Interplay of inorganic fertilizers on the relative abundance of effective microorganisms and methane emission in wetland soil

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Microbiology

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IN THE NAME OF ALLAH, THE MOST BENEFICENT & THE MOST MERCIFUL

"Proclaim! in the name of thy Lord and Cherisher, Who created – Created man, out of a (mere) clot of congealed blood: Proclaim! And thy Lord is Most Bountiful, He Who taught (the use of) the pen, Taught man that which he knew not" (Quran, 96:1-5).

"Say [unto them, O Muhammad]: Are those who know equal to those who know not? But only men of understanding will pay heed" (Quran, 39: 9)

The only legacy of the scholars is knowledge, so whoever takes from it, then he has indeed taken the ablest share (Jami`at-Tirmidhi 2682).

The saddest aspect of life right now is that science gathers knowledge faster than society gathers wisdom (Isaac Asimov, 1920-1992).

Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less (Marie Curie, 1867-1934).

DEDICATED
TO
MY MOTHER

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I **Mr. Mohsin Gulzar Barq** hereby state that my Ph.D. thesis titled **"Interplay of Inorganic Fertilizers on the Relative Abundance of Effective Microorganisms and Methane Emission in Wetland Soil"** is my own work and has not been submitted previously by me for taking any degree from Quaid-i-Azam University, Islamabad, Pakistan.

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LIST OF ACRONYMS/ABBREVIATIONS

N	Nitrogen
P	Phosphorus
K	Potassium
C	Carbon
Rs	Rice straw
GWC	Gravimetric water content
EC	Electrical conductivity
TN	Total nitrogen
TC	Total carbon
SOM	Soil organic matter
OM	Organic matter
AK	Available potassium
TEP	Total extractable phosphorus
CH_4	Methane
PMO	Potential methane oxidation
HTS	High throughput sequencing
PLFA	Phospholipid fatty acid
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
AP	Acid phosphatases
GLU	β-glucosidase
NAG	N-acetyl-β-glucosaminidase
MPP	Methane production potential
PCA	Principal component analysis
PC	Principal component
C/N	Carbon to nitrogen
CW	Constructed wetland
UV	Ultraviolet
BU	Bottom up
TD	Top down
NGS	Next generation sequencing
GHG	Greenhouse gas
GWP	Global warming potential
CFCs	Chlorofluorocarbons
CO_2	Carbon dioxide
MMO	Methane monooxygenase
AMMP	Anaerobic microbial methane production
CLPP	Community level physiological profiles
FAME	Fatty acid methyl esters
FISH	Fluorescent in situ hybridization

G+C	Guanosine plus cytosine
PCR	Polymerase chain reaction
KEGG	Kyoto encyclopedia of genes and genomes
KO	KEGG orthologs
COG	Clusters of orthologous genes
MOB	Methane oxidizing bacteria
MOP	Methane oxidation potential
KH_2PO_4	Potassium dihydrogen phosphate
KCl	Potassium chloride
Tg	Terra gram
Cl	Chloride
U	Urea
SRA	Sequence read archive
NCBI	National center for biotechnology information
QIIME	Quantitative insights into microbial ecology
OTUs	Operational taxonomic units
PD	Phylogenetic diversity
SE	Standard error
RDA	Redundancy analysis
CO	Carbon monoxide
C_0	Time zero control
C_f	Flooded control
C_{neg}	Non supplemented control with plant
GC	Gas chromatography
FID	Flame ionization detector
GP	Gram positive
GN	Gram negative
pNP	p-nitro phenyl
MUB	Modified universal buffer
THAM	Tris hydroxy aminomethane
CFA	Confirmatory factor analysis
tFAME	Total fatty acid methyl esters
TB	Total bacteria
TF	Total fungi
F/B	Fungi to bacteria
Cyc/pre	Cyclopropyl to precursor PLFA
Sat/mono	Saturated to monosaturated PLFA
CFI	Comparative fit index
SRMR	Standardized Root Mean Square Residual
GFI	Goodness of fit index
GB	General bacteria
ACT	Actinomycetes

GGP	General gram positive
AMF	Arbuscular mycorrhizal fungi
SF	Saprophytic fungi
g	Gram
mM	Milli molar
M	Molar
%	Percentage
^{o}C	Centigrade
nm	Nanometer
cm	Centimeter
mg	Milligram
h	Hour
ml	Milli liter
s	Seconds
µg	Microgram
mm	Milli meter
rpm	Revolution per minute
ppm	Parts per million

ABSTRACT

Abstract

The heterogeneity in fertilization (single or integrated) in paddy fields and wetland soil leads to dynamic unconformity in soil physicochemical properties, plant biomass, microbial community ecology, and their underlying physiological behavior, and enzymatic mechanisms. Paddy ecosystems comprise 11% of our arable land and are greatly influenced by composite environmental perturbations. The interplay among environmental factors and microbial community ecology is quite challenging and needs to be addressed via a contemporary perspective to attain sustainable ecosystems and agriculture practice.

The current study was devised to investigate the fluctuating influence of inorganic (NPK) and organic (rice straw) fertilizers in a single and integrated fashion on the soil physicochemical attributes, plant biomass, effective microbial community, and functional metabolic predictions. It employs microcosm-based experiments with agricultural paddy soil with rice plants (*Oryza sativa* var. Super basmati) and subsequent supplementation of NPK in three doses. Single and integrated supplementation of nitrogen (N) as $C(H_2N)_2O$, phosphorus (P) and potassium (K) as KH_2PO_4, K as KCl, and rice straw (Rs) with concentrations equivalent to 160 kg N ha^{-1}, 60 kg P ha^{-1}, 130 kg K ha^{-1}, and 1% respectively were made to make at least 16 different combinations (18 including two controls). Soil physicochemical properties i.e., gravimetric water content (GWC), pH, electrical conductivity (EC), total nitrogen (TN), total carbon (TC), soil organic matter (SOM), available K (AK), and total extractable P (TEP) were accessed. The microbial community structure was apprehended by 16S rRNA high throughput sequencing (HTS) and phospholipid fatty acid (PLFA) profiling. Using 16S rRNA HTS data, alpha diversity (Pielou's E and

Shannon) and beta diversity (Jaccard, Bray-Curtis) indices were reported. Furthermore, functional predictions were made employing phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) at different levels. Activities of acid phosphatase (AP), carbohydrate hydrolases i.e. β-glucosidase (GLU) and N-acetyl-β-glucosaminidase (NAG), and plant biomass variations were also studied. Additionally, methane production potential (MPP) of supplemented soils samples was measured. Moreover, in another experiment, Potential methane oxidation (PMO) rates were also monitored in flooded and non-flooded conditions against the different concentrations of $(NH_4)_2SO_4$ in wetland soil.

Our results indicated GWC, pH, TN, TC, C/N, and SOC to be the most influential indicators of microbial community ecology. HTS and PLFA analysis also revealed bacteria and archaea being more responsive compared to fungi. The outcomes revealed enhancement of community richness and diversity in all supplemented treatments compared to controls. *Proteobacteria*, *Actinobacteria,* and *Firmicutes* were the highly prominent phyla among bacteria. In different combinations, *Chloroflexi*, *Bacteroidetes,* and *Verrucomicrobia* showed positive while *Actinobacteria*, *Acidobacteria,* and *Gemmatimonadetes* showed negative tendencies contracted to controls. *Thaumarchaeota* and *Euryarchaeota* were dominant archaeal phyla and exhibited increasing and decreasing trends, respectively. The methanogenic genera i.e., *Methanobrevibacter*, *methaosaeta,* and *methanomassiliicoccus* showed a significant increase while *methanosphaerula* and *methanocella* showed decreasing trend. Overall, a discouraged methanogenic population was observed in U, K, UK, and UKRs which signifies the negative role of U and K in methane emission. The PICRUSt indicated microbial community shifts significantly towards amino acid, carbohydrate, energy,

and lipid metabolism while less towards glycan biosynthesis, synthesis of secondary metabolites, terpenoids, and biodegradation. Significantly enhanced activity of β-glucosidase (GLU) and urease was observed in a majority of treatments compared to acid phosphatase (AP) and N-acetyl-β-glucosaminidase (NAG).

The principal component analysis (PCA) signified microcosms with mixed combinations of fertilizer and controls as the most crucial variable regarding community structure. pH, C/N, SOM, and AK are identified as key factors in shaping bacterial and fungal abundance in different treatments. Our results inferred more disparity in the bacterial community than fungal. The effect of integrated fertilization especially UP and UPK was more promising regarding community ecology, methane metabolism, and plant growth.

Keywords: NPK fertilizers, paddy soil microcosms, high throughput sequencing, PICRUSt, methane metabolism, enzyme activities.

THESIS OUTLINE

Thesis outline

The critical role of the soil microbial community to attain ecologically sustainable agriculture has driven a proliferation to investigate the relationship between microbial community, nutrient recycling, and soil fertility. Being a temporary CW, occupying 11% of the global arable area, paddy soils introduce one of such examples in which relevant influencing factors need to be explored more than ever. Rice fields and wetland soils were established as robust sources and sinks of CH_4, thus making them a good experimental model for sustainable ecosystems and agriculture practices under different fertilization. **Chapter 1** briefly covers the overall introduction of the thesis. In **Chapter 2** detailed literature review regarding wetlands, paddy soils, different factors affecting microbial communities, methanogenesis, methanotrophy, 16S rRNA sequencing, PLFA profiling, and PICRUSt analysis are briefly discussed. **Chapter 3**, comprehends the published study concerning the short-term impact of inorganic and organic fertilization on archaeal and bacterial communities at the phyla and genera levels by metagenomic analysis. Functional predictions were made at level 2 (metabolism) and level 3 (energy metabolism) using 16S rRNA NGS data. In **Chapter 4**, the effect of bacterial, fungal, and archaeal (methanogenic) communities was investigated by PLFA profiling and 16S rRNA sequencing. Additionally, activities of CNP enzymes were also accessed. The influence of inorganic and organic fertilizers was analyzed on plant biomass and methane production potential. **Chapter 5** overlooks the role of different concentrations of $(NH_4)_2SO_4$ on type I and type II methanotrophs and their PMO rates under flooded and non-flooded conditions. Finally, **Chapter 6** presents an overall conclusion of the findings with the integration of the current study and future perspectives in **Chapter 7**.

INTRODUCTION

1. General Introduction

Soil plays a complicated and fundamental part in the terrestrial and wetland ecosystems by executing biotic and abiotic processes that evolve under environmental strains (Welc *et al.* 2012). Soil microbial communities in waterlogged conditions have become a prime emphasis of microbial ecologists during the past few decades concerning ecosystem functioning, stability, and sustainability. Paddy soil is distinctive from upland soil for its waterlogged condition and being a specific type of constructed wetland (CW), it is assessed as an important source and sink of methane (Kikuchi *et al.* 2007, Watanabe 2019). Rice paddies being distinctive in hydrology, water-dependent vegetation, and hydric soils represents one of the largest human-made wetlands (Ramsar Convention Secretariat 2013). In rice paddy agriculture practice, a key factor that affects crop yield, soil quality, and variation in microbial community composition in the paddy soil is the type and concentration of fertilizer applied (Greenberg *et al.* 2017). The physicochemical and biological behavior of paddy soil is quite different from upland soil due to flooded irrigation practice (Kikuchi *et al.* 2007, Kamaa *et al.* 2011) and its nutrient budget is dependent on supplementation of organic (e.g rice straw) and inorganic fertilizers e.g NPK respectively (Eo and Park 2016). NPK fertilization and rice straw have been known to induce variations of varying degrees in soil physicochemical properties as well as microbial community dynamics (Niswati *et al.* 2005, Pan *et al.* 2016, Kuppusamy *et al.* 2018). Significant variations have been reported in the microbial biomass and community composition after subsequent fertilization in upland and lowland soils (Esperschütz *et al.* 2007, Parrent and Vilgalys 2007). In addition to chemical fertilizers, the application of rice straw has been found to improve soil fertility and nitrogen uptake (Takahashi *et al.* 2003, Zheng *et al.* 2020).

Its application has also been found to increase methane emission by methanogens and the succession of bacteria responsible for straw decomposition (Jia *et al.* 2007, Rui *et al.* 2009). The dynamics of NPK and carbon (C) in soil are closely correlated, thus, evaluating the effects of fertilizers in various combinations on microbial communities in saturated soils is vital due to their active involvement in stimulating or inhibiting biogeochemical processes thus enabling ecosystems to function accordingly.

The role of saturated soils is very important concerning methane status and recycling (Kędzior and Dreger 2019) and are the major artificial sites of methanogenesis after natural wetlands. In saturated soils, from an environmental perspective, methanotrophs are more highlighted as consumers compared to methanogens as producers that are participating in the biogeochemical cycling of CH_4 in an antagonistic manner (Pazinato *et al.* 2010). Typically, aerobic soil is the only biological sink for the oxidation of methane by methanotrophic bacteria sequestering 10.0 to 30.0% CH_4 in wetland soils (Le Mer and Roger 2001, Griggs and Noguer 2002). Globally, paddy soils contribute 15–20% CH_4 emission (25-100 Tg/year) (Zhang *et al.* 2011, Dubey *et al.* 2014) and are predicted to increase up to 50% (145 Tg/year) by 2025 (Singh *et al.* 2018). Thus, it will lead to greater input of NPK fertilizers that may affect the energy metabolism of microbial communities, turning soil into a greater source of methane emission rather than a sink (Gulledge *et al.* 1997, Dubey 2005, Khush 2005). The global methane budget is summarized in Table 1.1 (Saunois *et al.* 2020).

The efficiency and sustainability of the terrestrial agroecosystem are highly reliant on microbial diversity and physiology that vary continuously with the nutrients' status (Wu *et al.* 2011, Wang *et al.* 2017). The substantial role of the soil microbiome in energy flow, nutrient cycling (Morris and Blackwood 2015), and soil quality

determinants are well documented (Wang *et al.* 2017). A minor shift in organic and inorganic content of soil may shift the microbial community dynamics and modify their underlying mechanisms (Zhong and Cai 2007, Ramirez *et al.* 2012, Eo and Park 2016) and ultimately lead to varying yield and soil quality (Su *et al.* 2015). In this context, the comparative abundance and role of specific microbe are considerably important (Mi *et al.* 2018).

Table 1.1: Global methane budget of natural sources, anthropogenic sources, and sinks. BU, bottom-up; TD, top-down.

Sources BU budget (Tg CH_4/year)				***Sinks TD budget (Tg CH_4/year)***	
Natural		***Anthropogenic***			
Wetlands	183	Fossil fuels	135	Tropospheric OH	519
Freshwater	122	Landfills and waste	68	Stratospheric loss	51
Geological	54	Rice cultivation	30	Tropospheric Cl	25
Wild animals & Termites	19	Enteric fermentation & manure	115	Soil uptake	30
Wildfire & Permafrost	4	Biomass & Biofuel burning	29		
Total Sources	759				
Total sinks	625				
Source-Sink imbalance	127				

Approaches employed to study soil microbial community ecology are 16S rRNA gene sequencing (Li, Liu, *et al.* 2019) and phospholipid fatty acid (PLFA) profiling which has been well established in recent years. Fluctuating enzyme activities have been reported in waterlogged soils that are involved in soil productivity and CNP cycling as a result of organic amendments (Gascó *et al.* 2016, Li *et al.* 2018). Soil enzymes being a vital component of biochemical functioning are suggested as a determinant of soil quality and their functionalities are associated with microbial biomass, nutrient availability, and plant growth (Liu *et al.* 2017, Zhang, Sun, Chen, *et al.* 2019). Soil enzymes like β-glucosidase (GLU), acid phosphatase (AP), N-acetyl-β-glucosaminidase (NAG), and urease are involved in CNP transformations and their

activities differed with the application of different fertilizers (Li *et al.* 2015, Zhang *et al.* 2016).

Considering the variability in type and application rate of fertilizers in paddy soil, it is vital to know the variation and dynamics of soil microbial community structure, function, and chemistry that affect the global methane burden. So, a microcosm-based experiment (Figure 1.1) 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 the bacterial, fungal, and archaeal communities in wetland/paddy soil. The impact of GLU, AP, NAG, and urease on soil community structure was also studied. Additionally, PICRUSt derived functional profiles i-e energy metabolism of contributing microbial community was predicted to estimate functional metabolism (methane metabolism). A correlation can also be suggested between the dynamics of microbial communities and physicochemical factors.

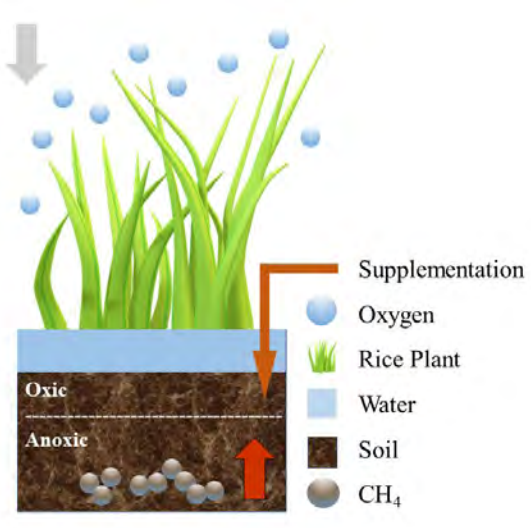

Figure 1.1: Paddy soil microcosm flooded with 5 cm of water and planted with rice plant.

Aims and Objectives

The study intends to evaluate the overall relative density and diversity of effective microbial communities under the influence of a different combination of organic and inorganic fertilizers in a controlled microcosm setup containing soil from a paddy field and wetland. The proposed study also includes optimizing a specific combination of inorganic and organic fertilizers to predict methane emission, predictive functional metabolism, and enzyme activities. The objectives are as follows.

- Development of microcosms containing paddy soil and indigenous *Oryza sativa* (var. Super Basmati).
- Supplementing planted microcosm with different combinations of inorganic and organic fertilizers viz. urea, potassium dihydrogen phosphate, potassium chloride, and rice straw to evaluate:
    1. Soil physicochemical properties of the paddy soil.
    2. Microbial community composition and diversity of effective microorganisms by PLFA and NGS.
    3. Functional metabolic predictions of the microbial community by PICRUSt.
    4. Impact of differential fertilization on CNP enzymes, the relative abundance of methanogens, methane emission, and plant biomass.
- To evaluate the relative abundance of type I and type II methanotrophs and their PMO rates under different concentrations of $(NH_4)_2SO_4$ in saturated and drained conditions.

LITERATURE REVIEW

2. Literature review

2.1. Wetlands

Wetlands, by definition, encompasses specific hydrology, vegetation, and hydric soils that transitioned between aquatic and terrestrial ecosystems (Craft 2015). Thus, wetlands are "edge" ecosystems and include all sorts of peatlands, marshes, swamps, and bogs (Neori and Agami 2017). The wetland hydrology can be permanently or intermittently flooded but the soil should be saturated long enough to support specific vegetation and processes distinctive to wetland ecology. Natural and man-made wetlands hosted some of the critical ecological processes like primary production, biodiversity, organic carbon storage, nutrient enrichment, and methane fluxes, yet covering roughly cover 6 % of the vegetative land area worldwide (Russi *et al.* 2012, Janse *et al.* 2019). During the past century, an estimated two-thirds of the natural wetland have been wiped out primarily due to human interventions (Wetlands International 2014). However, one of the important, major, and ever-growing types of man-made wetlands are rice paddies which lie within the very definition of constructed or man-made wetland (Ramsar Convention Secretariat 2013).

2.2. Paddy Soil

The competence, productivity, efficiency, and sustainability of the terrestrial agroecosystems are highly contingent on soil microbial ecology and physiology that varies continuously with the nutritional status, biochemical activities, and other ecological factors (Wu *et al.* 2011, Wang *et al.* 2017). Soil performs an indispensable and complex role in the terrestrial ecosystem by executing its transmuting abiotic and biotic processes (Welc *et al.* 2012). The vital role of soil microbiota in energy flow,

nutrient cycling (Morris and Blackwood 2015), and soil fertility are well documented (Wang *et al.* 2017). However, a minor shift in different factors like inorganic or organic contents of soil may shift the ecology and ultimately physiology of it (Zhong and Cai 2007, Ramirez *et al.* 2012, Eo and Park 2016).

Rice is the second most-cultivated basic crop intended for roughly 50% of the earth's inhabitants covering over 163 million ha cropland that represents 11% of the global arable land (Maraseni *et al.* 2018, Aslam Ali *et al.* 2019). Paddy field soil depicts a complex biosystem where a range of biogeochemical cycles occurs. Flooded irrigation of paddy soil makes it well distinguished from upland soil in soil quality, productivity, microbial ecology, and biological behavior (Kikuchi *et al.* 2007, Kamaa *et al.* 2011). At the same time, paddy soil is also a crucial source of methane emission with a fair impact of 15-20% of the overall anthropogenic methane production (Datta *et al.* 2009) which is likely to increase from 25-100 Tg to 145 annually by 2025 (Singh *et al.* 2018). The increase in rice demand with increasing population will lead to greater use of flooded irrigation, nitrogenous fertilizers, and methane metabolizing capabilities of waterlogged paddy soils.

2.3. Soil microcosms

Microcosms are reproducible and replicable, artificially bounded microclimatic subsets of a natural ecosystem with different trophic levels. Soil microcosms exampled as an excellent tool to study ecosystem’s functionality in a controlled environment to mimic ecological parameters. Microcosms are potentially beneficial and preferred in the following situations:

- Where the influence of individual ecological parameters needs to be investigated.

- In ecosystems where field analyses are laborious and time-consuming.
- In situations where investigations need to be reproduced.
- When intermediate studies are required before initiating full-scale experimentation.
- In hard access areas or where in-field experimentation is not possible.

Terrestrial or soil microcosms have been widely used to study different physicochemical or biological processes like biodegradation, bioaccumulation, bioremediation (Carbonell and Tarazona 2014, Maguffin *et al.* 2020, Pinto *et al.* 2020), gas measurements (Guo *et al.* 2020), microbial ecology and physiology (Ellis 2004, Nakagawa *et al.* 2020) and, nutrient recycling (Hoorman and Islam 2010).

2.4. Fertilization of Paddy soil

Different types of organic and inorganic fertilizers are applied to paddy soil in varying concentrations globally (Bååth and Anderson 2003). Since its nutrient budget is dependent on an artificial supply of organic and inorganic fertilizers, it demands a delicate balance between major (C, N, and P) and minor nutritional composition (Eo and Park 2016). A key factor that affects crop yield, soil quality, and variation in microbial community structure in the paddy field soil is the type and concentration of fertilizer applied (Greenberg *et al.* 2017). Evaluating the effect of individual fertilizers in a pure and mixed form on soil physicochemical, biological, and ecological behavior is vital due to their active involvement in stimulating or inhibiting biogeochemical processes, thus enabling ecosystems to function accordingly. Traditionally, paddy soil fertilization can be classified as inorganic (mineral) and organic fertilizers.

2.5. Inorganic fertilizers

In rice field agriculture, NPKs are primary mineral fertilizers while zinc (Zn) as zinc sulfate is used in lesser concentrations. The NPK dynamics are correlated with C and are primarily dependent on pH, soil moisture, temperature, and cultivation methodology. Considering rice cultivation, nitrogen is the most limiting nutrient (Luo *et al.* 2016) and its application as ammonium-based N fertilizers (urea and ammonium sulfate) substantially improves yields, however, N competence of paddy plants regarding integration efficiency is generally less (Qiao *et al.* 2012). Balancing nitrogen usage is also an important prospect to limit its toxicity because more than half of applied nitrogen is dissipated by volatilization, surface runoff, denitrification, and nitrate leaching. High N fertilization is known to decrease microbial diversity (Liang *et al.* 2008) and temporarily produce higher osmotic potential and toxic accumulation of ammoniacal N (Geisseler *et al.* 2017). P and K are not limiting nutrients and are essential in lesser concentrations compared to N. A delicate balance among these increase microbial ecology richness as well as crop yield (Su *et al.* 2015).

