional profiles were higher for amino acid and carbohydrate metabolism with the lowest values in samples C_0, C_f, and P and highest in UKRs and KP which suggested the overall trend of the community. The functional prediction for energy metabolism, lipid metabolism, metabolism of cofactors and
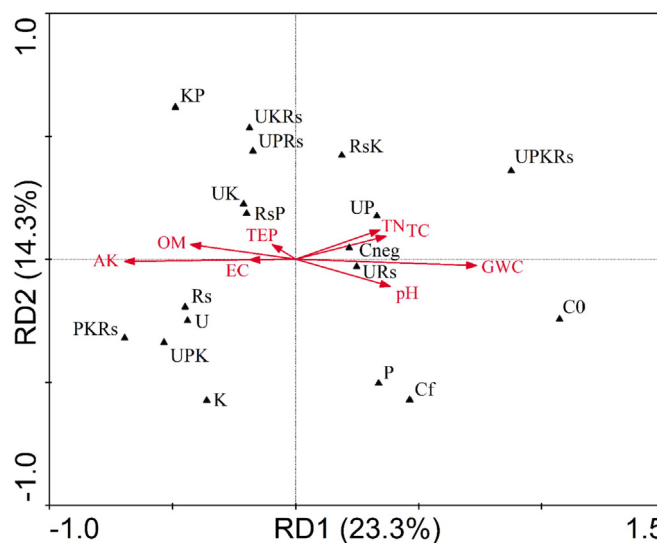
vitamins, and xenobiotic degradation also showed an increase as compared to control without plants i-e C_0 and C_f. An approximate two-fold increase was observed for C_{neg}, U, K, Rs, KP, RsK, UPK, UPRs, and UPKRs while a three-fold increase was observed for UP and PKRs.

At the third level, more pronounced differentiation was observed for carbon fixation pathways in prokaryotes, methane metabolism, nitrogen metabolism, and oxidative phosphorylation in KP, Rs, PKRs, and UP. The trend for variation against different samples was found to be synchronized with the second level. The shift in the abundance and composition of functional metabolism
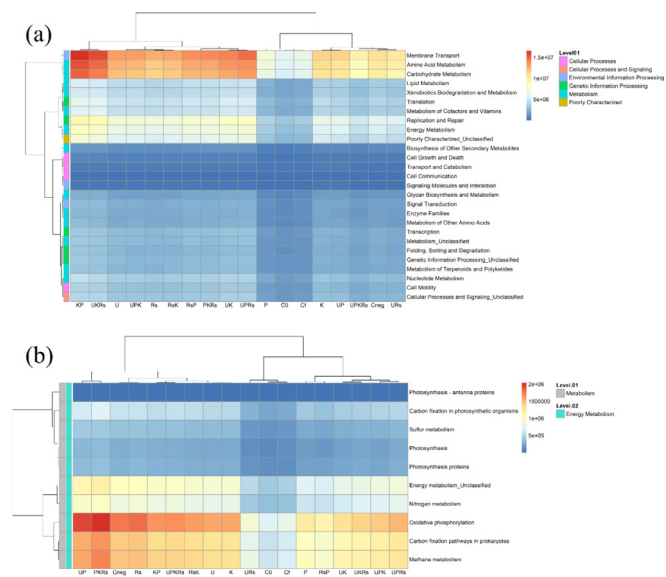
Fig 6. Heat map demonstrating PICRUSt derived hierarchical clustering of predicted functional profiles (un-scaled data) at a second level (Metabolism) (a) and third level (Energy metabolism) (b). KEGG database was used to predict functions at the second and third levels in PICRUSt (http://picrust.github.io/picrust/).

can explain a functional category. The heat map in Fig. 6b demonstrated methane metabolism which was observed highest in PKRs (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 responded higher for oxidative phosphorylation (21.76%), methane metabolism (18.26%), and carbon fixation pathways in prokaryotes (17.79%).

4. Discussion

As far as organic and inorganic supplementation of paddy soil is concerned, the current study characterized a comprehensive investigation of consequent microbial community variations and their functional metabolic prediction in paddy soil microcosm. It focused on quantification, diversification, and metabolic functional prediction at two different levels of two key microbial groups involved in methane metabolism i.e., bacteria and archaea whose functional metabolic predictions are reported rarely under given amendments. Thus, it gave us better insight into fertilizer usage concerning methanogens in the soil microbiome. Additionally, this study also covered some treatments that are non-conventional in rice agriculture to establish a comprehensive comparison between different combinations.

Soil physicochemical properties treated with various fertilizers have been reported to impact bacterial and archaeal community structures. Soil pH did not vary significantly in different treatments due to the flooded nature of the soil which stabilizes pH by inhibiting nitrification i.e., an acid-producing process (Mi et al., 2018). Although pH is known to be a considerate factor in shaping microbial communities (Shen et al., 2013), some studies have reported otherwise in clay loam (Zhang et al., 2015). After subsequent inorganic and organic supplementation, a significant increase in EC, AK, and OM was also observed which were strongly correlated to each other but poorly to pH in PCA biplot (Fig. 1) which also suggested the least role of pH in our study (Wang et al., 2017). The biplot showed a strong correlation between OM and AK; TC and TN; GWC, EP, and pH (Li et al., 2019b). Concerning different treatments, a strong association in microbial communities between KP, PKRs, and UK was observed, while the rest of the sample showed distinc-

tions of varying degrees. TC and TN have been shown to positively influence Rs, URs, RsP, UPRs, and UPKRs. No significant variation in TC, TN, and EP was observed, which are usually known to increase with straw application and NPK fertilization, 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. A better understanding can be established in the future by repeating the same study in paddy mesocosms.

The microbial community succession under the influence of NPK and rice straw is well documented in wetland ecology and rice fields (Iocoli et al., 2019), however, it failed to provide the comparative narrative. In particular, the control treatments i.e., C_0 and C_f were parted from C_{neg} as well as from other treatments suggesting a strong bacterial and archaeal shift due to rice nursery in C_{neg}. TC and TN seem to impact negatively or neutrally for most bacterial phyla while for archaeal phyla they were positively correlated except Thaumarchaeota. This exception can be supported by the fact that bacteria and archaea are less dependent on C and N sources compared to fungi which showed greatly varied responses under them (Schmidt et al., 2014). The total bacterial and archaeal population increased for every test sample as compared to control, however that increase was less evident in the case of bacteria (Lee et al., 2014). Flooded conditions have been known to impact bacterial communities moderately (Breidenbach and Conrad, 2014). Phylum Proteobacteria comprised the largest fraction of soil bacterial communities (Eo and Park, 2016; Zhan et al., 2018) both metabolically and genetically due to the copiotrophic lifestyle of paddy soil (Wang et al., 2018) and the prevalence of other dominant phyla i-e Actinobacteria, Firmicutes, Chloroflexi, Bacteroidetes, Acidobacteria, etc is also well documented (Breidenbach and Conrad, 2014; Itoh et al., 2013; Wang et al., 2018, 2017) and was as per our results (Chen et al., 2016). Bacterial phyla, Chloroflexi, Bacteroidetes, Planctomycetes, and Verrucomicrobia showed an increase in population size as compared to control while Actinobacteria, Acidobacteria, and Gemmatimonadetes showed a negative trend (Cederlund et al., 2014; Li et al., 2019b). Previous studies also report more response of bacterial diversity in the presence of inorganic fertilizer along with rice straw which satisfies our results for all cases except UPKRs (Chidthaisong et al., 1996; Conrad, 2002; Rath et al., 1998). In our case, Bacillus did not seem to be a very responsive genus for every combination except UPRs and UPKRs which indicated that rice straw in combination U and P may shift the functional dynamics of Bacillus. 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 (Cederlund et al., 2014; Nemergut et al., 2008; Wu et al., 2011) which was not in our case. One possible explanation could be the short-term nature of our study and can be investigated further in mesocosms. Additionally, RDA analysis showed a strong correlation of soil physicochemical properties i.e., GWC, pH, TC, and TN along the first axis that explained 23.3% variance (Fig. 5). It also showed time zero control (C_0), flooded control (C_f), and P well separated from all treatments. Since microbial diversity in the 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) which is a strong oxidant and acts as a potential biocide and known to obstruct nitrification even at low concentrations (Chowdhury et al., 2011; Vieira Megda et al., 2014). Additionally, rice straw incorporation has been reported extensively to stimulate bacterial communities in paddy soil and our results are as per it (Gong et al., 2009; Liu et al., 2009; Wu et al., 2011). 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. However, it satisfied our hypothesis regarding community variation under single and mix combinations of commonly used treatments.

The soil archaeal community in paddy soil is reported to be more stable unless influenced by temperature or the presence of organic matter such as rice straw (Breidenbach and Conrad, 2014; Peng et al., 2008). We found archaea (methanogens specifically) 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 (Borrel et al., 2011; Wang et al., 2010; Watanabe et al., 2006). There are controversies in the literature suggesting N-fertilization can stimulate (Banik et al., 1996; Shang et al., 2011) or inhibit (Dong et al., 2011; Xie et al., 2010) methanogenesis in wetland ecosystems but our results showed mutual cases for the most abundant group i-e Methanobacteriam. Most of the test samples showed an increasing trend except U, K, and UK treatments and reduced methanogenesis due to urea (Zou et al., 2005) and potassium is documented (Sheng et al., 2016).

A computational methodology to predict functional activities of microbial communities at the metabolism level was employed using PICRUSt. The idea was to evaluate marker genes of HTS to predict variation in functional metabolism under these treatments (Langille et al., 2013) since very few studies have predicted the prevalence and abundance of C, N, and P cycle-related genes under these conditions (Chen et al., 2020; Hartman et al., 2017; Hu et al., 2019; Kang and LeBrun, 2018). The hierarchical clustering at second level KEGG ortholog function prediction concerning metabolism showed microbial community response 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%). At energy metabolism level, specifically methane metabolism, which is confiscated by methanogenesis, the response 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 acetoclastic methanogens i.e., Methanosaeta (Wang et al., 2018) were also seemed to increase in our test samples.

5. Conclusions

The study comprehended the integrative use of organic and inorganic fertilizers which significantly alters the pH, EC, OM, and AK of the paddy soil. Regarding single fertilizers, the highest diversity was found in Rs and least for P as represented by OTUs, Faith's PD, and Shannon indices. While in mixed treatments the highest diversity was found in soil supplemented with KP and least in quadruple treatment i-e UPKRs which suggested a major role of K, P, and Rs in diversity. Overall, archaeal, and bacterial communities responded more to a combination of fertilizers compare to single treatments. Comparing both communities, the archaeal community was more responsive. The PICRUSt based energy and methane metabolism profiles indicated PKRs and UP as most alert and URs, C_0, and C_f as least responsive. Thus, in our suggestion, the application of KP together should be avoided in rice paddies and flooded soils. Adding to our conclusion regarding fertilizer usage, mixed fertilization can potentially increase the methane metabolism amongst the microbial community. The current results also implied that caution must be exercised in flooded agricultural systems regarding the usage of KCl to regulate methane emission. The amendments Rs, PKRs, and UP were found most responsive in

terms of methane metabolism and oxidative phosphorylation while least for nitrogen metabolism at the same time.

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