2.6. Organic fertilizers

The combination of mineral and organic fertilizer in rice agriculture is reported to increase soil fertility (Tian *et al.* 2015) and various types of organic amendments are applied globally among which rice straw (Rs), green manure, cattle manure, and spent mushroom compost are most common (Mi *et al.* 2018). Due to their distinct composition, their impact on soil physicochemical properties and biological behavior is also different. However, their specific interaction with mineral fertilizers in paddy soils is poorly studied. Overall, organic amendments are highly recommended in

addition to mineral fertilizers because numerous studies have suggested their role in enhanced microbial diversity, improved soil fertility, N uptake (Takahashi *et al.* 2003), P mobility and recovery (Mohanty, Paikaray, *et al.* 2006), increasing soil SOM (Agbede 2010), and crop yield (Yang *et al.* 2015, Qaswar *et al.* 2019).

2.7. Microbial ecology of paddy soil

The diversity, community structure, and biological activity of soil microorganisms are determined by multifaceted proximal factors such as pH, temperature, nutrient inflow, and soil moisture content (Brockett *et al.* 2012, Menéndez-Serra *et al.* 2019). The maintenance of soil productivity via energy flow, nutrient recycling by decomposing organic matter, and formation of soil aggregates are few underlying processes indispensable without archaea, bacteria, and fungi. The disparity in diversity and composition of microbial communities due to fertilization is well studied (Shen *et al.* 2013). Since, some vital processes like nutrient recycling by residue decomposition (Six *et al.* 2004), methanogenesis, methanotrophy, nitrogen fixation (Majeed *et al.* 2018), biodegradation (Nicolás *et al.* 2019), disease suppression, soil sustainability, and productivity (Newman *et al.* 2016) are microbe dependent, they will remain under critical status for investigation. The methanogenesis is exclusive to methanogens, identified only in domain archaea. The role of bacteria in controlling soil physiology and quality is evident by methanotrophy, decomposition, and nitrogen fixation. At the same time fungi are involved in decomposition, biodegradation, and fermentation (Zhang, Sun, Chen, *et al.* 2019). The *Proteobacteria, Actinobacteria, Chloroflexi* and *Acidobacteria* constantly outweighed concerning soil microbiota and most of the studies are in accordance with it. At different soil depths phylum *Proteobacteria* has been recognized as most dominant and it also outcompete other phyla in straw

decomposition (Li *et al.* 2017). Some reports has also identified it in improving soil fertility and plant growth (Chaudhry *et al.* 2012). The phylum *Chloroflexi*, being facultative anaerobe has been shown variations in abundance particularly during flooded period of rice cultivation (Chen *et al.* 2016). The bacterial phylum *Actinobacteria* played an important role in straw decomposition, changing functions related to soil conditions, because a variety of actinomycetes play an important role in degrading lignin and lignocellulose by excreting peroxidases and oxidases (Wu *et al.* 2020).

2.8. Determinants of microbial community ecology

Saturated soil ecosystems have been known as an anthropogenic source of methane metabolism and furnish aerobic and anaerobic conditions for methane production and oxidation. There has been a differential degree of variation within the community composition of effective microorganisms (archaea, bacteria, and fungi) and a particular environmental factor may be stimulatory, inhibitory, or may not affect the community at all. Fungal communities in paddy soil are less susceptible to structural or physiological variation due to waterlogged conditions (ZHANG *et al.* 2007, Chen *et al.* 2014). Environmental determinants of microbial community ecology can be of proximal nature that affects directly (soil water content, pH, temperature, micro and macronutrients, and SOM content, etc.) or site factors affecting indirectly such as location, regional climate, soil texture, land usage, and crop type (Brockett *et al.* 2012). The flooded conditions and organic amendments in paddy soils are pH stabilizers (Zhong *et al.* 2010, Rukshana *et al.* 2012), thus pH variations in waterlogged soils are usually narrow. The pH stabilization due to flooded nature inhibits acid-producing processes such as nitrification (Mi *et al.* 2018). Contradictory outcomes have been

reported previously regarding pH involvement in waterlogged soils. Few studies claimed pH to be one of the strongest contributors in distinguishing microbial ecology in flooded soil (Rousk *et al.* 2010, Kumar *et al.* 2018) while others did not found any significant influence of pH on microbial communities (Zhang *et al.* 2015). Such findings direct the investigators to focus on other contributing factors integrated with pH.

2.8.1. Temperature and soil moisture

Temperature and soil moisture content has been known as critical contributors in influencing community ecology and their impact is more profound in the case of methanogens and methanotrophs (Tian *et al.* 2011). Due to their involvement in shaping methanogenic and methanotrophic communities, they are considered key factors in methane emission and oxidation in hydric soils (Qiao *et al.* 2012). In general, it is a well-established fact that methanogens are most active under flooded conditions due to anaerobic environment while methanotrophic populations are decreased due to the same reason (Jia *et al.* 2007, Serrano-Silva *et al.* 2014).

2.8.2. Flooded conditions

Methanogenic communities keep manipulating through the rice-cultivation cycle with an insignificant increase at the preliminary stage and comparably become higher at the flowering stage. Their growing tendency has been defined (Singh *et al.* 2012) in the subsequent order: plantation of pre-plantation, post-harvest, tillering, ripening, and flowering stage. However, this shift in population is also precisely relatable to flooded and drainage conditions of the soil. This pattern of population shift is common in different soils and climatic conditions also, thus making flooding conditions a vital

determinant in shaping community structure. Few findings also report methanogen stability and survival during adverse drained conditions (Ma and Lu 2011).

2.8.3. Nitrogen Supplementation

N supplementation is unavoidable in rice field agriculture and it regulates GHG emission (Wu *et al.* 2009a), influencing crop yield, manipulates soil microbial ecology (methanogens or methanotrophs), and even alters soil physicochemical attributes. Generally, N supplementation stimulates methanogens and inhibits methanotrophs, and thus increases net CH_4 emissions (Datta *et al.* 2013, Bodelier and Steenbergh 2014). The influence of N fertilization on other communities such as denitrifying bacteria (that usually outcompetes methanogens for growth substrates) is also reported, however, their competence is dependent on the source utilized. The impact of N on a particular community also depends upon the type and concentration of applied fertilizer, for example, urea is more stimulatory for methanogens compared to ammonium sulfate. At lower CH_4 concentrations, NH_4 inhibits CH_4 oxidation and vice versa (De Visscher and Van Cleemput 2003).

2.8.4. P and K supplementation

Supplementation of P fertilizers influences community ecology by increasing N fixation, inhibiting methanogens, and stimulating PMO rates. Inhibition of methanogenesis is more pronounced in the case of the acetoclastic pathway thus decreasing net CH_4 emissions. Soil amendments with K fertilizers also inhibit methanogenesis in paddy soils, however (Wassmann *et al.* 2000) observed no substantial correlation between K-fertilizer and methanogenesis. (Conrad *et al.* 2008) reported a similar mechanism of inhibition by P and K fertilizers that execute their influence on the aerobic environment and root exudates. K fertilization in KCl form,

even in low concentrations, induces oxidizing environment and suppresses nitrification (Vieira Megda *et al.* 2014). The mechanism, interaction, and influence of P and K are still unclear and demand more attention.

2.8.5. Soil organic matter

Soil quality indicators (soil community structure and diversity) are sensitive to SOM content and its amendment in non-flooded soil induces CO_2 production while in flooded soils it enriches CH_4 production (Wang *et al.* 2018). Organic amendments integrated with NPK fertilization in poor quality soils is a promising and well-studied approach to improve soil quality and a good step towards sustainable agriculture practice (Yang *et al.* 2015, Mi *et al.* 2018). Although different types of organic supplementation are applied worldwide, their mechanism of action is perplexing, multifactor dependent, and poorly studied. Studies are continuously conducted to access their long-term impact in good quality and poor-quality soils which are crucial for C sequestering and stability (Liu, Sui, *et al.* 2019). (Hou *et al.* 2013) analyzed the influence of rice straw and wheat straw on the methane efflux from the soil in a three-year study. Four treatments containing rice straw, wheat straw, a combination of wheat and rice straw, and control were studied. The treatments containing wheat and rice straw showed an increase in methane emission but on the other hand they observed less methane emission in rice straw treatment and concluded that rice straw treatment could increase the productivity of rice with decreased methanogenesis. (Bhattacharyya *et al.* 2012) applied rice straw, green manure, and inorganic fertilizers in diverse consortiums on the soil and concluded that combination of rice straw and green manure caused a significant increase in methane production, however, the addition of rice straw with inorganic fertilizers increases the availability of carbon in the soil thus improving productivity in the fields.

(Kim *et al.* 2014) also reported enhanced CH_4 production after the supplement of air-dried and composted manure, however using composted manure showed a 50% decrease in methane as contrasted to air-dried manure.

(Aslam Ali *et al.* 2019) employed cohesive treatment of silicate fertilizers, composted animal manures, and Azolla biofertilizer. They concluded that the combination of these could reduce methane emission, enhance soil fertility, and enhance production thus suggested a substitute against conventional urea fertilizers. (Zhang *et al.* 2016) studied the impact of biochar with or without N fertilizer on rice yield as well as methane emission and concluded that it significantly increased the rice yields but an increase in CH_4 was also noticed.

2.9. Paddy ecosystem and methane emission

The anthropogenic emission of GHG (e.g., CO_2 and CH_4) has led to an escalation in the mean earth's surface temperature and climate changes during the previous 50 years. Methane gas with greater radiative efficiency and Global Warming Potential (GWP) as contrasted to CO_2, makes it 20-30 times more efficient than CO_2 as a GHG (Bernstein *et al.* 2007). With a retention time of approximately 10 years, methane is removed from the atmosphere through destruction in the stratosphere, oxidation in the troposphere by OH^- and soil biological processes (Boucher *et al.* 2009). More methane means more absorption of heat energy than the reflection from the earth's surface in the form of infrared radiation which slows the rate of energy evasion to space. Methane being a reactive gas changes the troposphere's configuration upon its reaction with hydroxyl radicals, falling its oxidative power and capacity to eradicate pollutants (for example chloro-fluoro carbons (CFCs)), which paves the way to produce other GHGs (ozone, CO, CO_2). Such processes generate water vapors in the stratosphere which damages

ozone layer, thus permitting the solar radiations towards earth's surface. Methane is eliminated in the troposphere through oxidation by OH• radicals with the production of water vapors, according to the reaction:

$CH_4 + OH^{\bullet} \rightarrow CH_3^{\bullet} + H_2O$

Additionally, CH_4 reacts with CFC-originated chlorine in the stratosphere as follows: $CH_4 + Cl^{\bullet} \rightarrow HCl + CH_3^{\bullet}$. Another mechanism of CH_4 eradication occurs in aerobic zone of wetland and upland soils which is brought about by microbial oxidation. The concentration of methane has been increased to 162% from the past 250 years and escalating at a rate of 1% each year. Petroleum systems and natural gas, landfills, agricultural activities, wastewater treatment, coal mining, mobile and stationary combustion, and some industrial procedures are included in the anthropogenic sources of methane (Stocker *et al.* 2013) and comprise 63% (566 Tg CH_4/year) of CH_4 emissions globally. Wetlands (marshes, swamps, and bogs) are also the foremost source of methane gas in the environment, producing 60-70% emission of all-natural sources or 100 Tg CH_4/year. Unaltered natural soils are the sink of methane and they emit less amount of CO_2 and sequester more carbon, but due to anthropogenic sources and agriculture activities, they are mostly a source of methane (Christiansen *et al.* 2012).

2.10. Methane Metabolism by microbial communities

Methanotrophs and Methanogens are two different communities of microorganisms present in the wetland or paddy field soil and are engaged in the biogeochemical progression of CH_4 (Pazinato *et al.* 2010) by working antagonistically. To estimate and predict methane metabolism, numerous investigations have been made to have an enhanced understanding of the operational response of methanogenic and

methylotrophic communities in paddy soil (Conrad 2007, Shrestha *et al.* 2010, Watanabe *et al.* 2013, Lee *et al.* 2014a).

In strict anaerobic conditions, methanogens use acetate or CO_2 as an electron acceptor to generate CH_4, while aerobic soil is the only biological sink of methane oxidation by methanotrophic bacteria (S. R. Mohanty l., 2006) sequestering 6% methane in the upland dry soils (i.e., forest and grasslands) and 10-30% in the wetland soils globally (Le Mer and Roger 2001, Griggs and Noguer 2002). Methane emission highly depends on methane oxidation by methanotrophs, and several mechanisms indicate that the addition of nitrogenous fertilizers inhibits methane oxidation. For example, competitive inhibition of methane monooxygenase (MMO) through ammonia can be one of the inhibitory mechanisms of methanotrophy at a cellular level. Moreover, oxidation of ammonia by methanotrophs also produces intermediate and end products such as hydroxylamine and nitrite which have a noxious impact on methanotrophs (Whalen *et al.* 2000). On the contrary, many authors explained that the addition of ammonia or nitrate fertilizer can enhance the methane oxidation rate due to change in the composition of the methanotrophic bacterial community (De Visscher *et al.* 2001) or nutrient availability enhancement (Veldkamp and O'Brien 2000), others also argued that methanotrophs simply need a nitrogen source and it acts as a limiting factor for methane oxidizers. (Bodelier and Laanbroek 2004) gave the hypothetical model for methane oxidation, in which they explained that nitrogen act as a limiting factor for methanotrophy, and a decrease in nitrogen concentration can shift the cycle from methane oxidation to nitrogen fixation. Methanogens are also affected by the addition of fertilizers, for instance, under the anaerobic conditions, the presence of other electron acceptors (i.e., SO_4^{-2}, Fe^{+3}, NO^{-2}, NO^{-3}) is used by certain types of sulfate-reducing,

iron-reducing, and denitrifying bacteria which outcompete methanogens for uptake of hydrogen and acetate (Borrel *et al.* 2011) and ultimately reduce the methane emission from soil. Some compounds inhibit methanogenesis, such as sulfate rich anaerobic environments in which inhibition takes place due to competition with sulfate reducers for substrates like acetate and hydrogen (Muyzer and Stams 2008). The sulfide produced through anaerobiosis also have toxic effect on the methanogens (Paula and Foresti 2009). From the above mechanisms and many research studies, it is still unclear how any fertilizer can affect methanogenesis and methanotrophy, since it depends on many other factors such as soil texture, pH, redox potential, organic matter, availability of mineral nutrients, and other accompanied microorganisms as well. Moreover, by applying fertilizers in different combinations can have also different effect on these processes which still need to be investigated and is an area of extensive research.

2.11. Methanogenesis

Methanogenesis results in CH_4 and CO_2 emission during the anaerobic microbial breakdown of organic matter in natural ecosystems with depleted electron acceptors such as oxygen, sulfate, ferric iron, and nitrate (Reim *et al.* 2017, CONRAD 2020). This anaerobic microbial methane production (AMMP) demands microflora of hydrolytic, fermentative, acetogenic, and methanogenic nature, which is quite common in rice paddies, natural wetlands, lake sediments, landfills, anaerobic digesters, and in the gut of some animals. The whole process of methanogenesis can be divided into five steps:

1. Breakdown of complex organic matter to monomers and the fermentation of monomers to simpler compounds like alcohols, low-C fatty acids (acetate, formate), hydrogen, and CO_2

2. Transformation of acetate into CH_4 and CO_2
3. Transformation of hydrogen and carbon dioxide into CH_4
4. Syntrophic transformation of short-chain fatty acids and alcohols to acetate, CO_2, and H_2
5. Fermentation of monomers to acetate only (heterotrophic acetogenesis) or transformation of H_2 and CO_2 to acetate (chemo lithotrophic acetogenesis)

Steps 1, 4, and 5 are usually accomplished by bacterial communities, while steps 2 and 3 are archaeal dependents. Steps 2 and 3 represent acetoclastic and hydrogenotrophic methanogenesis, respectively. Acetoclastic methanogenesis contributed to about two-thirds of the total CH_4 emission and the relative contribution of these two types are critically important to design mechanistic models to predict CH_4 emissions in the future (Bridgham *et al.* 2013, Xu *et al.* 2015, Vavilin *et al.* 2017).

Although methanogens have been found and studied in different environments, they are investigated extensively in paddy soils due to convenience and accessibility (Yuan *et al.* 2018, Jin *et al.* 2020). These environments still offer a huge research gap regarding microbial community structure and physiology under different stresses. *Methanobacterium, Methanobrevibacter, Methanosarcina, Methanoculleus, Methanogenium, Methanosaeta*, and *Methanospirillum* are the ecologically important and abundant methanogenic genera in paddy and other water logged soils (Daebeler *et al.* 2013). Various genera of methanogens are known to respond differently against a particular stress factor. Rice varietal variation with respect to methanogenesis has also been demonstrated (Le Mer and Roger 2001). Several factors have been reported to cause fluctuation in methanogens community composition and diversity such as gas diffusion, soil water content, nature of soils, soil physicochemical properties,

fertilization, substrate availability and, competition with de-nitrifiers and sulfate reducers (Zhang *et al.* 2007, Fazli *et al.* 2013).

2.12. Methanotrophy

The low-affinity oxidation of CH_4 in methanogenic environments (paddy soil, peatlands, landfills, etc.) is performed by methanotrophic bacteria called methanotrophs that occur in the aerobic layer of soil such as rhizosphere, inside roots, and submerged leaves. Syntrophic consortium of archaea and sulfate-reducing bacteria is responsible for anaerobic methane oxidation (Lee *et al.* 2014b). All methanotrophic bacteria are classified as type I or type II, depending upon the path they follow i.e., ribulose monophosphate cycle and serine pathway respectively (Rosenzweig and Ragsdale 2011). There is a disagreement in the literature regarding the third type of methanotroph, it is either reported as type X or subtype of type I (Hanson and Hanson 1996, Dubey 2005, Semrau *et al.* 2010). The CH_4 oxidation rate is multifactorial (crop cycle, temperature, CH_4 concentration, soil moisture content, plant biomass, geographic location, fertilizers and, soil flooding, etc.) can vary as broadly as 0-90% (Fey and Conrad 2000, Yue *et al.* 2007, Shrestha *et al.* 2010, Watanabe *et al.* 2013). Saturated soils are predominated by type II methanotrophs which can produce soluble methane monooxygenase and can oxidize as much as 80% of CH_4 diffused in the upper soil layer (Le Mer and Roger 2001). Inconsistent opinions are repeatedly reported in the literature about the impact of a particular rice variety on methanotrophy (Wu *et al.* 2009b, Win *et al.* 2012).

2.13. Interactions between methanogens and methanotrophs

The dynamic interaction exists between community composition, diversity and, the relative composition of methanogens and methanotrophs in a rice paddy. The factors responsible for their community ecology are the same as those controlling methanogens and methanotrophy. Their dynamic relative abundance can be positively or negatively correlated and integrated investigations involving environmental factors, methanogens, and methanotrophs are perplexing but urged, to develop a sustainable agriculture practice.

2.14. Methods to study soil microbial communities

While assessing microbial community ecology in different environments, microorganisms are assigned to different taxa, and the whole community is distributed among those taxa and characterized as functional groups (Liu *et al.* 2006). Traditionally, microbial community ecology studies are accomplished by subjecting the whole community to the stress of variable nature such as pH, temperature, and nutrients, etc. depending upon the study conducted. However, a major challenge existed for decades is the choice of optimal method to study microbial community ecology. The methodologies to measure microbial community ecology belong to either of the two categories i.e., 1) Biochemical based, such as plate count (Tabacchioni *et al.* 2000), community-level physiological profiles (CLPP), FAME, and PLFA analyses (Zelles 1999, Frostegård *et al.* 2011, Chowdhury and Dick 2012) or 2) Molecular based, such as nucleic acid hybridization and fluorescent in situ hybridization (FISH) (Thurnheer *et al.* 2004), guanine plus cytosine (G + C), and PCR based techniques. The current study used two of these advanced and very effective approaches to study community ecology i.e., PLFA and PCR-based amplification of highly conserved 16S rRNA followed by HTS. Nevertheless, PLFA profiling cannot contend with the rRNA gene-

sequencing regarding phylogenetic resolution since the latter results in better characterization of communities of diverse environments but doesn't provide enough evidence on the phenotype and the activity of the microorganisms under stress (Frostegård *et al.* 2011). 16S rRNA gene metabarcoding is well ahead of PLFA where the emphasis of the study is on the particulars of microbial ecology. The comparative aptitude of PLFA profiling and 16S rRNA gene metabarcoding procedures to answer several wider questions often asked by microbial ecologists, and whether outcomes can be directly associated, is experimental.

2.15. PLFA based community structure

Soil fatty acids are sensitive and specific indicators of microbial communities. Soil microbial community composition and diversity had been measured since the early 90's using PLFA profiling, which employs "signature" fatty acids associated with a specific group of microorganisms. PLFA method is not only rapid and inexpensive but also provides us a broad-scale quantitative overview of community composition, microbial biomass estimation, and shift in community structure over time and stress (Grayston and Prescott 2005, Willers *et al.* 2015). The actual method is primarily based on Blight and Dyer phospholipid extraction procedure, certain modifications have been made over the years to improve its sensitivity and specificity (BLIGH and DYER 1959, Frostegård *et al.* 2011). One of the key drawbacks of this method is the overlapping of some fatty acid biomarkers to specify a particular group of microorganisms and caution must be taken in structuring the community. For example, cy17:0 and cy19:0 biomarkers, which are misinterpreted as markers of gram-negative bacteria are also detected in few gram-positive bacteria (Schoug *et al.* 2008). Similarly, 18:2ω6,9 and 18:1ω9 are although good indicators of fungi but found in some other eukaryotes

including plants (Kaiser *et al.* 2010). PLFA biomarker, 16:1ω5, is an indicator of arbuscular mycorrhiza but is also found in bacteria. Despite these cautious and overlapping biomarkers, PLFA is comprehensive enough to study community ecology in different environments like landfills, fertilized soils (Pan *et al.* 2016, Kuppusamy *et al.* 2018, Zhang, Zheng, *et al.* 2019), anaerobic digester, aquifers (Green and Scow 2000), and compost (Steger *et al.* 2003). Table 2.1 represent different signature phospholipid fatty acid biomarkers present in different groups of organisms.

2.16. PCR amplification and HTS

With advancements in genomic analysis, broadening of reference databases, and more efficient HTS analysis, it is possible to describe the deeper taxonomic structure and phylogenetic diversity of microbial communities in different ecosystems. The microbial communities are examined using nucleic acid extraction, amplification of the V3-V4 hypervariable region of 16S rRNA, followed by subsequent sequencing. The primer pair 515F/806R, from Earth Microbiome Project, is well documented for 16S rRNA metagenomic library preparation from Illumina MiSeq (Caporaso *et al.* 2018). One of the few matrices used by PLFA and DNA barcoding is diversity, which is a function of evenness in PLFA while evenness and taxon richness in 16S rRNA gene metabarcoding (Orwin *et al.* 2018). While analyzing 16S rRNA gene metabarcoding information, matrices like proteobacteria: acidobacteria ratio (nutrient status), oligotrophic: copiotrophic ratio (comparative abundance at phylum and class level), and gram-positive: gram-negative ratio can well explain the cause of a shift in microbial communities (Collins *et al.* 2016, Orwin *et al.* 2018). 16S rRNA metabarcoding methodology along with advanced analysis like PICRUSt si summarized in Figure 2.1.

Table 2.1: Signature fatty acid biomarkers used as an indicator of specific microorganisms.

Fatty acid type & PLFA biomarker	***Indicator***	***Recent and actual citation***
Straight-chain saturated		(Zosso and Wiesenberg 2021)
14:0; 15:0; 16:0; 17:0; 18:0	General bacteria	(Zelles 1997)
Methyl-branched		(Zhang, Sun, Li, *et al.* 2019)
10Me16:0; 10Me17:0; 10Me18:0	Actinomycetes (actinobacteria)	(Kroppenstedt 1985) (Vestal and White 1989)
Monounsaturated		(Yan *et al.* 2018)
14:1ω5c; 15:1; 15:1ω6c 16:1ω7t; 16:1ω9c; 16:1ω11c; 17:1; 18:1ω5c; 19:1ω9c; 20:1ω9c; 19:1ω12c; 20:1ω9t; 22:1ω9c; 22:1ω9t	Gram-negative bacteria	(Wilkinson 1988) (Zelles 1997)
16:1ω5c	Arbuscular mycorrhizal fungi	(Pacoysky and Fuller 1988)
16:1ω7c	Gram-negative bacteria	(Wilkinson 1988)
	Cyanobacteria; diatoms	(Ahlgren *et al.* 1992)
16:1ω5t; 16:1ω8c	Type I methanotrophs	(Nichols *et al.* 1985)
17:1ω8; 17:1ω5	Sulphate-reducing bacteria	(Kaneda 1991)
18:1ω7c	Cyanobacteria; diatoms	(Ahlgren *et al.* 1992)
	Gram-negative bacteria	(Wilkinson 1988)
18:1ω7t	Gram-negative bacteria	(Zelles 1997)
18:1ω8c	Type II methanotrophs	(Nichols *et al.* 1985)
18:1ω9c	Cyanobacteria; green algae	(Ahlgren *et al.* 1992)
	Fungi	(Vestal and White 1989)
Hydroxy substituted		(Ferrari *et al.* 2018)
2OH 12:0; 3OH 12:0; 2OH 14:0; 3OH 14:0; 2OH 16:0; 2OH 18:0	Gram-negative bacteria	(Parker *et al.* 1982)
Cyclopropyl saturated		(White *et al.* 2020)
cy17:0; cy19:0	Gram-negative bacteria	(Wilkinson 1988)
Terminally branched		(Maarastawi *et al.* 2019)
a13:0; i13:0; i14:0; i15:0; a15:0 i16:0; a17:0; i17:0; a18:0; i18:0	Gram-positive bacteria	(O'Leary, W.M. and Wilkinson 1988)
Polyunsaturated		(Wang *et al.* 2019)
18:2ω9c	Saprotrophic fungi	(W. 1986)
18:3ω3	Fungi	(Zelles 1997)
18:2ω6c; 18:3ω6c	Saprotrophic fungi	(W. 1986)
16:2ω4; 16:2ω6; 16:2ω7; 16:3ω3; 16:3ω4; 16:4ω3; 16:4ω1; 18:4ω3; 18:5ω3; 20:4ω6; 20:5ω3; 22:5ω3; 22:6ω3	Cyanobacteria; diatoms; green algae	(Volkman *et al.* 1989, Ahlgren *et al.* 1992)

2.17. PICRUSt

One of the few advantages of employing amplicon-based next-generation 16S rRNA sequencing is the use of bioinformatics tools to predict the functional attributes of microbial communities (Sansupa *et al.* 2021). Few of such tools are operational annotation of prokaryotic taxa (FAPROTAX) (Louca *et al.* 2016), Tax4Fun (Aßhauer *et al.* 2015), and PICRUSt (Langille *et al.* 2013, Douglas *et al.* 2019). PICRUSt approach, with the most citations, utilizes rRNA sequencing data and reference databases (KEGG and COG) to predict ecological functions of the microbiome and thus serve as a powerful predictive and hypothesis-generating tool. The PICRUSt methodology is shown in Figure 2.2.

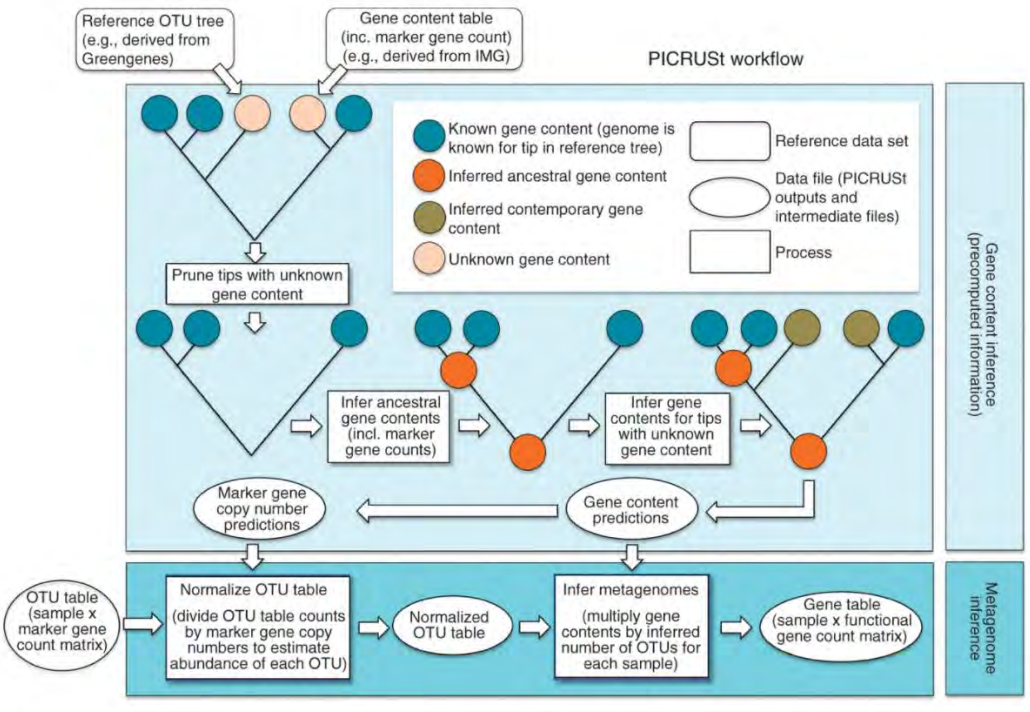

Figure 2.2: PICRUSt Methodology (Langille *et al.* 2013).

2.18. Methane Oxidation Potential

Mitigation of methane emissions from flooded soils has been a point of concern and seeks more studies to attain methane mitigation techniques. One of the key factors to focus on is measuring the capability of methane-oxidizing bacteria (MOB) to transform methane into CO_2. MOBs, also known as methanotrophs are differentiated from methylotrophs in utilizing CH_4 and C1 compounds respectively as a sole carbon and energy resource (Semrau *et al.* 2010). The oxidation of methane in MOBs is brought about by the methane monooxygenase enzyme. The methane oxidation by MOB is regulated by environmental variables such as pH, nutrient availability, temperature, and soil moisture content. However, N is considered to be the regulatory element of methane oxidation (Bodelier and Laanbroek 2004). Incubation studies employing diverse environmental samples such as wetland and forest soil (Reddy *et al.* 2019), landfill soil (Park *et al.* 2005), and boreal soil (Whalen and Reeburgh 1996) have reported methanotrophs as mesophilic with their maximum oxidation rates within a temperature range of 25-35 ^{o}C (Sadasivam and Reddy 2014). In paddy soils, both varieties of methanotrophs (type I and type II) have been identified and their population tends to be many folds greater in the rhizosphere region contrasted to bulk soil (Frenzel 2000, Dubey *et al.* 2014). Previously, studies have been conducted to estimate PMO of methanotrophs in NPK fertilized microcosms (Mohanty *et al.* 2007, Jugnia *et al.* 2012, Shrestha *et al.* 2012) and the oxidation rate measurements were made.

2.19. Methane Production Potential

An alternative approach to mitigate methane emission from submerged soil is to overlook methanogenesis carried out by methanogens. The contribution of rice paddies

among anthropogenic methane sources is somewhere around 11-19% of the global methane emission (Kim *et al.* 2018, Yuan *et al.* 2020). The vital aspect of methanogenesis is the anaerobic decomposition of organic matter by methanogens, thus shifting the dynamics of soil organic matter content and methane emission. In rice paddy, the addition of organic fertilizer plays a crucial part in soil fertility and is usually recommended to minimize fertilizer supplementation to achieve sustainable agriculture. However, the addition of such organic supplementation (cattle manure, rice straw, etc.) provides a readily available C source in flooded ecosystems. In contrast, organic compost is known to deliver promising mitigation of methane emission in the paddy field by several studies (Pramanik and Kim 2014, Ho *et al.* 2015, Kim *et al.* 2018). The impact of rice straw, which is a readily available C source, in methane emission is however misleading since it is reported to induce specific methanogens (*Methanosarcinaceae*) while suppressing others at the same time (rice cluster I methanogens) (Conrad and Klose 2006). In wetland rice paddies the impact of soil organic C is yet to be determined since the methane production potential is yet known to increase, decrease or remain unaffected (Conrad 2020). Thus it demands more investigations to study the methane production potentials at microcosms and mesocosm levels under different stress indicators by the easier incubation methods (Singh *et al.* 2012).

2.20. Biological activities in paddy soil

Extracellular microbial enzymes have been known as a very delicate indicator of a variety of ecosystem functions as decomposition, fertilization, crop rotations, eutrophication, and heavy metal pollution (Dick 2015). Additionally, they are fairly straightforward to determine, have microbial biological significance, are receptive to

environmental strain, and respond promptly to fluctuations in land management (Yakovchenko *et al.* 1996, Dick *et al.* 2015a). With the advancements in soil enzymology, great interest has been developed in the use of extracellular enzymes as biological indicators of soil quality as well as particular reactions of the overall microbial community (Frankenberger and Dick 1983). Both long-term N addition and temperature have a profound effect on extracellular enzyme activity. To monitor alteration in soil quality in terms of soil organic matter, β-Glucosidase activity can be considered in recycling soil organic matter content. In addition to β-Glucosidase, another enzyme involved in polysaccharide turnover is N-Acetyl Glucosaminidase. Moreover, the dynamics of P transformation are still unknown under the influence of soil density, pH values, and organic C and N. Acid phosphatases catalyze the mineralization of organic phosphorous which is commonly found in surface soils. It is predicted that N fertilization may have a significant effect on acid phosphatases and urease.

CHAPTER 3: VARIATION IN ARCHAEAL AND BACTERIAL COMMUNITY PROFILES AND THEIR FUNCTIONAL METABOLIC PREDICTIONS UNDER THE INFLUENCE OF PURE AND MIXED FERTILIZERS IN PADDY SOIL

3. 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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3.1. 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.

Keywords: NPK fertilizers, Paddy soil, microcosm, High throughput sequencing, PICRUSt, Methane metabolism

Declaration of Interest

None

CRediT author statement

Mohsin Gulzar Barq: Methodology, Writing- Original draft preparation, Software. Muhammad Mubashar Hassan: Methodology, Investigation. Humaira Yasmeen: Validation. Asim Shahzad: Validation. Noshaba Hassan Malik: Formal analysis. Nicola Lorenz: Resources, Data Curation. Abdulaziz Abdullah Alsahli: Visualization. Richard Dick: Resources, Supervision. Naeem Ali: Conceptualization, Supervision.

3.2. 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 (Wu *et al.* 2011, Wang *et al.* 2017). 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 (Pan *et al.* 2016, Kuppusamy *et al.* 2018, Fang *et al.* 2021). Though 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 (Zhang *et al.* 2011, Dubey *et al.* 2014) 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, Zhong and Cai 2007, Ramirez *et al.* 2012, Eo and Park 2016). 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, Liu, *et al.* 2019) 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).

3.3. Materials and Methods

3.3.1. 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 ºC till further experimentation. Aseptic conditions were maintained wherever necessary. The soil had a pH of 8.05, total nitrogen (TN) 0.17%, and total carbon (TC) 1.40%.

3.3.2. 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 3cm 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 3.1).

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 ^{o}C in a greenhouse facility through the 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 ^{o}C till further analysis.

Table 3.1: 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

3.3.3. 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 ºC 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).

3.3.4. 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 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.

3.3.5. 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.

3.3.6. 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 16S rRNA reads from amplicon with 97% similarity with the taxonomy of resulting Operational Taxonomic Units (OTUs) (Bolyen *et al.* 2019, Jiang, Adebayo, *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).

3.3.7. 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).

3.3.8. 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 (Bolyen *et al.* 2019) 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.

3.4. Results

3.4.1. Physicochemical properties of soil

Soil pH varied between 7.68-8.28 in four fertilizer regimes, and it varied significantly with different combinations of amendments (Table 3.2). Soil EC significantly increased in all treatments compared to control and varied between 275-645 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 Figure 3.1. 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 influencing most of the samples. The biplot showed a strong correlation between TC and TN; TEP, OM, EC, and AK.

Table 3.2: Physicochemical properties of soil against all treatments. Values represent means (n = 3) with SE. Different lowercase letters represent difference at p < 0.05. EC = electrical conductivity, TC = total carbon, TN = total nitrogen, OM = organic matter, AK = available potassium, TEP = total extractable P, 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 (dS/cm)***	***TN (%)***	***TC (%)***	***OM (%)***	***AK (mg/kg)***	***TEP (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±0.00a	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±0.07g	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±0.03h	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±5.55g	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±0.10k	9.83±0.37fg	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±0.20g	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±0.29h	0.36±0.03a

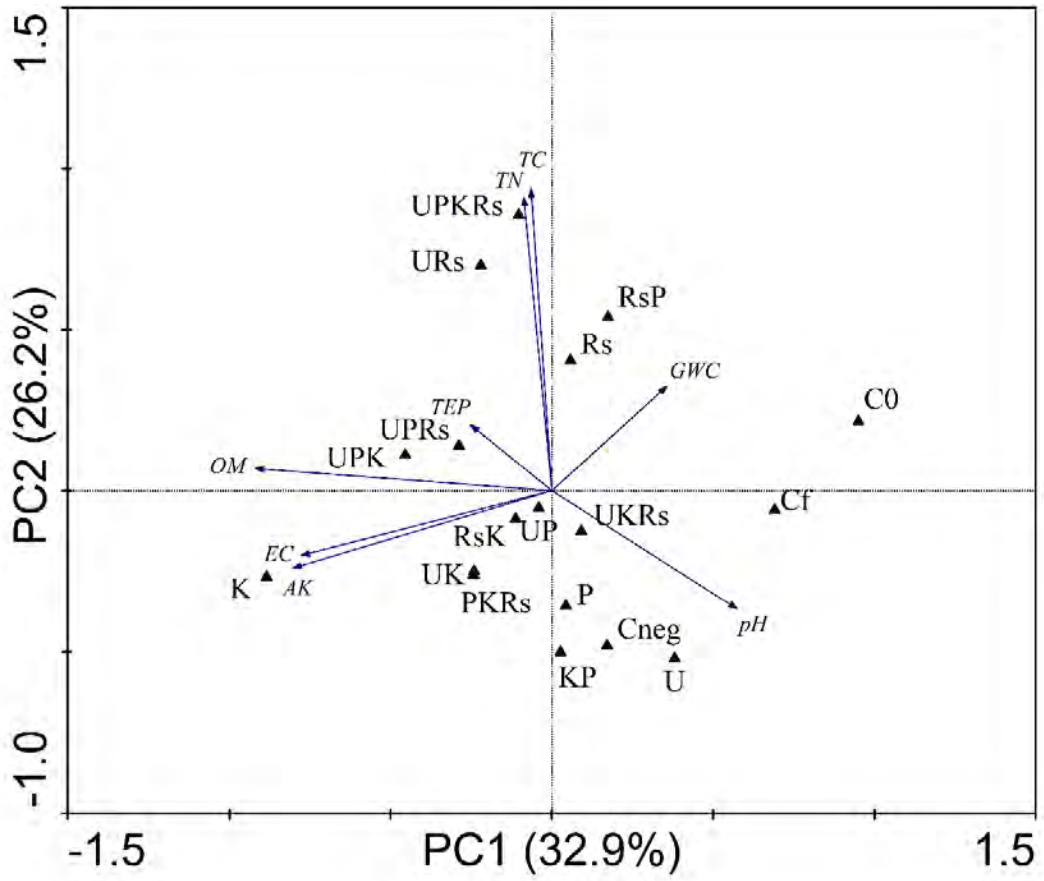

Figure 3.1: Principal component analysis (PCA) showing correlation biplot of first two PCs between explanatory soil treatments and loadings (blue lines). Total carbon (TC), total nitrogen (TN), gravimetric water content (GWC), electrical conductivity (EC), organic matter (OM), available potassium (AK), and total extractable phosphorus (TEP).

3.4.2. 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 bacterial phyla and genera is shown (Figure 3.2 and 3.3). *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 93-97, 1-6, and 0.2-0.9% respectively (Figure 3.4 and 3.5). The dominant archaeal genera gave ~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* 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.

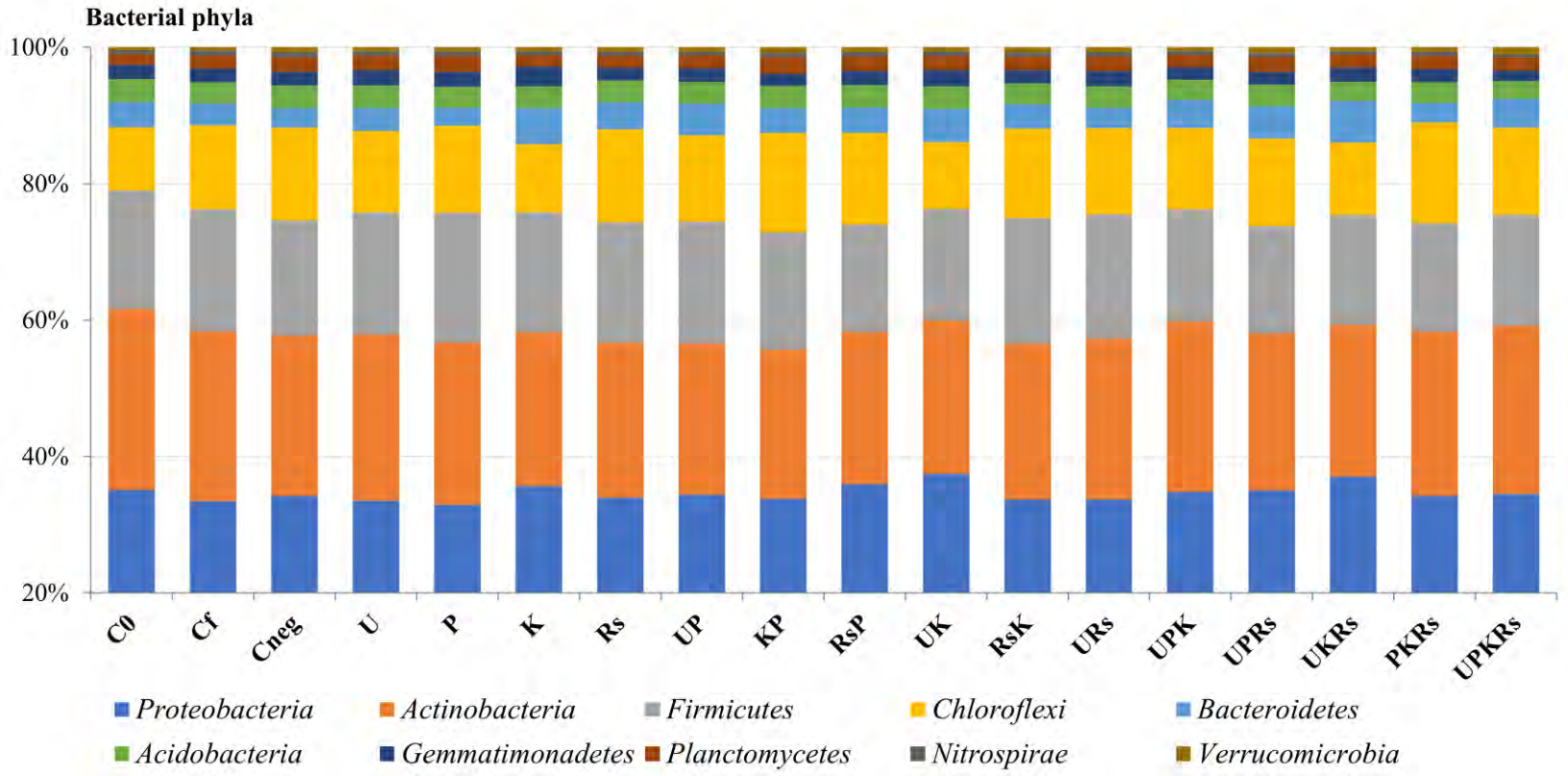

Figure 3.2: ***Relative abundance of major bacterial phyla that accounts for ≈ 99% of the bacterial community in all*** *treatments. Symbols for different treatments are shown in Table 3.1.*

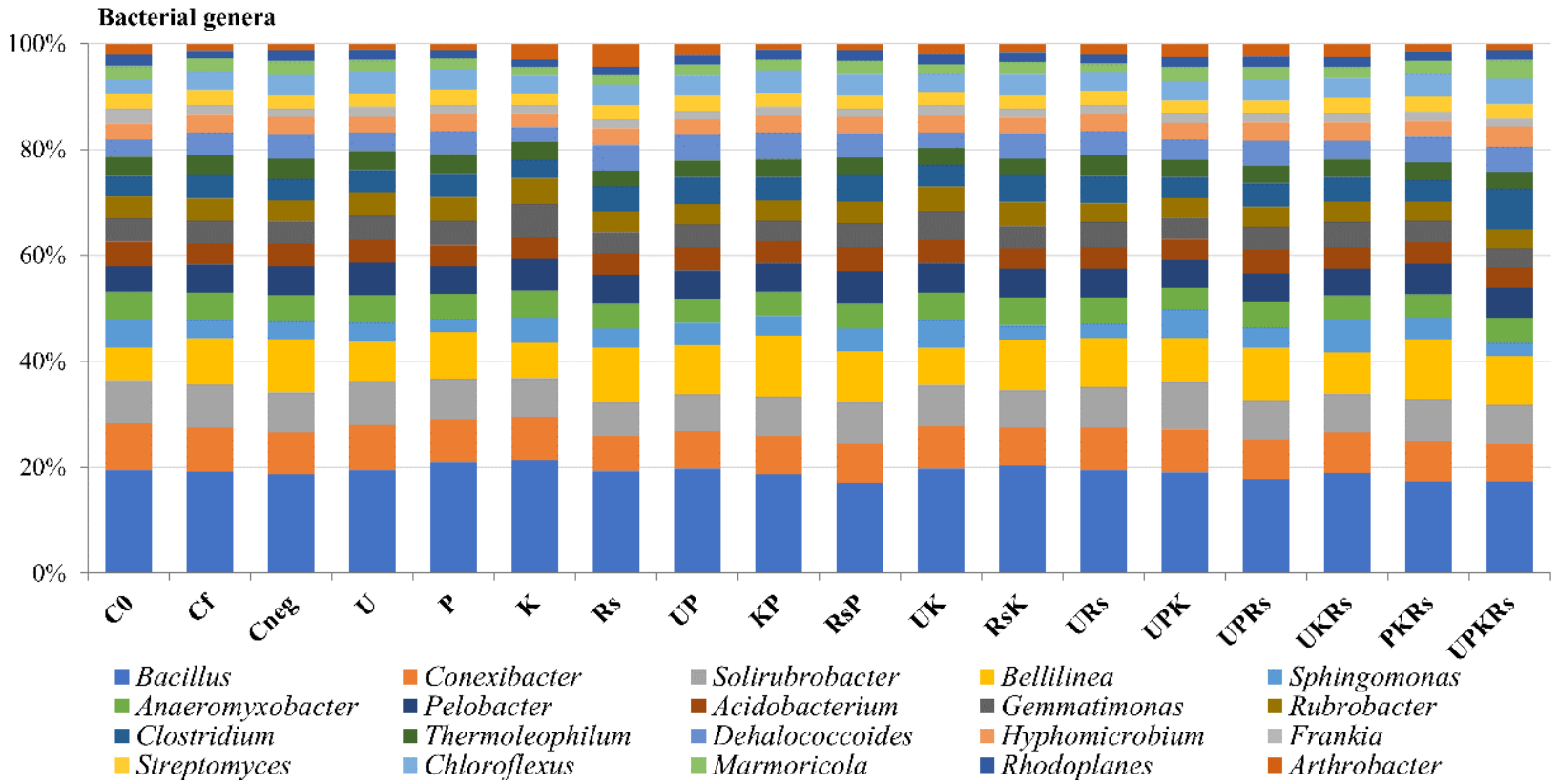

Figure 3.3: ***Relative abundance of major bacterial genera that accounts for ≈ 99% of the bacterial community in all treatments.***

Symbols for different treatments are shown in Table 3.1.

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 (Figure 3.6). 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 compositions explained 23.3% and 14.3% variance for RD1 and RD2 axes, respectively (Figure 3.7). The biplot showed the highest 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 significant correlation with the first axis.

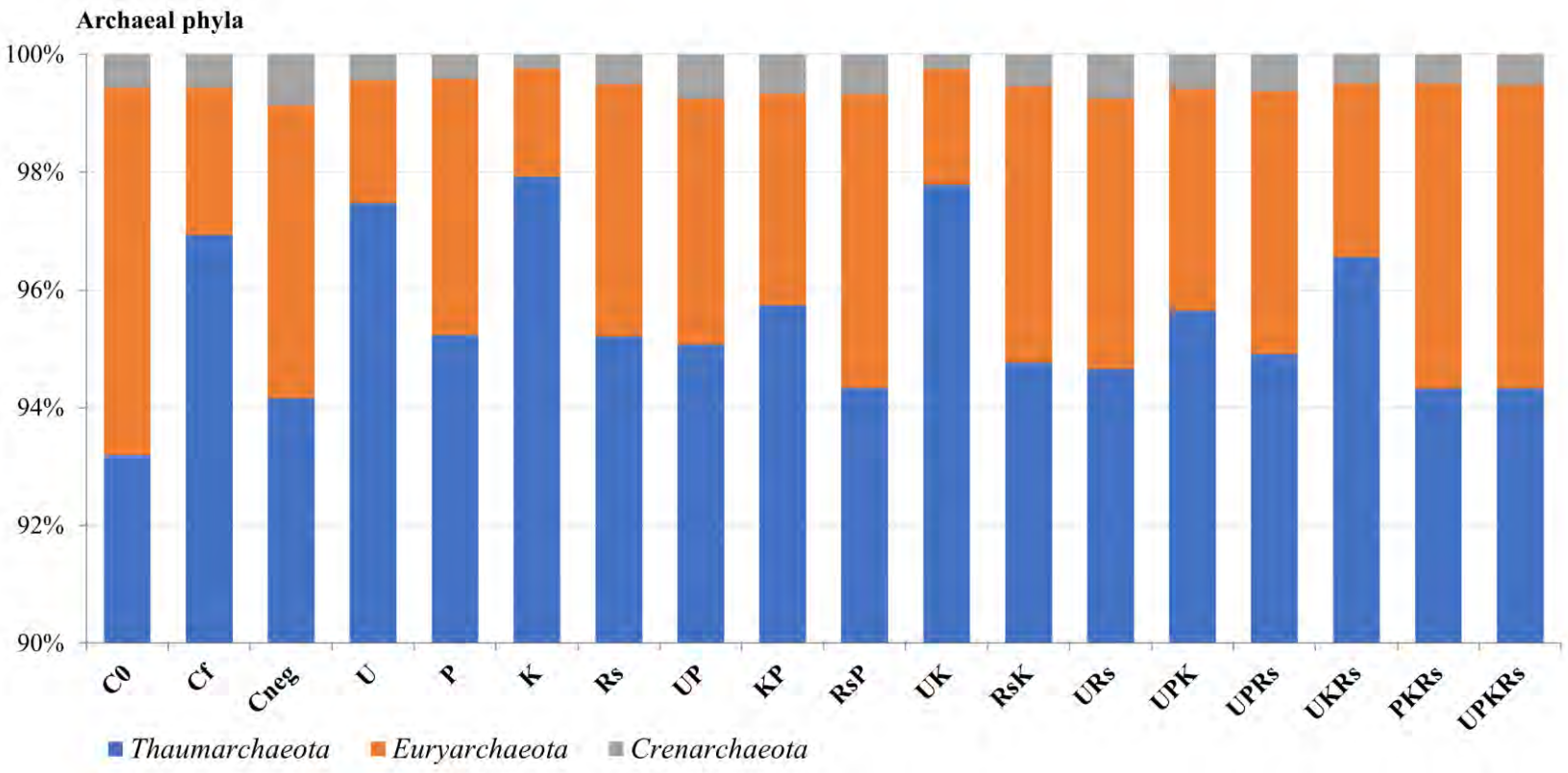

Figure 3.4: Relative abundance of archaeal phyla in all treatments. Symbols for different treatments are shown in Table 3.1.

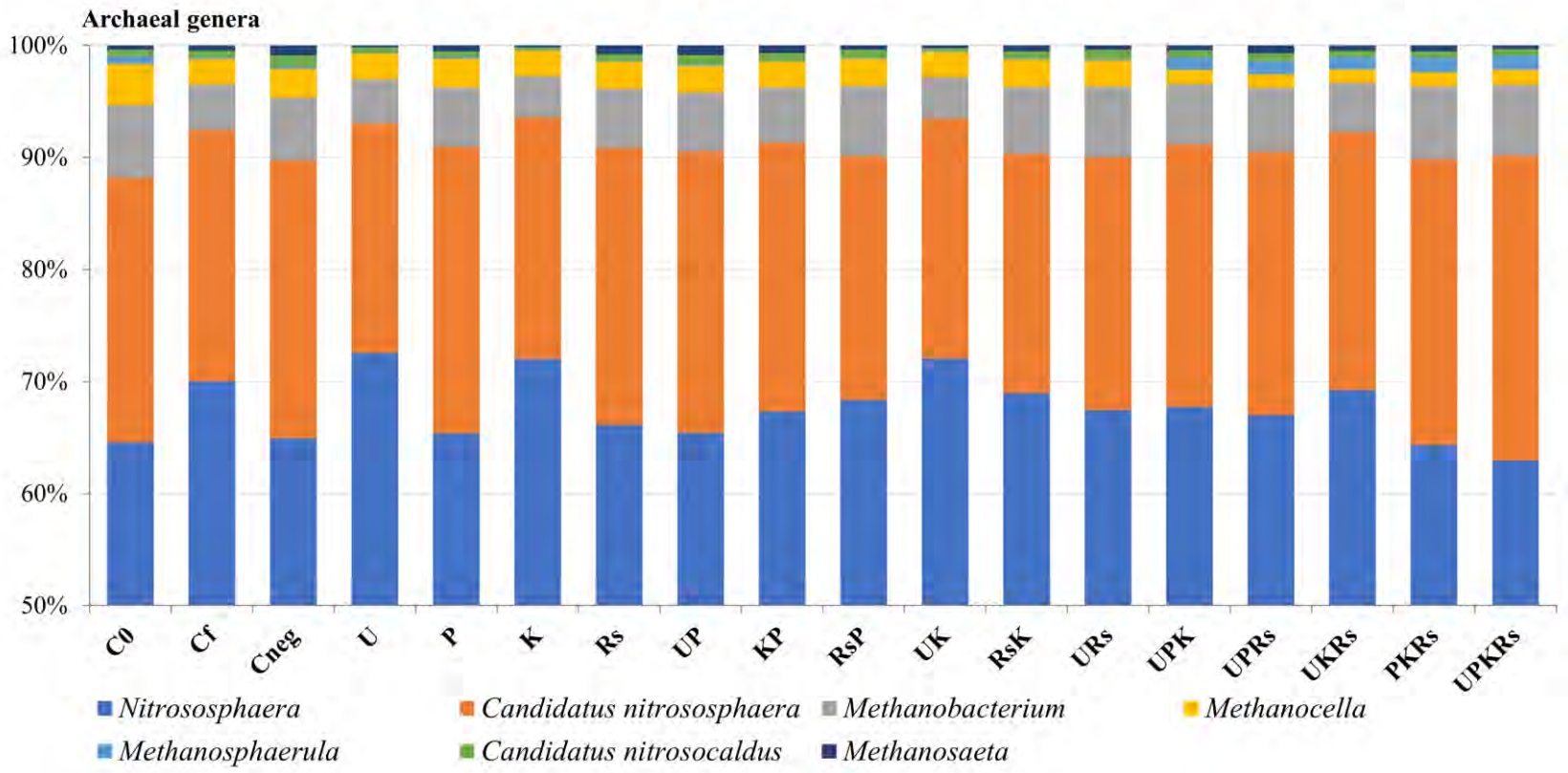

Figure 3.5: Relative abundance of archaeal genera in all treatments. Symbols for different treatments are shown in Table 3.1.

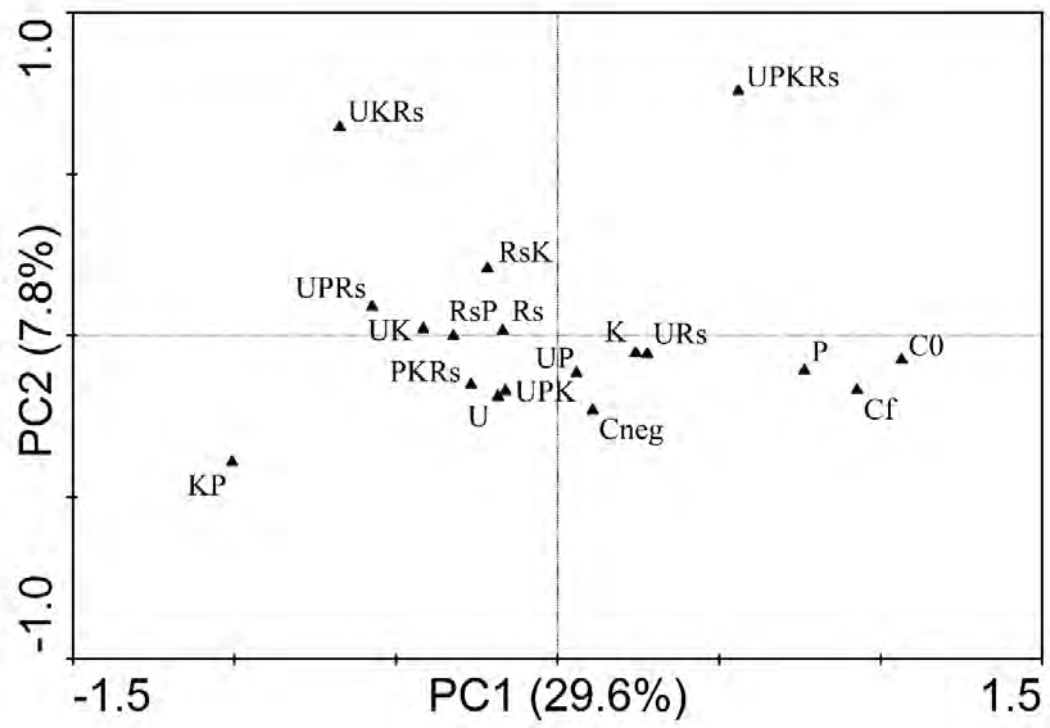

Figure 3.6: Principal component analysis (PCA) showing a plot of the first two PCs for all amplified 16S rRNA amplicons targeting archaeal and bacterial communities. The symbols for each treatment are explained in Table 3.1.

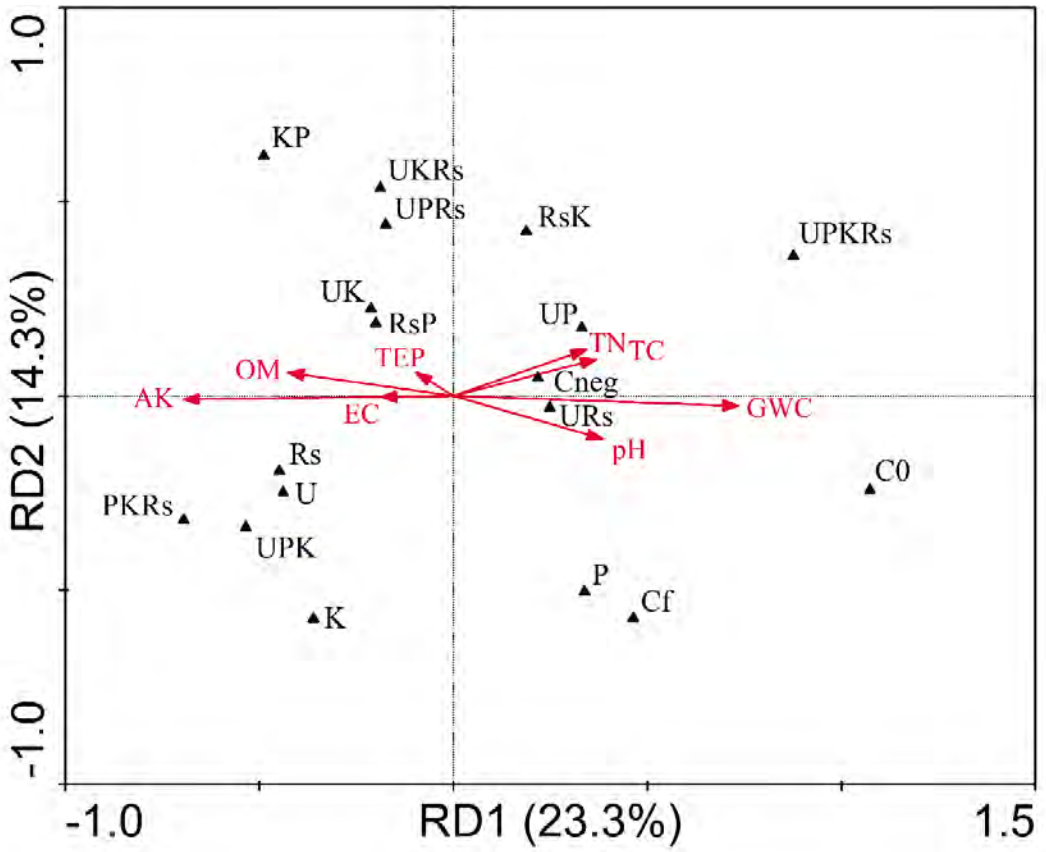

Figure 3.7: Biplot of redundancy analysis (RDA) showing community succession in all samples and their relation to environmental variables i.e., pH, Total carbon (TC), total nitrogen (TN), gravimetric water content (GWC), electrical conductivity (EC), organic matter (OM), available potassium (AK) and total extractable phosphorus (TEP).

3.4.3. 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 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 3.3). Other α-diversity indices i.e., Pielou's E and Shannon's are shown in the appendix (A1).

Table 3.3: ***Observed OTUs and Faith's PD index for all treatments.*** *OTU = Operational Taxonomic Units, PD = phylogenetic diversity. Symbols for different treatments are explained in Table 3.1.*

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

3.4.4. 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 Figure 3.8. 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 can explain a functional category. The heat map in Figure 3.9 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%).

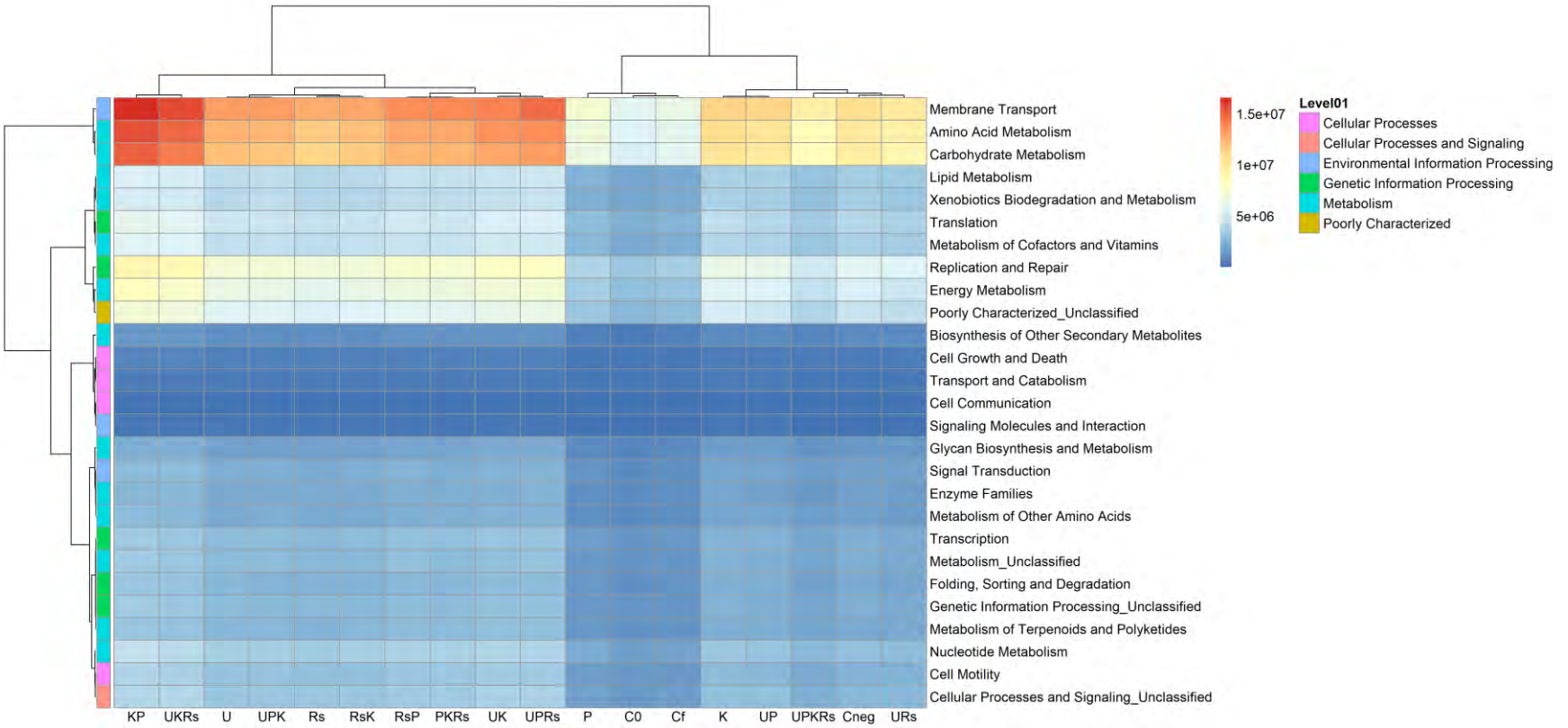

Figure 3.8: Heat map demonstrating PICRUSt derived hierarchical clustering of predicted functional profiles (un-scaled data) at a second level (Metabolism). Row annotations represent predicted functions at level one. KEGG database was used to predict functions at the second and third levels in PICRUSt (http://picrust.github.io/picrust/).

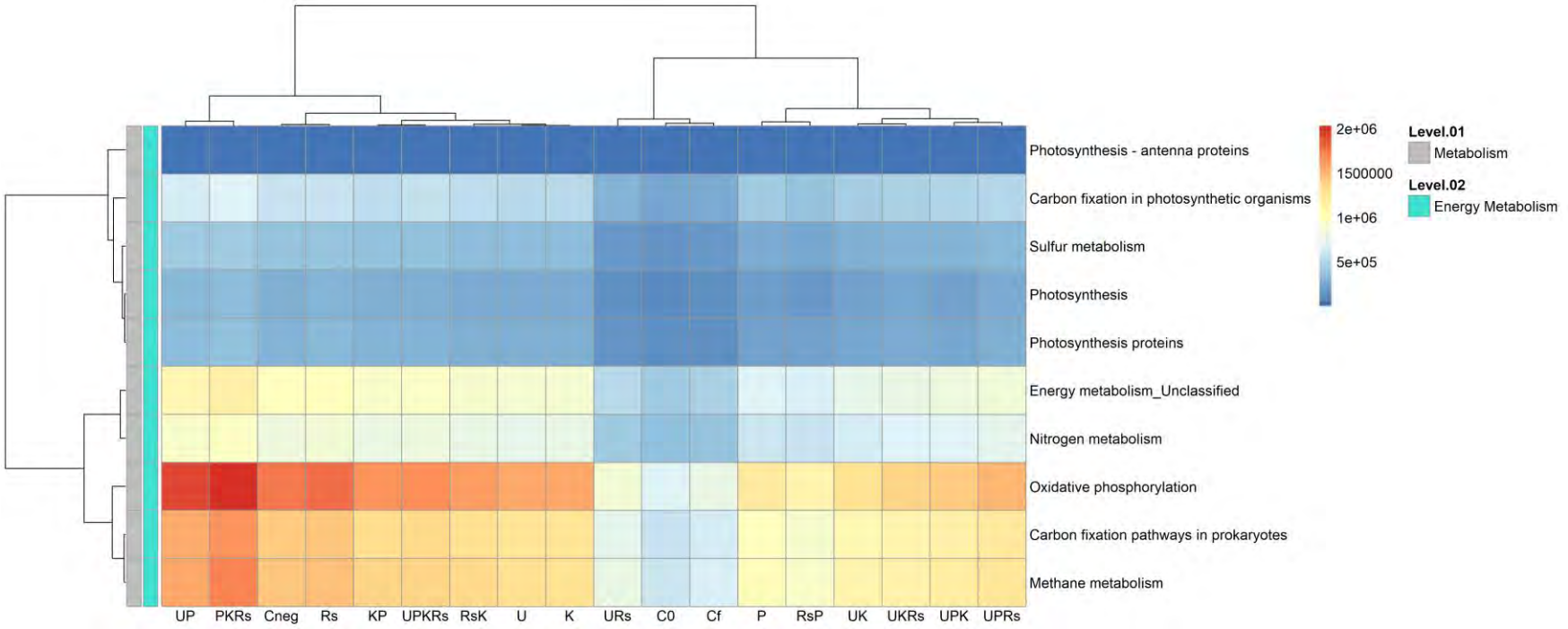

Figure 3.9: Heat map demonstrating PICRUSt derived hierarchical clustering of predicted functional profiles (un-scaled data) at a third level (energy metabolism). KEGG database was used to predict functions in PICRUSt (http://picrust.github.io/picrust/).

3.5. 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 (Hoshino *et al.* 2011). 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 (Figure 3.1) 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, Wang, *et al.* 2019). Concerning different treatments, a strong association in microbial communities between KP, PKRs, and UK was observed, while the rest of the sample showed distinctions 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.* 2014a). 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 are also well documented (Itoh *et al.* 2013, Breidenbach and Conrad 2014, Wang *et al.* 2017, 2018) 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, Liu, *et al.* 2019). 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, Rath *et al.* 1999, Conrad 2002). 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 (Nemergut *et al.* 2008, Wu *et al.* 2011, Cederlund *et al.* 2014) 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 (Figure 3.7). 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 (Peng *et al.* 2008, Breidenbach and Conrad 2014). 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 (Watanabe *et al.* 2006, Wang *et al.* 2010, Borrel *et al.* 2011). There are controversies in the literature suggesting N-fertilization can stimulate (Banik *et al.* 1996, Shang *et al.* 2011) or inhibit (Xie *et al.* 2010, Dong *et al.* 2011) 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 (Hartman *et al.* 2017, Kang and LeBrun 2018, Hu *et al.* 2019, Chen, Tian, *et al.* 2020). 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.

3.6. 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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CHAPTER 4: ENZYME ACTIVITIES AND PLFA BASED MICROBIAL COMMUNITY STRUCTURE OF WATERLOGGED PADDY SOIL UNDER THE INFLUENCE OF SINGLE AND MIXED NPK FERTILIZERS

4. Enzyme activities and PLFA based microbial community structure of waterlogged paddy soil under the influence of single and mixed NPK fertilizers

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4.1. Abstract

Fertilization in the paddy ecosystem is diverse and their influence on soil physicochemical characteristics, microbial community structure, enzymatic efficacy, and plant growth is quite challenging. Flooded microcosms were developed using $CO(NH_2)_2$, KH_2PO_4, KCl, and rice straw (Rs) and planted with 26 days old nurseries of *Oryza sativa.* The microbial community structure and stress were accessed employing phospholipid fatty acid (PLFA) biomarkers. Activities of acid phosphatase (AP), urease, and carbohydrate hydrolases i.e. β-glucosidase (GLU) and N-acetyl-β-glucosaminidase (NAG), and plant biomass were estimated. Significant increase in PLFA profiles in all treatments was observed as compared to control. Abundant PLFAs accounted for bacteria while the fungal community remained comparatively more stable. Significantly enhanced activity of GLU was observed in a majority of treatments compared to acid AP and NAG. The principal component analysis (PCA) signified microcosms with mixed combinations of fertilizer and controls as the most crucial and variable regarding community structure. Redundancy analysis (RDA) concluded gravimetric water content (GWC), total nitrogen (TN), total extractable phosphorus (TEP), and GLU as key factors in shaping microbial community structure. The network analysis also presented the overall interactions of all environmental variables against treatments. Our results inferred more disparity in the bacterial community than fungal. The effect of integrated fertilization especially in UPK was more promising regarding community ecology and plant biomass.

Keywords: NPK fertilizers, Microbial community ecology, Paddy soil, Phospholipid fatty acid analysis (PLFA), Enzyme activities.

4.2. Introduction

Soil microbial communities in waterlogged conditions have become a vital interest of microbial ecologists in recent decades as to ecosystem functioning and stability. Paddy field soil is distinct from upland soils being habitually in waterlogged conditions and is an important source and sink of methane metabolism (Cheng *et al.* 2000, Kikuchi *et al.* 2007). Rice is the second most-produced staple crop supporting approximately 50% of the earth's population and covering over 163 million ha cropland which accounted for 11% of the global arable land (Maraseni *et al.* 2018, Aslam Ali *et al.* 2019). A key factor that affects crop yield, soil quality, and variation in microbial community ecology of paddy soil is the type and concentration of fertilizer applied (Greenberg *et al.* 2017). Evaluating the effects of fertilizers in various combinations on soil microbial communities is imperative due to their active involvement in stimulating or inhibiting biogeochemical processes that enable ecosystem functioning (Barq *et al.* 2021). The microbial community impacts plant growth and soil quality through its involvement in residue decomposition, suppression of disease, production of phytochromes, nutrient cycling, and long-term soil sustainability (Wu *et al.* 2011, Berendsen *et al.* 2012, Newman *et al.* 2016). Various fertilizers with different concentrations are being used globally and the establishment of their role in the soil is quite challenging. Variations in community ecology and microbial biomass after subsequent inorganic amendments in upland and lowland soils are well reported (Wasaki *et al.* 2005, Burke *et al.* 2006, Esperschütz *et al.* 2007, Parrent and Vilgalys 2007). In addition to chemical fertilizers, the application of rice straw has been extensively investigated to enhance soil fertility and nitrogen uptake by plants (Takahashi *et al.* 2003). Its application has been found to increase methane emission by methanogens and the succession of bacteria responsible

for straw decomposition (Jia *et al.* 2007, Rui *et al.* 2009). The dynamics of nitrogen, phosphorus, potassium (NPK), and carbon (C) in soil are closely correlated and primarily dependent on temperature, moisture, soil-forming characteristics, and human activities (cultivation). Variable enzyme activities have been reported in waterlogged soils that are involved in soil productivity and CNP cycling after organic amendments (Gascó *et al.* 2016, Li *et al.* 2018).

The phospholipid fatty acid (PLFA) profiles vary under different stress factors such as fertilizer application and waterlogged conditions. Consequently, the soil productivity and fertility can be predicted through PLFA biomarker and individual fatty acids that reveal the structure of microbial community with an estimated abundance of various microbial groups (Stark *et al.* 2007, Helgason *et al.* 2010, Ai *et al.* 2012). Soil enzymes being a vital component of biochemical functioning are recommended as an aspect of soil quality and their activities are related to microbial biomass, nutrient accessibility, and plant growth (Liu *et al.* 2017, Zhang, Sun, Chen, *et al.* 2019). Soil enzymes like β-glucosidase (GLU), acid phosphatase (AP), N-acetyl-β-glucosaminidase (NAG), and urease are involved in CNP transformations of soil organic content and other nutrients, and their activities varied with inorganic and organic fertilizer application (Dick and Tabatabai 1993, Li *et al.* 2015, Zhang *et al.* 2016).

From the above mechanisms and many research studies, it is still unclear how microbial community dynamics respond with different sources of C, N, P, and K since it depends on many other factors such as soil texture, pH, electrical conductivity (EC), soil organic matter (SOM), availability of mineral nutrients and other accompanied microorganisms as well. To understand the effect of different variables such as soil pH, EC, OM,

availability of N, P, and K on the microbial community dynamics in paddy soil, a short-term microcosm-based experiment was established. The hypotheses of the study are (1) The relative effect of single and mixed NPK fertilizers and rice straw on relative bacterial and fungal communities (2) how responsive are microbial communities in terms of their enzymes i.e., GLU, AP, NAG, and urease; and (3) to analyze the relation of microbial community dynamics with soil physicochemical properties, enzyme activities, and plant biomass.

4.3. Materials and Methods

4.3.1. Soil Description

Bulk soil (non-calcareous, silty clay loam, isohyperthermic Udic Haplustalfs) was procured from 10 - 20 cm depth from an experimental paddy field in Gujranwala, Pakistan (32°19'N and 74°20'E) in early August 2018. The sampling site was 226m above sea level with a hot semi-arid climate (BSh), having annual rainfall and an average annual temperature of 578 mm and 23.9 °C respectively. Paddy soil samples were transported in zip lock bags to an experimental facility with minimum possible contamination and stored at -20 ^{o}C till further experimentation. Aseptic conditions were maintained wherever necessary. The soil had a pH of 8.07, 0.16% total carbon (TC), and 1.39% total nitrogen (TN). Total extractable phosphorus (TEP) and available potassium (AK) were calculated as 0.31 and 6.60 mg/kg, respectively.

4.3.2. Development and supplementation of Microcosms

Triplicate microcosms were established with 2.2 kg of experimental soil slurry in the 64 oz polyethylene pots (20cm height and 15cm diameter) and an anoxic environment was developed by flooding up to 3cm of water that was maintained throughout the

experiment. Each microcosm apart from control is planted with a 26-day old nursery of rice plants (Oryza sativa var. Super Basmati). Thirteen triplicate combinations were established using N as $CO(NH_2)_2$, P and K as KH_2PO_4 (31% P, 39% K), K as KCl (39% K, 35.5% Cl), and rice straw (Table 4.1), while no supplementation was done for two controls (with plant and without plant). Supplementation was done using 50 ml solution of respective fertilizer (per liter: 2.3 g N as urea, 0.87g P and 1.08g K as KH_2PO_4, 1.85g K and 1.68g Cl as KCl), and 1% rice straw (1% of soil dry weight) at day 0, 5 and 30 as basal dressing followed by two top dressings. These amendments correspond to 160 kg of N as urea, 61 kg P as KH_2PO_4 and 128 kg K as KCl per ha (Shrestha *et al.* 2010) which are common in rice agriculture. Microcosms were set in the greenhouse for 45 days with an average temperature range of 20–25 ^oC. Bulk and rhizospheric soil samples from each microcosm (0 – 10 cm depth) were sampled after 45 days and stored at -20 ^oC till further assessment.

Table 4.1: Description of controls and amendments with abbreviations for each developed microcosm.

Microcosm	***Treatment model***	***Symbol***
1	No Treatment, Non-Flooded	C0
2	No Treatment, Flooded	Cf
3	No Treatment, Flooded + Plant	Cneg
4	$CO(NH_2)_2$ + Plant	U
5	Rice Straw + Plant	Rs
6	Rice Straw + KH2PO4 + Plant	RsP
7	Rice Straw + KCl + Plant	RsK
8	$CO(NH_2)_2$ + Rice Straw + Plant	URs
9	$CO(NH_2)_2$ + KH_2PO_4 + KCl + Plant	UPK
10	$CO(NH_2)_2$ + KH2PO_4 + Rice Straw + Plant	UPRs
11	$CO(NH_2)_2$ + KCl + Rice Straw + Plant	UKRs
12	KH_2PO_4 + KCl + Rice Straw + Plant	PKRs
13	$CO(NH_2)_2$ + KH_2PO_4 + KCl + Rice Straw + Plant	UPKRs

4.3.3. Soil physicochemical assessment

The soil moisture content was evaluated using the standard gravimetric method (Baldoncini *et al.* 2019). The pH and EC were calculated using the soil/water 1:1 (v/v) ratio. TC and TN were estimated by dry combustion at 1800 ºC using Vario Max CN Analyzer and soil particle distribution was analyzed by hydrometer method (Bouyoucos 1962) and the textural category was designated according to US textural classification. TEP was measured using Mehlich 3 test (Mehlich 1984).

4.3.4. PLFA based bacterial and fungal community analysis

Triplicate samples were subjected to PLFA profiling of microbial communities by the protocol described (BLIGH and DYER 1959, Chowdhury and Dick 2012). Concisely,

phase mixture (1:2:0.8 v/v/v) of chloroform, methanol, and citrate buffer respectively (0.15 M, pH 4.0) was used to extract lipids from sample soils followed by fractionation into neutral, glyco- and phospholipids on silicic acid columns (Supelco, Sigma). The phospholipids fractions were subjected to mild alkaline methanolysis and examined by Hewlett-Packard 5890 Series II gas chromatography (Palo Alto, CA) equipped with an HP Ultra 2 capillary column and flame ionization detector (FID-GC). The measurements were performed with MISystem, (version 4.5, MIDI Inc, Newark, DE), using the TSBA 40 method. Signature PLFA biomarkers were used to establish a quantitative measurement of the relative abundance of a microbial group according to fatty acid nomenclature defined (Frostegård *et al.* 1993). The Gram-positive bacteria (GP) were represented by iso- and anteiso-branched saturated fatty acids (i14:0, i15:0, i16:0, i17:0, a17:0) (Kaur *et al.* 2005), whereas Gram-negative bacteria (GN) were characterized by cyclopropyl (cy17:0, cy19:0), mono-unsaturated (16:1ω7c) and the straight-chain fatty acids (14:0, 15:0 and 17:0) (Kourtev *et al.* 2002). The fungal biomass is indicated by PLFA 18:2ω6, 9.

4.3.5. Enzyme Activities

Treated soil samples were analyzed for GLU, AP, and NAG activities using 1 g of air-dried soil. The respective p-nitrophenyl (pNP) substrates used in those assays were 5 mM pNP-β-D-glucoside, 5mM pNP-phosphate, and 1mM pNP-N-acetyl-β-D-glucopyanoside. For β-glucosidase activity and acid phosphatase activity, the buffer was modified universal buffer (MUB) with pH 6.0 and pH 6.5 respectively (Page 1982, Weaver *et al.* 1994). For NAGase assay, acetate buffer with pH 5.5 is used. The reaction for each enzyme was terminated with tris-hydroxy aminomethane (THAM) with pH 12 and 0.5 M $CaCl_2$ to deprotonate the pNP, producing a yellow color after incubation at

37 °C for 1 hour. Samples were filtered, and absorbance of the filtrate was measured at 415 nm on a Biotek spectrometer (Biotek, Winooski, VT) and compared with a developed pnp standard curve. The urease activity is determined by the ammonium method (Kandeler and Gerber 1988) following the protocol described by (Dick 2015). About 5 g of soil was treated with substrate and borate buffer followed by incubation at 37 ºC for 2 hrs. After incubation, the reaction is terminated by KCl (2M) and HCl (0.01M) solution, shaken in a rotary shaker and filtered by N-free filters. The standard curve is prepared by ammonium chloride standards at 0, 1.0, 1.5, 2.0, and 2.5 µg N per ml. the color reaction is created by adding 5 and 2 ml of sodium salicylate-sodium hydroxide and sodium dichloroisocyanurate solution respectively. The filtrate is diluted to factor ten and the absorbance was measured at 660 nm on a Biotek spectrometer (Biotek, Winooski, VT).

4.3.6. Methane production potential

To measure methane production potential of supplemented and incubated soil samples, 20 g of 0.2 mm diameter air-dried soil samples were taken in 125 ml Wheaton serum bottles. Soil slurry was made by adding 20 ml distilled water. The bottles were sealed with butyl rubber stoppers and crimped with aluminum caps. The bottles were incubated in dark at 25 ºC while shaking horizontally at 150 rpm. Acetylene was added that acts as an inhibitor of methane oxidation. The measurements were taken in triplicate at a three-day interval including T0. The CH_4 produced was measured by gas chromatograph (Shimadzu GC-2010; Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionization detector (FID), and the gas production (in µg CH_4/kg soil/day) was calculated using the following equation (Mujiyo *et al.* 2017).

$$CH_4\ production = (C_{24} - C_0) \times \frac{Vhs}{WS} \times \frac{MW}{Vm} \times \frac{Tst}{Tst + T}$$

Where:

C_0 – CH_4 concentration at hour 0 (ppm)

C_{24} – CH_4 concentration at hour 24 (ppm)

V_{hs} – headspace volume (ml)

WS – weight of soil sample (g)

MW – molecular weight of CH_4 (16.123 g)

V_m – CH_4 volume at standard conditions (273.2 K) = 22.41 l

Tst – temperature standard conditions (273.2 K)

T – air temperature of incubation (°C)

4.3.7. Relative abundance of methanogens

In order to detect the presence of methanogens in different treatments, universal primers 515F/806R (Caporaso *et al.* 2018) targeting the 16S rRNA V4 region, were used. The primer sequence and PCR conditions are given in Table 4.2. The resulting amplicons are sequenced using Illumina MiSeq Platform and the sequences are deposited in Sequence Read Archive (SRA) of NCBI (National Center for Biotechnology Information) under the BioProject PRJNA627288 with accession numbers SAMN14661259 to SAMN14661276.

Table 4.2: Primer pair with its sequence and PCR conditions used to generate amplicons

Primer	***Sequence***
515F	5'-GTGCCAGCMGCCGCGGTAA
806R	5'-GGACTACHVHHHTWTCTAAT
PCR Conditions	***Time and temperature***
Initial denaturation	3 mins, 94 ºC
Denaturation	45 secs, 94 ºC
Annealing	1 min, 50 ºC
Elongation	90 secs, 72 ºC
Final elongation	10 mins, 72 ºC
Number of cycles	40

4.3.8. Effect on plant growth and biomass

The effect of different amendments in pure and mixed form (double, triple, and quadruple) on paddy plants was also analyzed. Whole plants were oven-dried at 65˚C for 72 h and weighed. The height of the whole plant was also measured on days 0, 15, 30, and 45.

4.3.9. Data Analysis

The data of environmental variables were analyzed in each case by Multivariate Analysis of Variance (MANOVA) followed by Post HOC test (Tukey's HSD, $P < 0.05$) using SPSS (IBM SPSS Statistics for Windows, Version 26.0., Armonk, NY, USA). The heterogeneity of species data i.e., the beta-diversity correlation between different treatments was accessed by principal component analysis (PCA) and redundancy analysis (RDA) and in Canoco 4.5 (Microcomputer Power, Inc., Ithaca, USA) and software R (version 4.0.5). The Monte Carlo permutation test (499 permutations) was

used to predict the statistical significance of RDA. Confirmatory factor analysis (CFA) was performed in R (version 4.0.5, package; Lavaan and semPaths) to develop a fit model between soil physicochemical properties, PLFA biomarkers, enzyme activities, and plant biomass to study the relationship between them. Network analysis was performed in Gephi 9.0.2.

4.4. Results

4.4.1. PLFA based soil microbial community structure

In total, 27 PLFA biomarkers were identified and used to characterize soil microbial community (Table 4.3). Distinct amendments significantly influenced total fatty acid methyl esters (tFAME), total bacteria (TB), total fungi (TF), actinomycetes, GP, and GN abundance compared to time zero control i-e C_0. Overall, higher indices were found in URs, UPRs, and UPKRs while lower RsK and C_0. The fungal PLFA significantly increased in all treatments except RsK and this increase was more profound in URs. A similar trend is found for actinomycic PLFA. The fungi to bacteria (F/B) ratio, however, remained insignificant (Table 4.4). Considering GP/GN, each of the controls had significantly higher ratios compared to other treatments. The cy/pre and sat/mono were also significantly higher in time zero control (Table 4.4). Absolute abundances (in nmol g^{-1} soil) of total PLFA biomarkers and individual fatty acids were subjected to multivariate analysis by PCA (Fig. 4.1) to access the variation in the microbial community under different fertilizer amendments. The first principal component (PC) signified 69.1% of the total variation, whereas the second PC contributed 22.7% of the overall variance. The time zero control (C_0), non-amended controls (C_{neg} and C_f), UPRs, UPKRs, and URs showed substantial variation from other treatments along PC1.

Table 4.3: Values represent amounts of total FAME (tFAME), total bacterial PLFA (TB), total fungal PLFA (TF), actinomycic PLFA, gram-positive (GP), and gram-negative (GN) PLFAs (nmol g-1) in different treatments. Data signify means ± standard error, (n=3). Different lower-case letters show significant differences ***at P < 0.05 (Tukey's Post HOC test).***

Treatment	***tFAME***	***TB***	***TF***	***Actinomycetes***	***GP***	***GN***
C0	34.80±1.35a	29.66±1.09a	5.09±0.51a	7.70±0.61a	19.58±0.09a	8.54±0.06a
Cf	47.38±1.85ab	46.17±2.27ab	8.36±0.40b	8.90±0.95b	27.04±1.67ab	11.80±0.81b
Cneg	54.93±4.29b	45.56±3.45b	8.76±1.20b	11.43±0.78bc	30.90±2.08b	14.16±1.19ab
U	55.49±0.31b	45.27±0.04b	9.92±0.34b	10.27±0.28b	27.10±0.73ab	15.68±0.83ab
Rs	54.87±2.19b	44.91±2.13b	9.63±0.37b	8.63±0.57b	25.38±2.78ab	16.89±0.93ab
RsP	50.81±0.88bc	41.09±0.40b	9.74±0.35b	10.07±0.34b	24.02±0.62ab	15.02±1.09ab
RsK	35.01±3.33a	28.25±2.86a	6.48±0.44a	8.09±0.55a	16.34±1.85a	10.64±1.05b
URs	84.77±4.98c	58.36±7.96bc	19.08±0.50c	11.20±0.54bc	33.65±1.55b	12.54±7.23b
UPK	53.90±3.24b	45.40±2.16b	9.03±0.79b	11.32±0.20bc	26.94±2.21ab	14.41±0.85ab
UPRs	44.48±2.01ab	52.30±6.12ab	11.67±1.73b	11.88±2.12bc	30.41±3.20b	17.74±2.40ab
UKRs	54.42±1.58b	44.93±1.32b	9.34±0.55b	10.39±0.27b	26.08±1.42ab	16.49±1.44ab
PKRs	52.49±3.11ab	46.51±2.16b	9.04±1.04b	10.00±1.12b	22.86±2.79ab	15.02±2.54ab
UPKRs	79.62±5.13c	48.51±12.96c	14.67±1.40c	14.61±1.31c	39.54±3.85b	25.06±3.46c

Table 4.4: Values represent ratios of fungi to bacteria (F/B), gram-positive to gram-negative bacteria (GP/GN), cyclopropyl to precursor PLFAs (cyc/pre), and saturated to monounsaturated PLFAs (sat/mono) in different treatments. Data signify means ± standard error, n=3. Different lower-case letters show ***significant differences at P < 0.05 (Tukey's Post HOC test).***

Treatment	***F/B***	***GP/GN***	***cyc/pre***	***sat/mono***
C0	0.19±0.05a	2.29±0.01a	0.23±0.08a	1.11±0.07a
Cf	0.21±0.00a	2.30±0.13a	0.16±0.01ab	0.90±0.08ab
Cneg	0.23±0.02a	2.19±0.07a	0.16±0.02ab	0.85±0.04ab
U	0.22±0.01a	1.74±0.14ab	0.15±0.01ab	0.85±0.07ab
Rs	0.22±0.01a	1.53±0.23ab	0.14±0.01b	0.83±0.06ab
RsP	0.23±0.01a	1.62±0.15ab	0.16±0.01ab	0.77±0.05b
RsK	0.22±0.01a	1.54±0.12ab	0.15±0.01ab	0.86±0.05ab
URs	0.24±0.02a	1.89±0.51b	0.14±0.02b	0.78±0.04b
UPK	0.22±0.01a	1.87±0.04ab	0.15±0.00ab	0.83±0.04ab
UPRs	0.21±0.01a	1.73±0.07ab	0.15±0.01ab	0.90±0.05ab
UKRs	0.21±0.01a	1.62±0.22ab	0.15±0.01ab	0.84±0.06ab
PKRs	0.23±0.01a	1.56±0.17ab	0.15±0.01ab	0.77±0.04b
UPKRs	0.21±0.00a	1.60±0.12ab	0.14±0.01b	0.79±0.05b

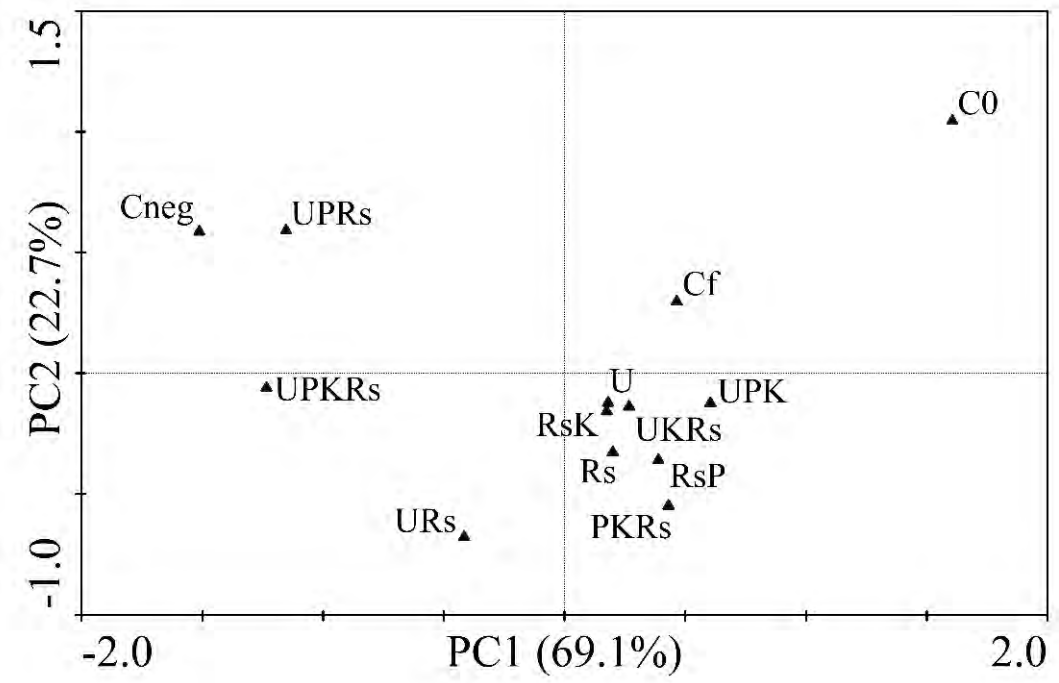

Figure 4.1: Ordination plot of principal component analysis (PCA) of the identified PLFA biomarkers from soils with different amendment applications (n=3). Each symbol (triangle) represents an individual sample (microcosm) and is explained in Table 4.1.

4.4.2. GLU, AP, NAG, and urease activities

Activities of GLU, AP, NAG, and urease in all treatments are shown in Figures 4.2, 4.3, and 4.4 respectively. GLU action varied from 0.337 to 0.826 pNP µmol g^{-1} h^{-1}. Overall, there was an increasing trend of GLU activity for a majority of the treatments except for RsP and UK which showed a significant decrease and accounted for 0.337 and 0.430 pNP µmol g^{-1} h^{-1} respectively. No significant variation in activity was observed for P, K, and UPK as compared to either of the controls. AP activity varied from 0.808 to 1.932 pNP µmol g^{-1} h^{-1} for Rs and PKRs, respectively. Compared to control, significantly higher activity of AP was observed for PKRs and a significantly

decreasing trend for K, Rs, UK, and UPK. Lastly, the activity of NAG significantly increased in UPKRs. The treatments K, RsP, UK, RsK, and URs showed a significant decrease as compared to flooded non-planted control (C_f) and remained insignificantly different as compared to C_0. The urease activity was significantly higher in U, UPK, and UPRs treatments. In UPKRs however, urease activity decreased despite urea supplementation (Figure 4.5).

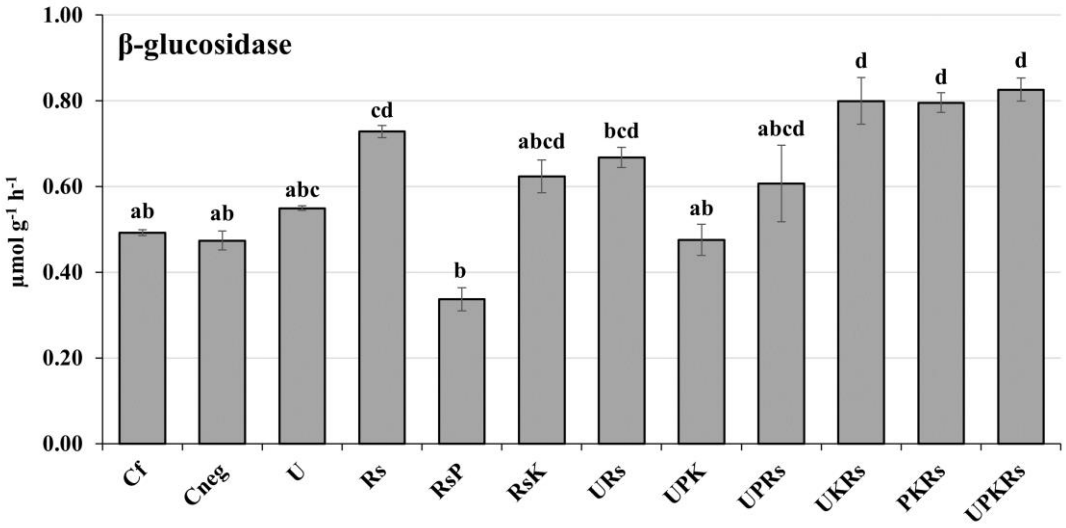

Figure 4.2: Activity of GLU as the mean pNP in soils under different treatments. Values represent means and error bars represent SE (n = 3). Different letters ***denote significantly different means p<0.05 (Tukey's Post Hoc test). pNP, p-nitrophenol; GLU, β****-glucosidase. Symbols for different treatments are shown in Table 4.1.*

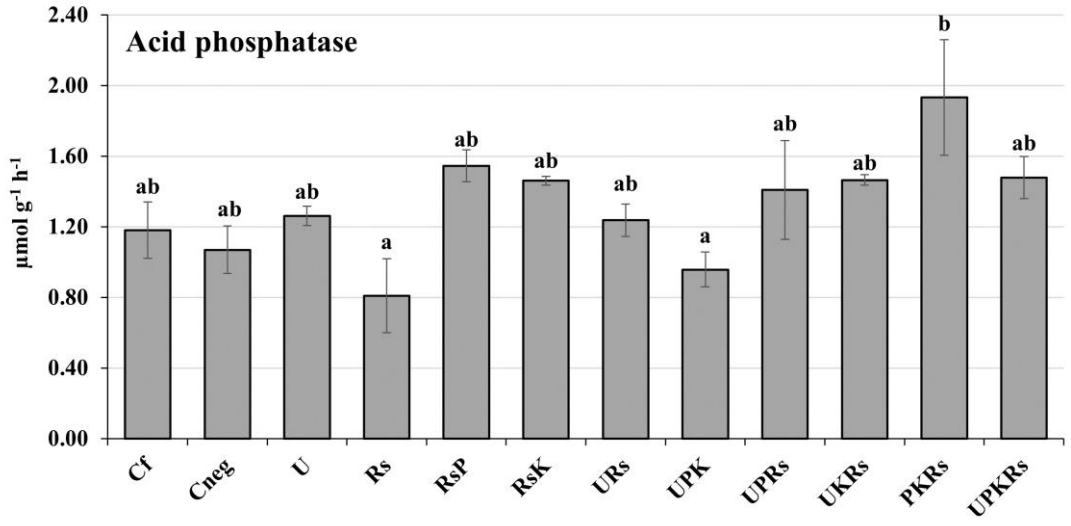

Figure 4.3: Activity of AP represented as the mean pNP in soils under different treatments. Values represent means and error bars represent SE (n = 3). ***Different letters specify significantly different means p<0.05 (Tukey's Pos****t Hoc test). pNP, p-nitrophenol; AP, acid phosphatase. Symbols for different treatments are shown in Table 4.1.*

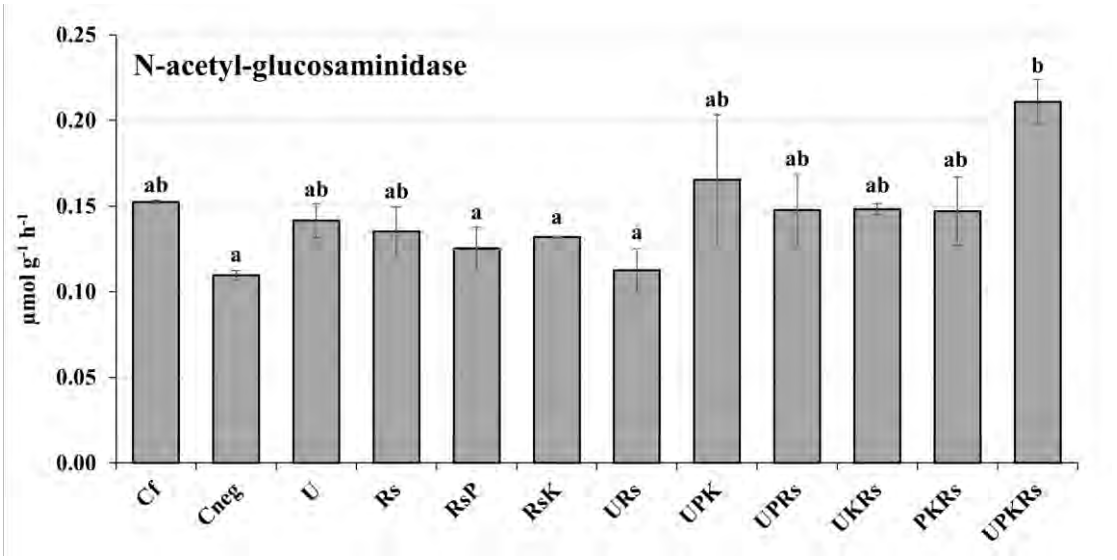

Figure 4.4: Activity of NAG represented as the mean pNP in soils under different treatments. Values represent means and error bars represent SE (n = 3). ***Different letters indicate significantly different means p<0.05 (Tukey's Post Hoc*** *test). pNP, p-nitrophenol; NAG, N-acetyl-****β****-glucosaminidase. Symbols for different treatments are shown in Table 4.1.*

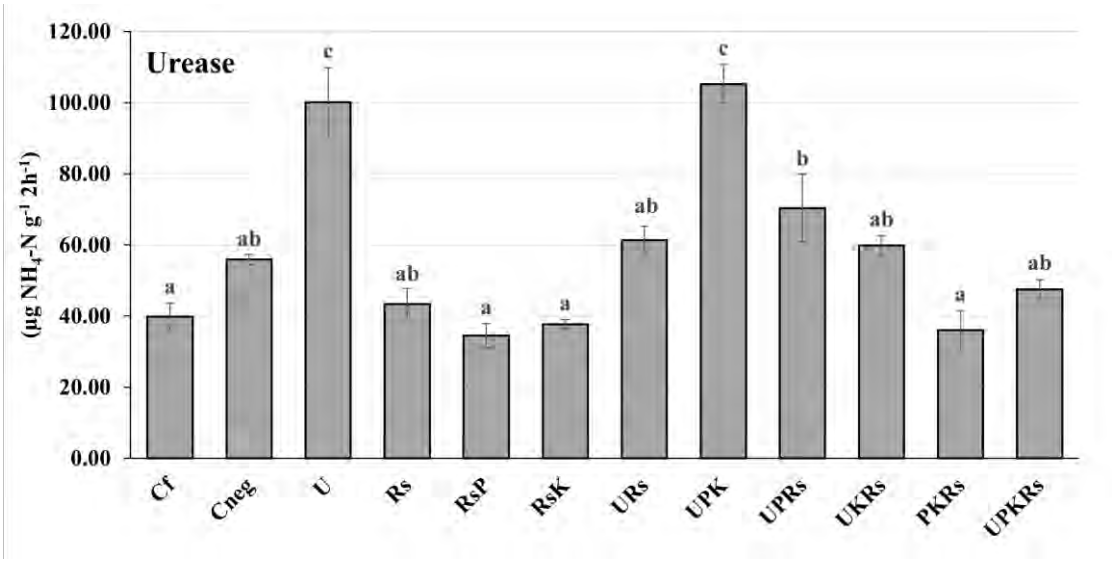

Figure 4.5: Urease activity represented as the mean pNP in soils under different treatments. Values represent means and error bars represent SE (n = 3). ***Different letters indicate significantly different means p<0.05 (Tukey's Post Hoc*** *test). Symbols for different treatments are shown in Table 4.1.*

4.4.3. Methane production potential

Significant variations in MPP were observed in different treatments as shown in Figure 4.6. The overall range of MPP varies from 0.30-0.68 µg CH_4/kg soil/day. Significantly higher values of MPP were observed in treatments containing Rs except in UKRs, while lower values were observed in the case of C_0, C_{neg}, U, UPK, and UKRs were reported.

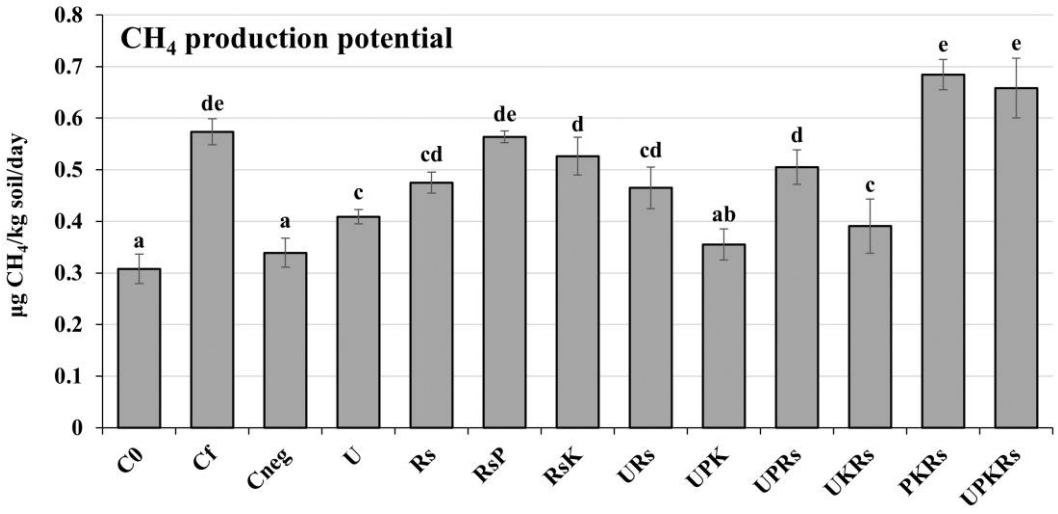

Figure 4.6: Mean methane production potential (n=9) in different treatments.

4.4.4. Relative abundance of methanogens

The total and relative abundance of methanogens are shown in Figures 4.7 and 4.8 respectively. Compared to time zero control there was a significant decrease in *methanocella* in all treatments. Overall, all methanogenic genera tend to decrease in C_f, U, and UKRs. *Methanobrevibacter*, *methanosaeta,* and *methanomassiliicoccus* showed increasing while *methanosphaerula* and *methanocella* showed decreasing trend in all treatments. The relative abundance of methanogens was in accordance with their MPP values in Figure 4.6 except for C_0.

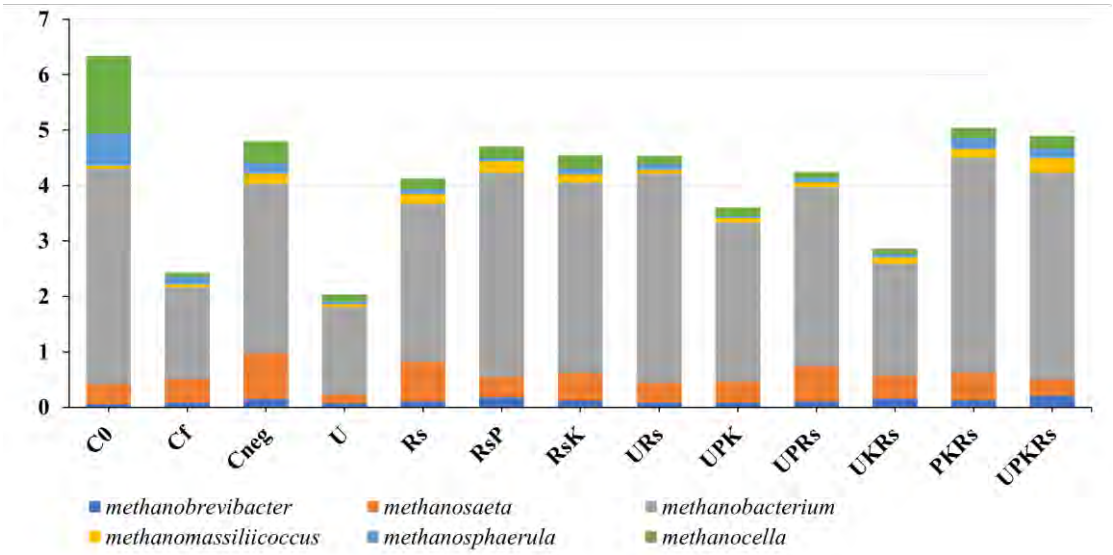

Figure 4.7: Total abundance of different methanogenic genera of the archaeal community in supplemented soil samples. The data is based on 16S rRNA V4 region HTS.

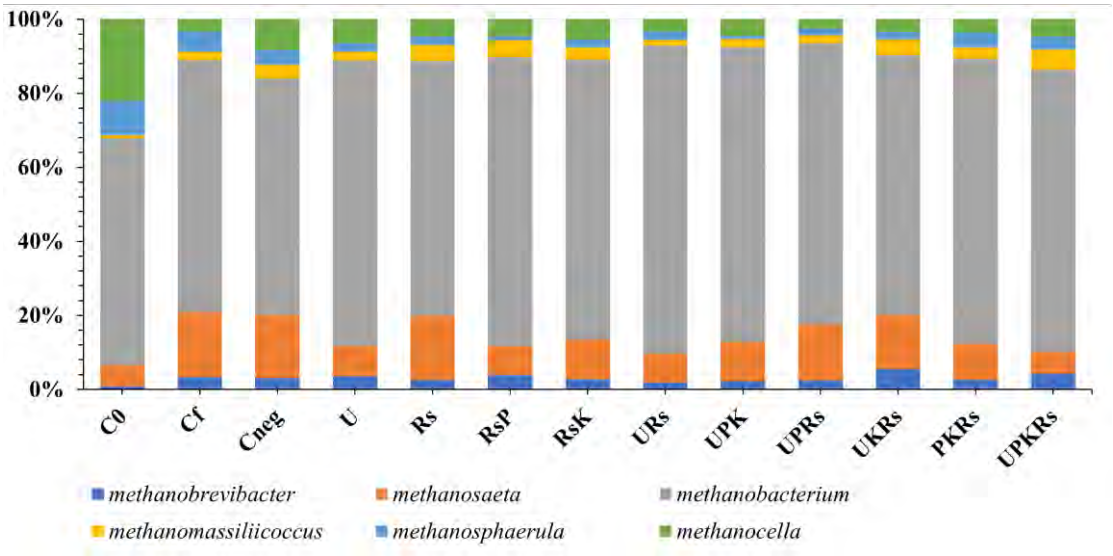

Figure 4.8: Relative abundance of different methanogenic genera in supplemented soil samples. The data is based on 16S rRNA V4 region HTS.

4.4.5. Plant biomass and height

The plant growth rate was measured at days 0, 15, 30, and 45 (data shows final readings only). A significant increase in growth was observed for UPK, accounted for an almost 250% increase as compared to day 0 (Figure 4.9). A significantly lower growth rate was shown only in PKRs and UPKRs as compared to control. The variation was similar on days 15 and 30. Overall, the treatments C_{neg}, Rs, RsP, RsK, UKRs, PKRs, and UPKRs showed less than a 200% increase. The same trend was seen for an increase in plant height also (Figure 4.10).

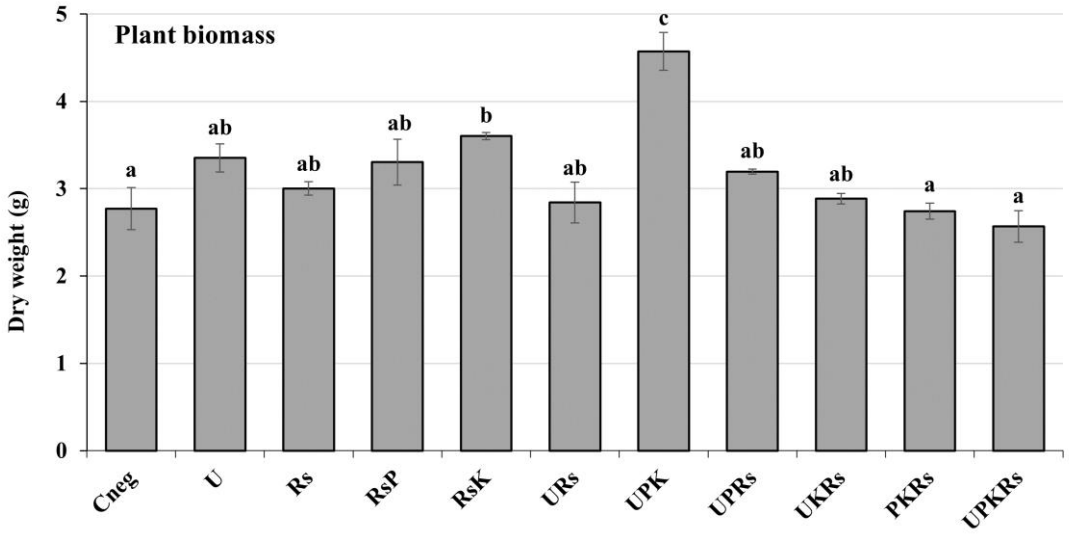

*Figure 4.9: Dry weight (biomass) of the whole plant under control (Cneg) and different treatments. Values represent means and error bars represent SE (n = 3). **Different letters specify significantly different means p<0.05 (Tukey's Post** Hoc test). Symbols for different treatments are explained in Table 4.1.*

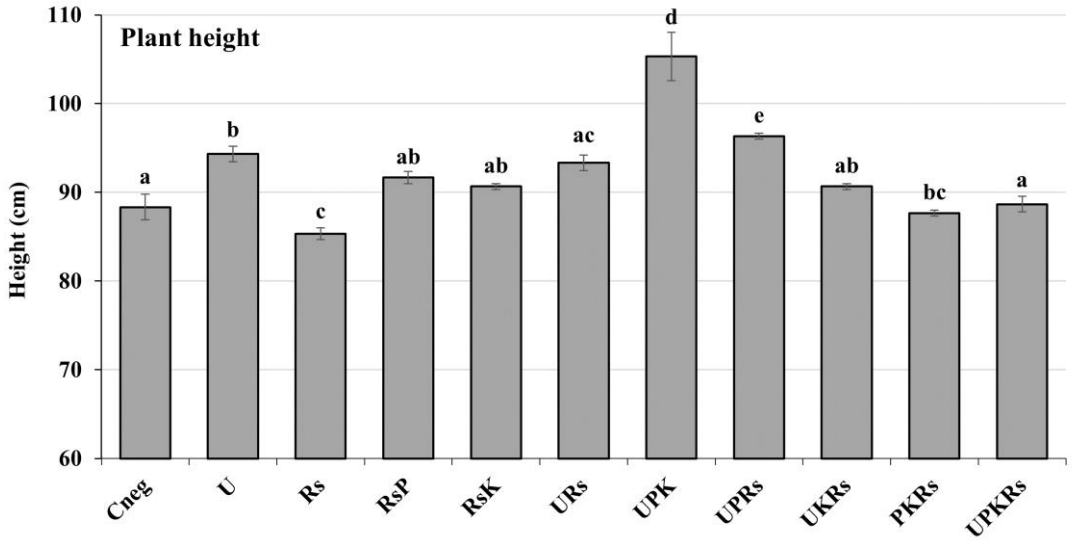

Figure 4.10: Final height of the whole plant under control (Cneg) and different treatments. Values represent means and error bars represent SE (n = 3). ***Different letters indicate significantly different means p<0.05 (Tukey's Post Hoc*** *test). Symbols for different treatments are explained in Table 4.1.*

4.4.6. Controls on soil microbial community

The soil physicochemical characterization and enzyme activities as environmental variables that might influence the bacterial and fungal communities were analyzed by redundancy analysis RDA demonstrated in Figure 4.11. The RD1 and RD2 axes described 72.2% and 13.0% variance, respectively. The significant contributors in shaping microbial community were found to be AK ($P < 0.05$, $F = 2.48$, $R^2 = 0.18$), TN ($P < 0.05$, $F = 3.08$, $R^2 = 0.50$), GLU ($P < 0.05$, $F = 2.57$, $R^2 = 0.62$), AP ($P < 0.05$, $F = 2.50$, $R^2 = 0.72$) and GWC ($P < 0.05$, $F = 4.13$, $R^2 = 0.83$).

The impact of important environmental variables on overall bacterial and fungal communities was also accessed by confirmatory factor analysis (CFA) as shown in Figure 4.12. The final fil model was statistically significant ($\chi^2 = 15.475$, $p = 0.346$, CFI

= 0.983, SRMR = 0.185, GFI = 0.794). The CFA model inferred that all communities were well addressed by pH, C/N, OM, AK, and EC.

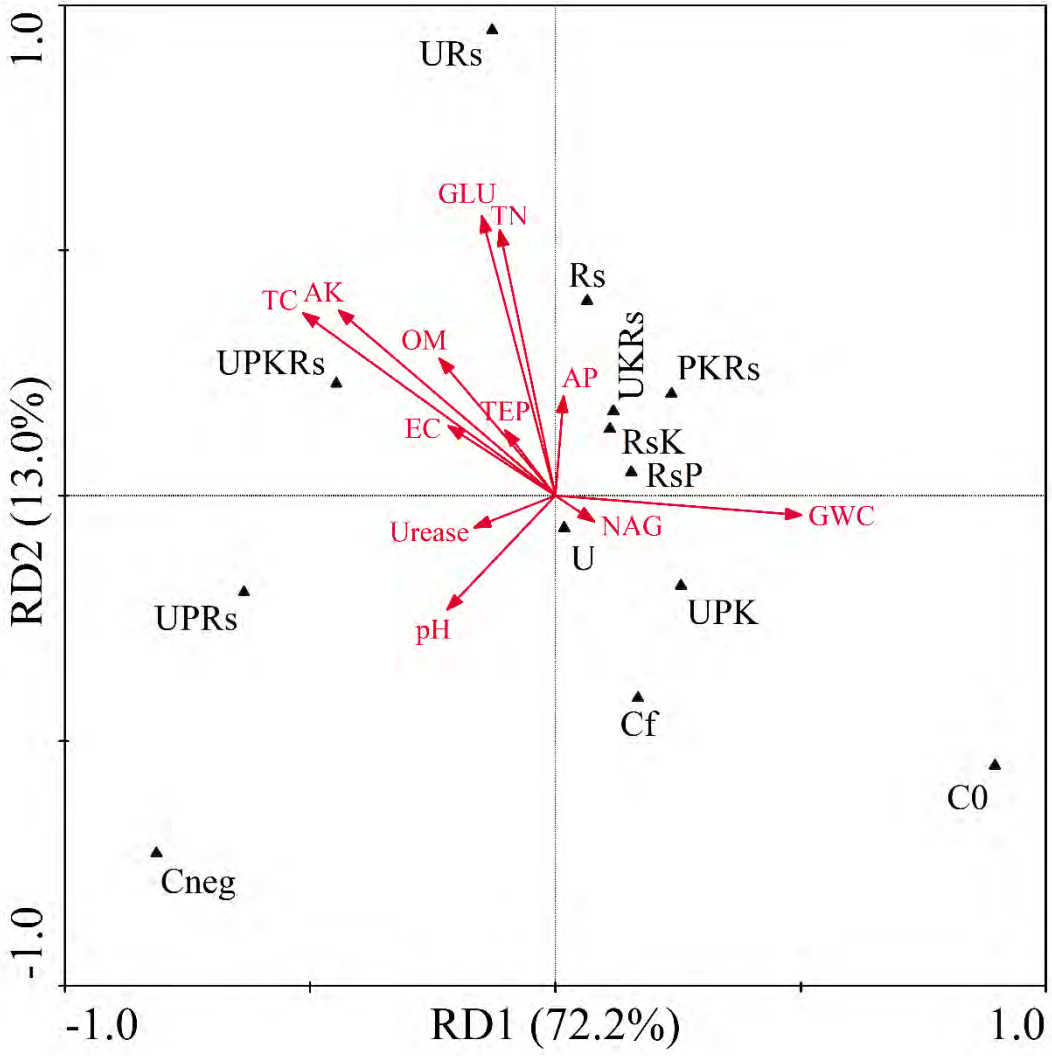

*Figure 4.11: Triplot ordination of Redundancy analyses (RDA) of the correlations among soil physicochemical characterization and enzyme activities with individual PLFA biomarkers. Red arrows represent explanatory variables and their correlations with treatments. GWC, gravimetric soil water content (w/w); EC, electrical conductivity; AK, available potassium; OM, organic matter; TC, total carbon, TN; total nitrogen; TEP, total extractable phosphorus; NAG, N-acetyl-**β-glucosaminidase; AP, acid phosphatase; GLU, β-**glucosidase.*

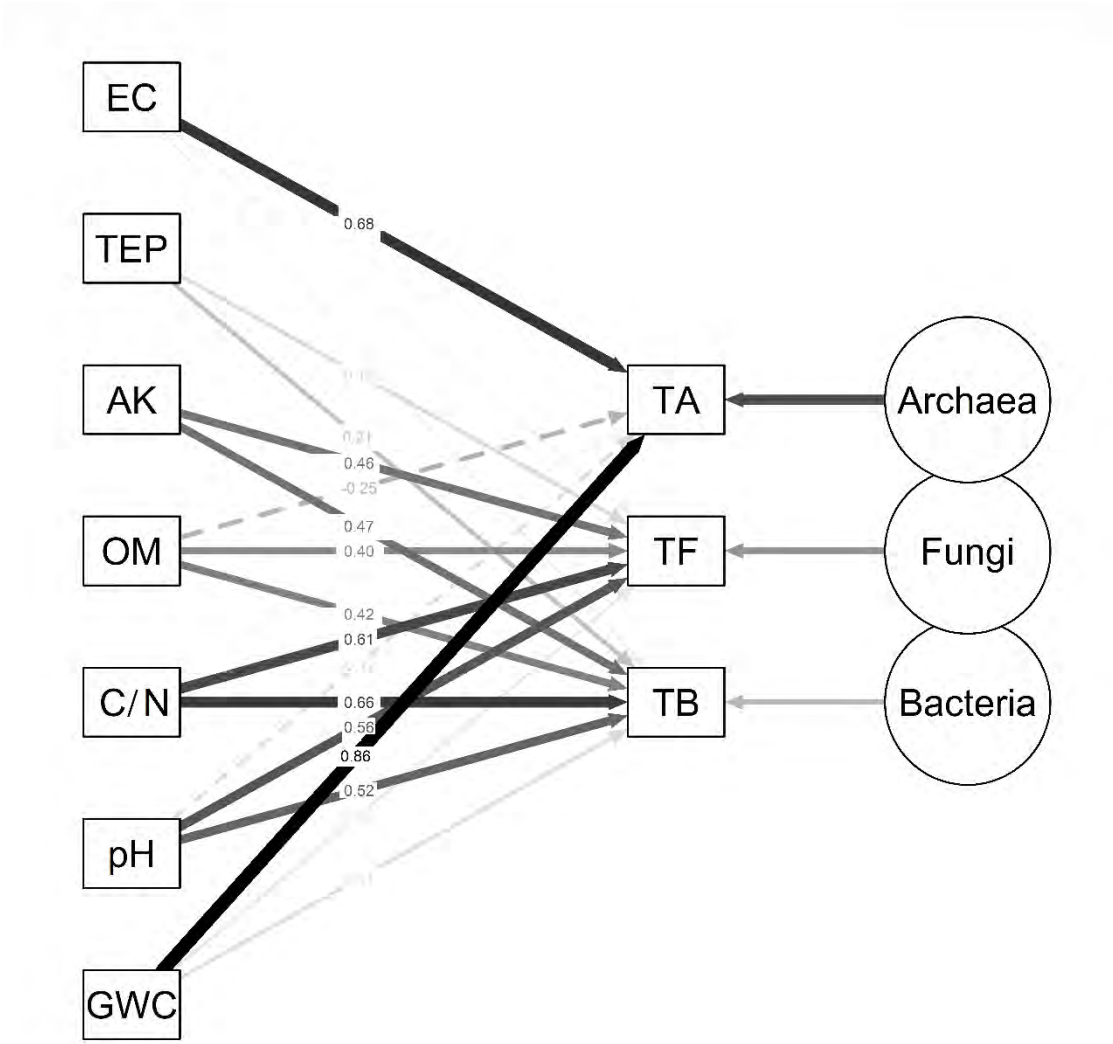

Figure 4.12: Confirmatory factor analysis (CFA) of important environmental variables (GWC, pH, C/N, OM, AK, TEP, and EC) on methanogenic archaea (TA), bacterial (TB), and fungal (TF) communities. The developed CFA model was based on regressions between soil physicochemical properties and microbial abundance. Values on arrows represent standardized direct path coefficients. GWC, gravimetric soil water content (w/w); C/N, carbon to nitrogen ratio; OM, organic matter; AK, available potassium; TEP, total extractable phosphorus EC, electrical conductivity; TF, total fungi; TB, total bacteria.

4.4.7. Controls on enzyme activities and plant biomass

The enzyme activities were also accessed by the CFA model by regressing plant biomass on enzyme activities, and enzyme activities on tFAME and GP/GN as shown in Fig 4.13. The final mode fitted statistically well (χ^2 = 6.856, p = 0.032, SRMR = 0.079, GFI = 0.851). It depicts tFAME strongly impacted NAG and urease activities while GP/GN had a positive impact on urease while the strong negative impact on GLU and AP. F/B had a strong negative influence on GLU and NAG. Among enzymes, only urease had a strong impact on plant biomass. GLU has also been reported to be a significant contributor by RDA.

The network analysis has been shown in figure 4.13. It implies EC, plant height, tFAME and TB (total bacteria) to be the most significant factors amongst different treatments. Color variations in nodes are based on modularity while edges represent correlation weights.

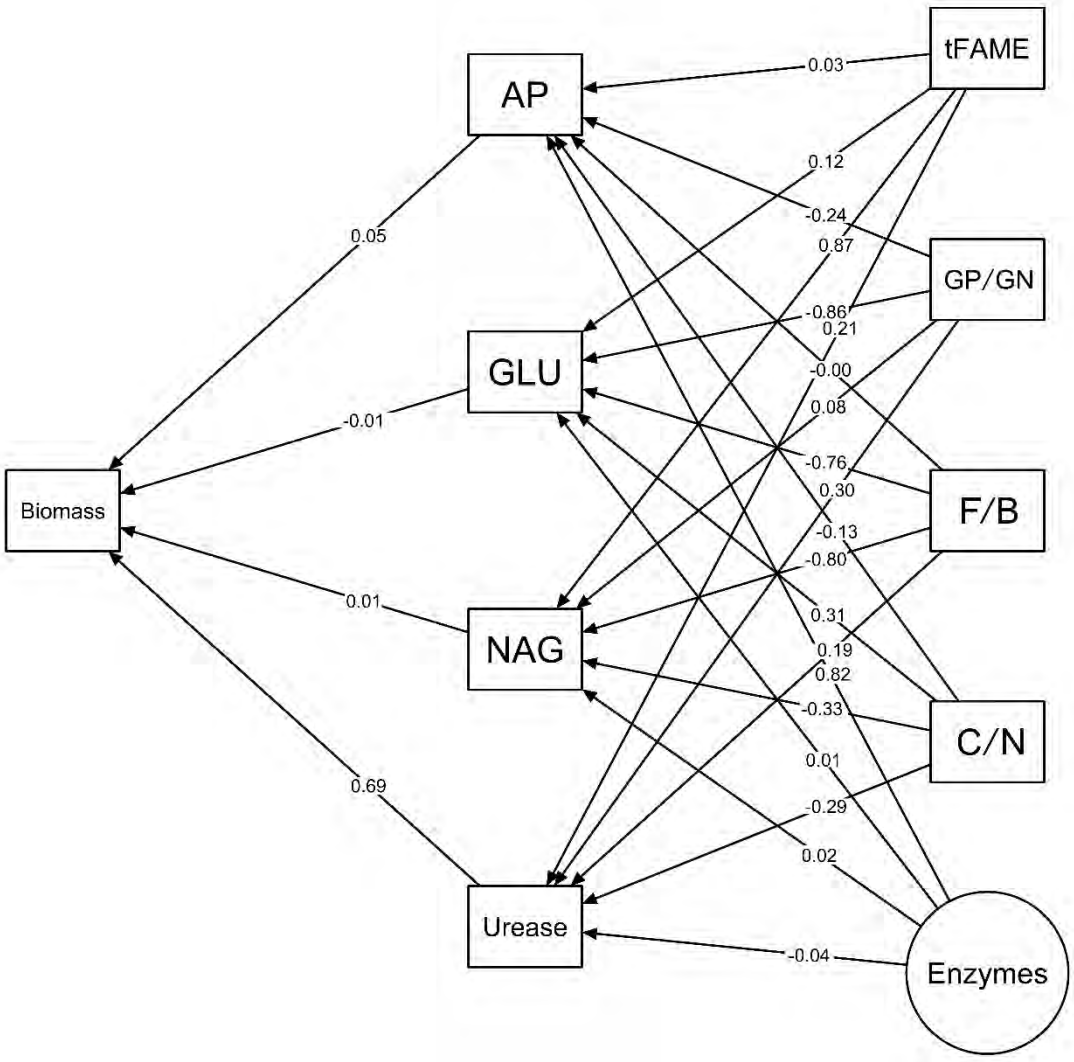

Figure 4.13: Confirmatory factor analysis (CFA) of enzymes regressed on tFAME, GP/GN, F/B, and C/N. The plant biomass regressed on all enzymes. The latent variable (Enzymes) is explained by four observed variables (indicators) i.e., GLU, AP, urease, and NAG. Values on arrows represent standardized direct ***path coefficients. GLU, β****-glucosidase; AP, acid phosphatase; NAG, N-acetyl-****β****-glucosaminidase; tFAME, total fatty acid methyl esters; GP/GN, gram-positive to gram-negative; F/B, fungal to bacterial ratio; C/N, carbon to nitrogen ratio.*

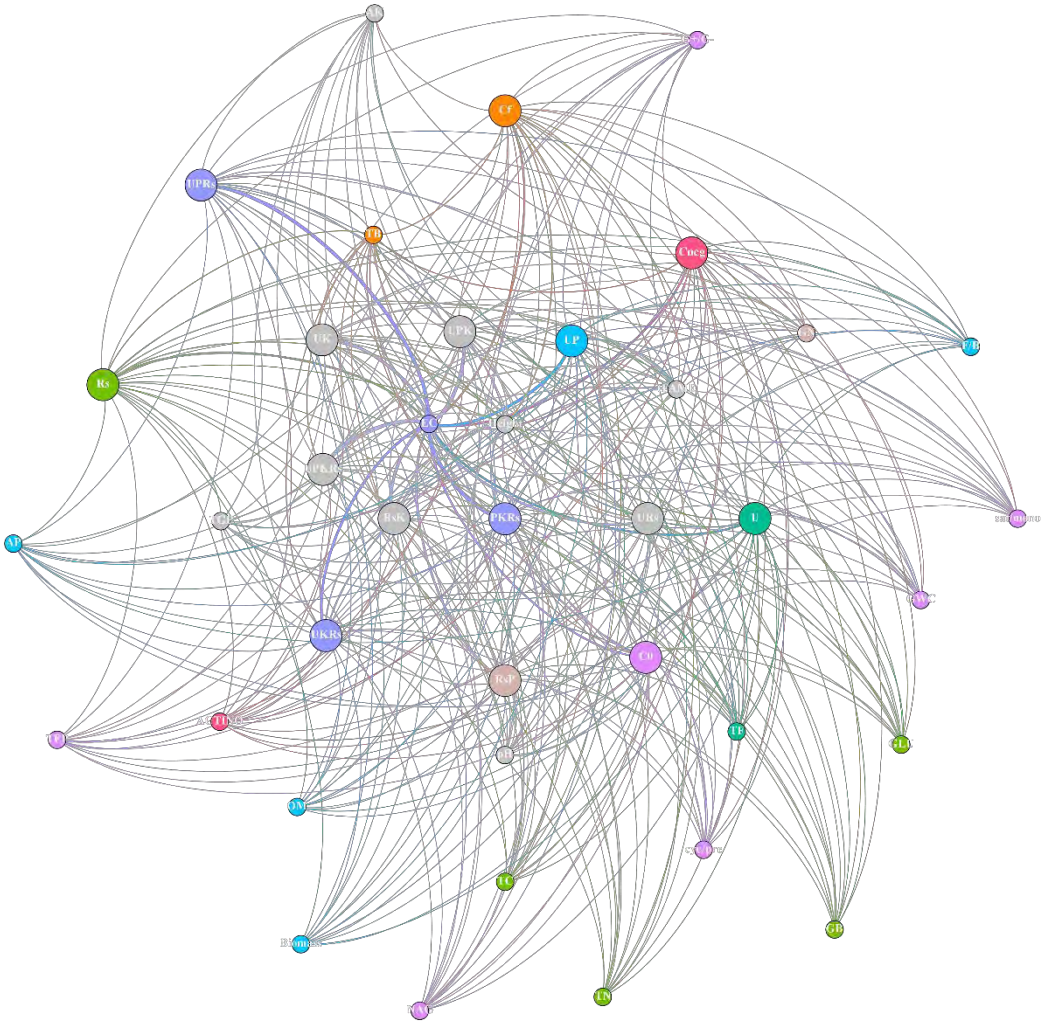

Figure 4.14: Network analysis shows correlation between environmental variables, enzymes, plant height, plant biomass, and microbial PLFA against different treatments.

4.5. Discussion

The spatial differentiation and composition of soil microbial ecology in flooded soils are attributed to ecological conditions, soil quality, and soil properties which in turn are based on inorganic and organic fertilization. The current study probed the impacts of pure and mixed combinations of inorganic fertilizers and organic material on soil physicochemical properties, microbial community structure, enzyme activities, and biomass of rice plants. Significant variations in nutrient status of the soil (EC, OM, and AK) due to organic amendments (rice straw) were observed, which is well documented to enhance nutrient availability, microbial composition, and biological activity of soil in long term fertilization (Potthoff *et al.* 2001, Liu *et al.* 2009, Yuan *et al.* 2013). We observed a substantial increase in microbial abundance, GLU activity, plant growth, and biomass in the treatments containing rice straw with any combination of inorganic fertilizer. Additionally, straw incorporation was reported to increase soil P and C contents in the long-term fertilization experiments (Malý *et al.* 2009, Wu *et al.* 2020), it was though insignificant in our study probably due to short-term experimentation, however, C/N was a more important contributor in our study. Overall, our RDA and CFA results (figure 8, 9) suggested GWC, TN, AK, pH, C/N, and OM be significantly influential in composing bacterial and fungal communities in flooded paddy microcosms. Previous studies have also acknowledged these variables in shaping the structure of a microbial community (Liu, Sui, *et al.* 2019).

Microbial tFAME was significantly elevated in all treatments except RsK when contrasted to time zero control (C_0) and flooded control (C_f). A similar trend was shown by GB, ACT, GGP, GN, AMF, and SF. The PLFA biomarker-based PCA indicated distinct differentiation of C_0, C_f, C_{neg}, UPRs, UPKRs, and URs (Fig. 4.1) from the rest

which was closely correlated. CFA analysis showed C/N to be the strongest indicator for bacterial ($R^2 = 0.66$) and fungal ($R^2 = 0.61$) communities (Figure 4.12). The overall methanogenic, bacterial (GB, GGP, ACT, GP, and GN), and fungal (AMF and SF) abundance is represented by TA, total bacteria (TB), and total fungi (TF) respectively in Figure 4.12. Other important indicators in driving microbial communities were pH, OM, and AK. RDA analysis signified GWC, TN, and AK as significant contributing indicators to drive microbial community (Figure 4.11). Although variance explained by pH and OM were > 0.80, it was insignificant according to the RDA model. Some previous studies suggested pH to be the strongest contributor in microbial community structure (Rousk *et al.* 2010, Shen *et al.* 2013, Kumar *et al.* 2018), however, others found no correlation between them in clay loam soil (Zhao *et al.* 2014, Zhang *et al.* 2015). Organic amendments have been known to stabilize the pH of water-logged soil (Zhong *et al.* 2010, Zhang *et al.* 2015) which is per our study, however, due to the narrow overall variation of pH in our samples, the possibility of other factors in pH stabilization can't be overlooked. One possible justification for pH stabilization could be flooded conditions that inhibit the acid-producing process i-e nitrification (Rukshana *et al.* 2012, Mi *et al.* 2018). The overall microbial community was dominated by GP, followed by GN bacteria and ACT (Watanabe *et al.* 2006), indicated by fatty acids (15:0, 15:0 anteiso, 16:0 iso, 17:0 iso,17:0 anteiso), (16:1 ω7c, 17:0 cyclo, 18:1 ω7c) and (16:0 10Me, 17:0 10Me, 18:0 10Me) respectively (Frostegård and Bååth 1996). The actinomycic PLFA has been reported to decrease from early to a later stage of vegetation (Li *et al.* 2012) and our results revealed significant higher abundance in the treatments containing UPRs and UPKRs and lower or insignificant abundance in a majority of treatments which is in agreement with previous studies (ZHANG *et al.*

2007, Li *et al.* 2012). The GP/GN ratios were higher similar in each control but significantly lower in all amended soils which suggests the role of fertilization in lowering GP/GN ratios in flooded soils. Additionally, since waterlogged conditions are less favorable for fungi and paddy soil is known to contain lesser biomass, their PLFA profiles were significantly less varied in our study (ZHANG *et al.* 2007, Chen *et al.* 2014). The F/B ratios in our results were higher in all treatments compared to control but insignificant which is a common case in soil under flooded conditions (Drenovsky *et al.* 2004). Additionally, the cy:pre ratio remained less in all treatments as compared to time zero control (C_0) which indicated that microbial community has responded indifferently to stress, which is significantly evident in Rs, URs and UPKRs. At the same time cy:pre ratio remained the same compared to flooded control (C_f) which recognized flooding conditions to be another contributor in microbial community structure (Bossio *et al.* 1998, Rinklebe and Langer 2006). The PC1 axis, which accounted for 69.1% variation, differentiates C_0, C_{neg}, UPRs, and UPKRs from other treatments due to lower values of gram-positive and gram-negative in controls and higher in UPRs and UPKRs treatments (Fig. 4.1).

Soil biological activity is principally involved in nutrient recycling and is possibly most sensitive to environmental fluctuations. Significant higher activities of GLU were found in all treatments except RsP and UPK that can be explained due to non-uniform substrate supply which in succession affect a microbial community. GLU activity overall responded negatively against GP/GN (R^2 = -0.40) and slightly positive with tFAME (R^2 = 0.12) in CFA. The RDA too, recognized GLU ($P < 0.05$, $F = 2.57$, R^2 = 0.62) and AP ($P < 0.05$, $F = 2.50$, $R^2 = 0.72$) to be most important regarding microbial community (Fig. 4.11). The soils with more OM have been previously known to

respond with higher GLU activities (Martinez and Tabatabai 1997, Kader *et al.* 2017). GLU activity has been also known to improve in N application and in combination with P, they rapidly mineralize straw residue (Poeplau *et al.* 2016, Zhu *et al.* 2018). The AP activity was less variable in most treatments and significantly lower activity was found in Rs and UPK treatments while higher only in PKRs. The AP activity has been known to increase with rice straw and soil depth while decrease with mineral fertilizer which did not in our case. This disagreement may be due to the limited environment in microcosm and short-term experimentation and should be further studied in plot-based mesocosms to mimic large-scale paddy agroecosystem. AP activity, like GLU, is negatively influenced by GP/GN. Our data represent the least variable activity for NAG, although decreases significantly in URs and increase only in UPKRs. NAG activity, however, was most strongly influenced by GP/GN and tFAME as compared to GLU and AP. GLU and AP also have a negligible negative impact on plant biomass. The urease activity is generally an N mineralization of soil and its production is associated with bacteria, plants, and animals. (Dick *et al.* 2015b) suggested that bacteria are predominant urease producers which is the expected scenario in our case. However, urease activity is kept changing during rice cultivation and its significantly higher activity was observed only in U and UPK. Although UPKRs were also supplemented with U and Rs, no significant difference was found in these treatments compared to control. More or less the same results were found in treatments with P and K, suggested that urease activity varies in P and K presence.

Apart from other factors involved, NPK treatment has been known to increase rice grain yield and above-ground biomass (Lan *et al.* 2012) and our results showed significantly higher growth of rice nurseries in all treatments except in Rs, PKRs, and UPKRs

compared to control. The maximum growth was observed for UPK. Since P in our study represent KH_2PO_4, it gave us ample evidence of UPK essentiality in shaping bacterial and fungal communities in rice agriculture without compromising the plant growth.

4.6. Conclusions

The fertilizer amendments in different combinations had challenging associations and varying effects on soil physicochemical properties, PLFA profiles, enzymes activities, and plant biomass. The PCA explained most variation in PLFA profiles in C_0, C_{neg}, UPRs, and UPKRs as compared to the rest of the treatments. According to CFA, pH, C/N, OM and AK were the most influential on the bacterial and fungal abundance in different treatments. Overall, the bacterial community was more responsive with variable amendments while the fungal community remained stable despite the stress. The least insignificant increase of tFAME was observed in RsK treatment and it demands further study to investigate the possible inhibitory effect of KCl when combined with Rs or U. GLU was also found to be most crucial regarding its impact on community structure while tFAME impacted NAG more than any other. Our findings also implied that UPK treatment accounted for better plant growth without altering bacterial and fungal communities and resulted in increased biomass and better yield.

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Authors' contributions: Mohsin Gulzar Barq: Methodology, Writing- Original draft preparation, Formal analysis. Somia Saleem: Methodology, Investigation. Nathan Lee: Resources, Data Curation. Richard Dick: Resources, Supervision. Naeem Ali: Conceptualization, Supervision.

CHAPTER 5: INTERACTIVE EFFECTS OF WATER SATURATION AND AMMONIUM SULFATE CONCENTRATIONS ON RELATIVE ABUNDANCE AND METHANE OXIDATION COMPETENCE OF METHANOTROPHS

5. Interactive effects of water saturation and ammonium sulfate concentrations on relative abundance and methane oxidation competence of methanotrophs

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5.1. Abstract

Methane being the second most abundant greenhouse gas (GHG) and a bacterial metabolite plays a crucial role in climate control and the ecosystem. The global methane budget tends to increase during the past 50 years and thus a delicate balance is required between its sources and sinks. Submerged wetland soil and drained upland soil are the important sources and sinks of methane, respectively. Methanogenic and methanotrophic communities, fertilizers, redox potential, ammonium, oxygen, and temperature are the other factors that can shift the flux in either way. The natural and supplemented nitrogen (N) dynamics can be inhibitory, enhancing, or neutral in effect regarding methane emission or oxidation. The current study analyzes the effect of different concentrations of nitrogen source i.e., $(NH_4)_2SO_4$ in flooded and non-flooded conditions on soil properties and methane oxidation by evaluating the diversity and abundance of methanotrophs. The experimental setup was planned for 45 days during which $(NH_4)_2SO_4$ progressively decreased. Type I methanotrophs significantly increase with increased $(NH_4)_2SO_4$ concentration in flooded conditions from 0-1 mg/l but they started to decline by a further increase in $(NH_4)_2SO_4$ concentration, while type II methanotrophs outnumbered type I in both conditions. The potential methane oxidation (PMO) rates were higher in non-flooded conditions while decreasing and increasing trend was observed in flooded and non-flooded conditions respectively for pH. TC and C/N were the most influential variable for the relative abundance of type I and type II methanotrophs.

Keywords: Greenhouse gases, Methane, Ammonium sulfate, wetland soil, Methanotrophs, potential methane oxidation.

5.2. Introduction

Amongst different GHG, methane is the second most abundant greenhouse gas (GHG) after carbon dioxide (Nguyen and Lee 2021) with greater radiative efficiency, trapping 25-30 times more long wave radiation than CO_2 and contributing one-third of the radiative forcing on the atmosphere (Yuan *et al.* 2018). The contribution of submerged wetland ecosystems to the annual global methane emission has been estimated to be about 55% (Tiwari *et al.* 2020) while that of non-flooded upland soil is regarded as the only biological methane sink with a comparatively little contribution to global methane consumption (Tveit *et al.* 2019). The biological CH_4 sources and sinks are attributed to the existence and functional metabolism of communities of methanogens and methanotrophs and hydrology of the ecosystem (Denman *et al.* 2007, Silvey *et al.* 2019). Moreover, redox potential, oxygen, minerals, soil profile, its depth, seasonal variations (Bodelier and Laanbroek 2004, Martinez-Cruz *et al.* 2015), and varying nitrogen species influence methane oxidation.

Wetland areas with periodic air exposure exhibited reduced CH_4 effluxes as compared to constantly flooded regions (Dalva *et al.* 2001). The high efflux of methane has been attributed to reduced oxidation-reduction potential maintained under drenched conditions. The methane oxidation is typically higher in frequently flooded regions and winter season, and it is reduced with soil depth. Nevertheless, enhance methanogenesis than methane oxidation was reported when water was drained from the Welsh peat land (Alfadhel 2020). Most the investigations of CH_4 fluxes are carried out in natural ecosystems such as wetlands or closely controlled paddy fields (Zhang *et al.* 2018, Jiang, Qian, *et al.* 2019, Liu, Zang, *et al.* 2019). A common censure on constructed wetlands is that they lack dynamic hydrology and are often continuously been swamped

for disposal and treatment of domestic and industrial effluent due to their cost-efficacy and environmentally friendly nature (Brooks *et al.* 2005, Hu *et al.* 2020). However, they are considered to be a source of emission of greenhouse gases like; CH_4, N_2O, CO, and CO_2 (Tai *et al.* 2002, Søvik and Kløve 2007, Chen, Zhu, *et al.* 2020), which is approximately 10 times larger than the natural wetland.

The N supplementation in the form of inorganic fertilizers in different ecological systems has been reported to produce inhibitory, stimulatory, or no effect at all (Bodelier and Laanbroek 2004). Supplementation of urea or $(NH_4)_2SO_4$ in planted microcosms is reported to increase methanotrophic bacteria up to 3-fold compared to unfertilized or unplanted soils respectively (Wei *et al.* 2016, Liu, Zang, *et al.* 2019). The combined effects of N application and drained conditions of soil results in great variations in regulating factors with depth of study regions. The detailed mechanisms underlying nitrogenous input effects are still not fully understood and controversial depending on the soil pH and the chemical form in which N was applied. The restoration of wetland in some cases can however elaborate the role of specific fertilizer with respect to CH_4 flux in the intermittently flooded soils along with high input of nitrogenous material. The current study hypothesized a significant shift in the relative abundance of type I and type II methanotrophs under different concentrations of $(NH_4)_2SO_4$ in drained as well as non-flooded conditions in wetland soil.

5.3. Materials and Methods

5.3.1. Soil Sampling

Soil samples were collected from a permanently flooded vegetation-free site of constructed wetland at Olentangy River Wetland Research Park (ORWRP), The Ohio State University, Columbus, Ohio (40.01°N, -83.01°E; 226 m above sea level). A total

of 20 intact soil cores (10 cm × 8 cm) at a depth of 0-8 cm were collected randomly to get the typical wetland soil. The soil cores were separately taken in polyethylene bags and immediately transported to the laboratory for further experimentation. Aseptic conditions were maintained wherever necessary.

5.3.2. Physicochemical characterization of wetland soil

Soil samples were characterized for various physicochemical properties including pH, texture, redox potential, gravimetric water content, total C (TC), total N (TN), nitrate (colorimetric method), and ammonium (colorimetric method). Spasmodic analysis of soil samples was done at 0, 10, 20, and 30th day during incubation with ^{13}C labeled methane.

5.3.3. Soil amendments in flooded and non-flooded state

Soil-based PVC microcosms (10 cm dia × 12 cm height) with a drain hole were developed and amended with six concentrations of $(NH_4)_2SO_4$-N (0, 0.5, 1, 10, 25 & 50 mg N l^{-1}) in flooded and drained conditions. Besides, the non-amended control was only treated with deionized water. All microcosms were incubated for 10 days at 25-30 ^oC and water contents were maintained throughout the period. The soil samples were taken from the cores of both experimental setups for soil physicochemical characterization and further incubated with ^{13}C labeled -CH_4.

5.3.4. Incubation and labeling experiments with ^{13}C-methane

The treated soil samples were weighed (10 g each) in Petri dishes (5 cm) and incubated at 25 ^oC into plexiglass with labeled CH_4 ($^{13}CH_4$ with 99% purity, Cambridge Isotope Laboratories, Inc., Mass.) and unlabeled CH_4 between 60 and 100 ppm. Aerobic conditions, moisture saturation, and methane concentration in the head space were

monitored and maintained. Flow rates of $^{13}CH_4$ and $^{12}CH_4$ were sustained using flowmeters (Dwyer Instruments, Inc., IN, USA). The secondary effects of $^{13}CO_2$ are minimized during incubations by using 1M NaOH solution placed within incubating chambers. Triplicate soil samples, for both conditions and for each treatment, were incubated for 30 days. Soil samples were drawn for PLFA evaluation.

5.3.5. Extraction and quantification of PLFA

The PLFAs of treated soil were extracted according to the method of Bligh and Dyer (BLIGH and DYER 1959) with modifications (Chowdhury and Dick 2012) and analyzed via gas chromatography (Agilent, CA, USA). In brief, soil lipids were extracted with Bligh and Dyer reagent (1:2:0.8 v/v: chloroform, methanol, and citrate buffer) and separated into neutral, glyco, and polar lipids on a silicic acid column (Supelco Inc., USA). Fatty acid methyl esters (FAMEs) were obtained following esterification of fatty acids using alkaline methanolysis at 37 ºC and their analysis were carried out on a gas chromatograph (Agilent Technologies GC 6890). Helium was used as a carrier gas and temperature ramping was set from 120 to 260 ºC at a rate of 5 ºC per minute). The GC was equipped with a flame ionization detector (FID). During GC analysis, methyl nonadecanoate (C19:0-ME, Supelco Inc.) was used an as internal standard. MIDI Sherlock® Microbial Identification System (MIDI MIS) (MIDI, Inc., Newark, Delaware, USA) was used for identifying resulted peaks and integrating areas. The Peak chromatographic responses from GC were interpreted into nmol responses using the internal standard 19:0 fatty acid.

5.3.6. ^{13}C analysis of PLFA

Isotopic ratios of FAMEs were determined by gas chromatograph equipped with 30 m 0.25 mm i.d. HP-5ms fused silica capillary column and coupled with combustion isotope ratio mass spectrometer (GC-C-IRMS, Thermo Finnigan, CA, USA). The carrier gas, column temperature, program, and injector temperature were identical to the previously described. Reference CO_2 of known isotopic composition was injected three times at the start and end of each run. Peaks were simultaneously detected in Faraday collectors at *m/z* 44 ($^{12}C^{16}O_2$), 45 ($^{13}C^{16}O_2$ and $^{12}C^{17}O\,^{16}O$), and 46 ($^{12}C^{16}O^{18}O$ and $^{12}C^{17}O\,^{16}O$, $^{12}C^{17}O\,^{17}O$), amplified and corrected for the presence of ^{17}O at mass 45 using the Craig correction (Craig 1957). The isotopic composition of individual fatty acids is reported in the delta (δ) notation relative to the international internal standard Vienna Pee Dee Belemnite (VPDB), a South Carolinian carbonate rich in ^{13}C. The isotopic composition of fatty acids was corrected using exogenous methyl carbon obtained from methanol while of methanol ($\delta^{13}C$ = - 47.1 ‰, n=4) was determined by bulk stable isotope analysis (Pichlmayer and Blochberger 1988). To expresses precise GC-IRMS results in δ, the following notation was applied:

- $\delta\ (‰) = [(R_{FAME}/R_{PDB}] - 1) \times 10^3$ (1)

where R_{FAME} and R_{PDB} represents $^{13}C/^{12}C$ isotopic ratios of sample and VPDB, respectively. The higher $\delta\ ^{13}C$ represents higher ^{13}C in a sample. The isotopic fractionation of C during the transformation of substrate to product under consideration is expressed (Hayes *et al.* 1999), using the epsilon (ε) notation

- $\varepsilon = (\alpha_{A/B} - 1) \times 10^3$ (2)

where,

- $\alpha_{A/B} = (1000 + \delta_A)/(1000 + \delta_B)$ (3)
- $\alpha_{A/B}$ denotes factor of fractionation, δ_A denotes $\delta^{13}C$ of the substrate, and δ_B represents $\delta^{13}C$ of the fatty acid under consideration.

- **Calculation of the isotope ratios of the fatty acids.**

The correction of resulted FAME isotopic ratios with reference to methyl moiety isotopic ratios were made using following equation:

$$\delta^{13}C_{FA} = [(C_n + 1) \times \delta^{13}C_{FAME} - \delta^{13}C_{MeOH}]/ C_n \quad (4)$$

where:

$\delta^{13}C_{FA}$ represent $\delta^{13}C$ from equation (1),

C_n is the length of fatty acid in term of carbon number,

$\delta^{13}C_{FAME}$ represents $\delta^{13}C$ of the esterified fatty acids (FAMEs), and

$\delta^{13}C_{MeOH}$ represents $\delta^{13}C$ of the methanol used for the methylating reaction (Goodman and Brenna 1992, Abrajano *et al.* 1994).

To calculate ^{13}C enrichment ($\Delta\delta^{13}C$ ‰) of PLFAs, the values of unlabeled PLFA $\delta^{13}C$ were subtracted from the labeled ones. To compute incorporated ^{13}C into PLFA from the total PLFA, the fractional abundance ^{13}C in control (F_c) in enriched (F_e) PLFAs were calculated according to following equation:

Incorporation = $(F_e - F_c)$ $[PLFA]_c$.

5.3.7. PMO

The measurement of PMO rates was made as per the modified method reported (Roy Chowdhury *et al.* 2014) after (Crossman *et al.* 2004) and (Sundh *et al.* 1995). In brief, 10 ml of CH_4 with 99.99% purity was to prepare standard curves in a

polytetrafluoroethylene (PTFE) sampling bag. Calibration standards were prepared at different concentrations using suitable CH_4 volume in serum bottles and sealed with butyl rubber stoppers after being purged with nitrogen gas. PMO rates were measured for each sample treatment in triplicates using 15 g of soil and 15 ml of deionized water in Wheaton serum bottles. The samples were shaken for 1 hour and refilled with air in three cycles. The final concentration of 0.35% (v/v) CH_4 was created by injecting 0.32 ml of standard CH_4. The samples were shaken horizontally at 150 rpm at 25 ^oC for 8 hours and the gas phase was measured every one hour. A gas chromatograph (ShimadzuGC-2010, Kyoto, Tokyo) equipped with RT-QPLOT column and helium as a carrier gas, was used to quantify CH_4 and PMO rates were expressed as nmol CH_4 g^{-1}. The PMO rates are reported as nmol CH_4 g^{-1} dry weight soil h^{-1}. A gas chromatograph (Shimadzu GC-2010, Kyoto, Tokyo) equipped with a flame ionization detector (FID), an RT-QPLOT column, and helium as carrier gas was used to measure CH_4. The flow rate of hydrogen and helium used were of ultra-purity grade and their flow rate was maintained at 45 ml min^{-1} and 450 ml min^{-1}.

5.3.8. Statistical analysis

Multivariate analysis of variance (MANOVA) followed by Post HOC test (Tukey's HSD, $P < 0.05$) was performed wherever necessary using SPSS (IBM SPSS Statistics for Windows, Version 26.0., Armonk, NY, USA). Multivariate analysis of the data was performed using Principal Component Analysis (PCA) with standardization at samples and centered by specie function using Canoco for Windows (version 4.5) and drawn by CanoDraw (ter Braak and Šmilauer 2002). Redundancy analysis (RDA), using physicochemical properties of soil as species and PMO rates, type I and type II methanotrophs as environmental variables, was performed in Canoco for Windows

(version 4.5) with Monte Carlo permutation test (499 random permutations) and standardization with specie. Confirmatory factor analysis (CFA) was performed in R (package; Lavaan and semPaths).

5.4. Results

5.4.1. Physicochemical properties of soil

The pH of soil samples was monitored at 0, 7, 15, and 30th day (data shown for 30th day) of incubation and it varied significantly under flooded and non-flooded states (Table 5.1). Concisely, the decreasing trend for flooded conditions and increasing trend for the non-flooded state was observed with an overall range that lies within 6.89-7.84. Different concentrations of $(NH_4)_2SO_4$ resulted in an insignificant rise in pH in both flooded and non-flooded states. The Eh of soil samples varied insignificantly with different concentrations of NH_4-N under flooded (range = -88 to -126) and non-flooded conditions (range = -6 to -43). In the case of flooded soil, a decreasing trend was observed after 15 days. For non-flooded conditions, the mean value was twofold higher. Generally, an increase in concentrations of NH_4-N in treated soils resulted in decreased Eh. The NH_4-N concentration constantly depleted significantly in flooded states and insignificantly in the non-flooded state. Under the flooded condition, it slightly increased and then declined afterward in samples treated from 0-1 mg/L NH_4-N while it decreases by 28 and 60% at 25 and 50 mg/L of NH_4-N respectively. NH_4-N depletion rates were higher and varied from 85 – 96 % under un-flooded conditions. A significant and insignificant increase in TC and TN respectively was observed with 50 mg/L treatments. Similarly, C/N ratios were significantly different in high concentration treatments. According to PCA, pH and Eh were found strongly correlated with each other while weakly with NH_4-N (Figure 5.1). The PCA biplot showed 57.3 and 40.3%

variation at PC1 and PC2, respectively. TC, TN, and C: N was however found strongly correlated according to PCA biplot. A strong correlation between pH and EC was observed while EC and TN remained insignificantly different in different concentrations. The same concentrations of NH_4-N seem to vary only in flooded conditions. TC and C: N differ significantly in F50 and NF50 treatments. The PCA biplot showed considerable variation in flooded and non-flooded treatments with lower concentrations of NH_4-N.

5.4.2. Distribution of methanotrophs

Overall, type II methanotrophs were more abundant than type I. In flooded conditions, the increase in NH_4-N concentration up to 1 mg/L resulted in an increase in methanotrophs however further increase in concentration (1 to 50 mg/L) not only resulted in their decline but also type II methanotrophs outcompete type I (Figure 5.2). In non-flooded conditions (Figure 5.3), type II methanotrophs were significantly higher than Type I overall but they showed a consistent decrease from 70-30 nmol g^{-1} dwt. soil with increased concentration. Type I methanotrophs increased up to 25 mg/L but a decline was observed at 50 mg/L concentration. The maximum amount of Type I methanotrophs was 28 nmol g^{-1} dwt. soil at 25 mg/L concentration. Different concentrations of NH_4-N and water saturation significantly affected the abundance of both types of methanotrophs after 30-day incubation as shown in the PCA biplot which explained 86.5 and 12.9% variance at PC1 and PC2 respectively (Figure 5.4). The loadings also showed a weak correlation between PMO, type I methanotrophs, and type II methanotrophs.

Table 5.1: Measured soil physico-chemical properties of soil, PMO rates and PLFA based abundance of type I and type II methanotrophs at day 45 in flooded and non-flooded conditions. Values represent means ± standard error, n = 3. Different lower-case letters indicate significant difference at p <0.05 (Tukey's Post HOC test).

	pH	***Eh***	***NH4-N***	***TN***	***TC***	***C: N***	***PMO***	***TYPE I***	***TYPE II***
		mV	*µg/g dws*	*%*	*%*		*nmol CH_4 g^{-1} dws h^{-1}*	*nmol g-1 dws*	*nmol g-1 dws*
F0	6.89± (0.01) a	-88± 42.19) a	9.93± (1.19) a	0.28± (0.02) a	4.21± (0.25) a	0.07± (0.00) a	13.91± (1.08) abc	19.46± (6.97) a	18.77± (1.53) a
F0.5	7.00± (0.06) a	-91± (31.01) a	12.53± (0.99) ab	0.29± (0.01) a	4.36± (0.32) a	0.07± (0.00) a	15.44± (1.16) ab	33.09± (14.29) a	28.46± (3.87) ab
F1	6.97± (0.02) a	-118.33± (21.14) a	12.92± (0.43) ab	0.27± (0.01) a	3.96± (0.34) a	0.07± (0.00) a	12.01± (1.15) abc	31.98± (8.02) a	41.54± (14.16) ab
F25	7.05± (0.02) a	-123± (22.55) a	13.86± (0.41) b	0.30± (0.00) a	4.13± (0.24) a	0.07± (0.00) a	10.49± (1.52) bc	23.74± (8.07) a	38.56± (7.04) ab
F50	7.01± (0.09) a	-126.67± (10.91) a	11.15± (1.28) ab	1.31± (0.03) b	5.73± (0.18) a	0.23± (0.01) b	7.65± (1.29) c	14.40± (5.37) a	31.62± (5.65) ab
NF0	7.60± (0.14) b	-8± (40.72) a	1.59± (0.62) c	0.26± (0.09) a	3.78± (1.26) a	0.07± (0.01) a	17.78± (0.88) ab	13.67± (2.14) a	70.43± (14.46) b
NF0.5	7.66± (0.07) b	-6.33± (12.02) a	1.09± (0.22) c	0.28± (0.09) a	4.07± (1.36) a	0.07± (0.00) a	16.93± (1.40) ab	6.51± (1.26) a	47.12± (10.46) ab
NF1	7.84± (0.03) b	-11.33± (42.26) a	1.91± (0.29) c	0.28± (0.09) a	3.88± (1.29) a	0.07± (0.00) a	13.37± (2.36) abc	15.12± (6.46) a	45.61± (5.83) ab
NF25	7.76± (0.01) b	-38± (30.14) a	0.75± (0.20) c	0.26± (0.09) a	3.71± (1.24) a	0.07± (0.00) a	15.96± (0.72) ab	28.05± (12.79) a	36.76± (11.62) ab
NF50	7.61± (0.06) b	-43± (21.79) a	1.30± (0.53) c	1.11± (0.37) c	5.34± (1.78) a	0.21± (0.02) b	16.80± (0.52) ab	17.93± (5.77) a	30.62± (7.45) ab

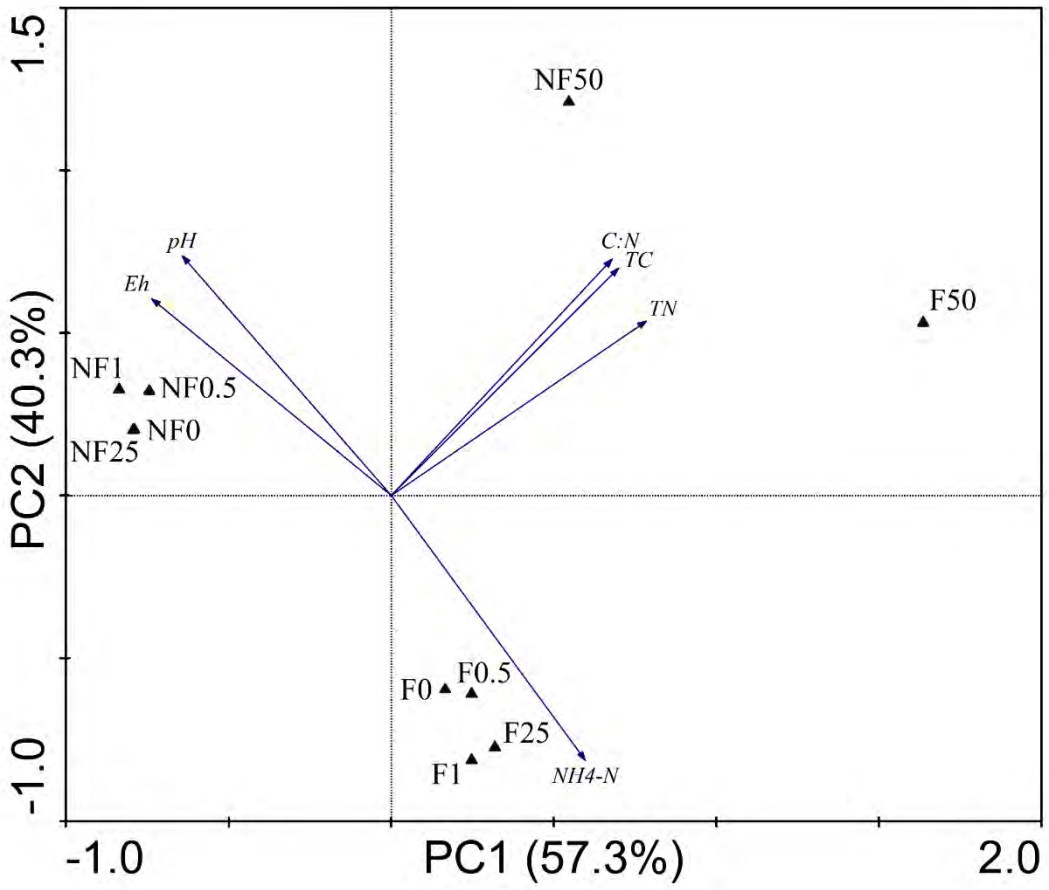

Figure 5.1: PCA biplot of the soil physicochemical properties against different concentrations of NH_4-N in flooded and non-flooded conditions.

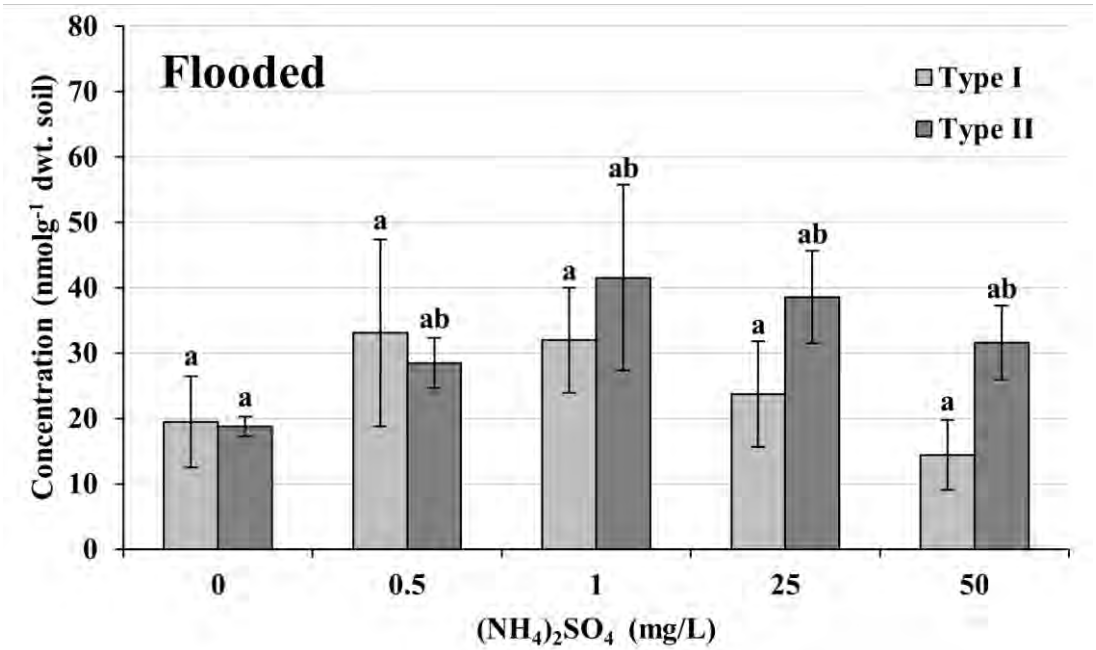

Figure 5.2: Relative abundance of type I and type II methanotrophs in flooded conditions at varying concentrations of NH_4-N. Different lowercase letters indicate significantly ***different means p<0.05 (Tukey's Post Hoc test).*** *Values represent means (n = 3).*

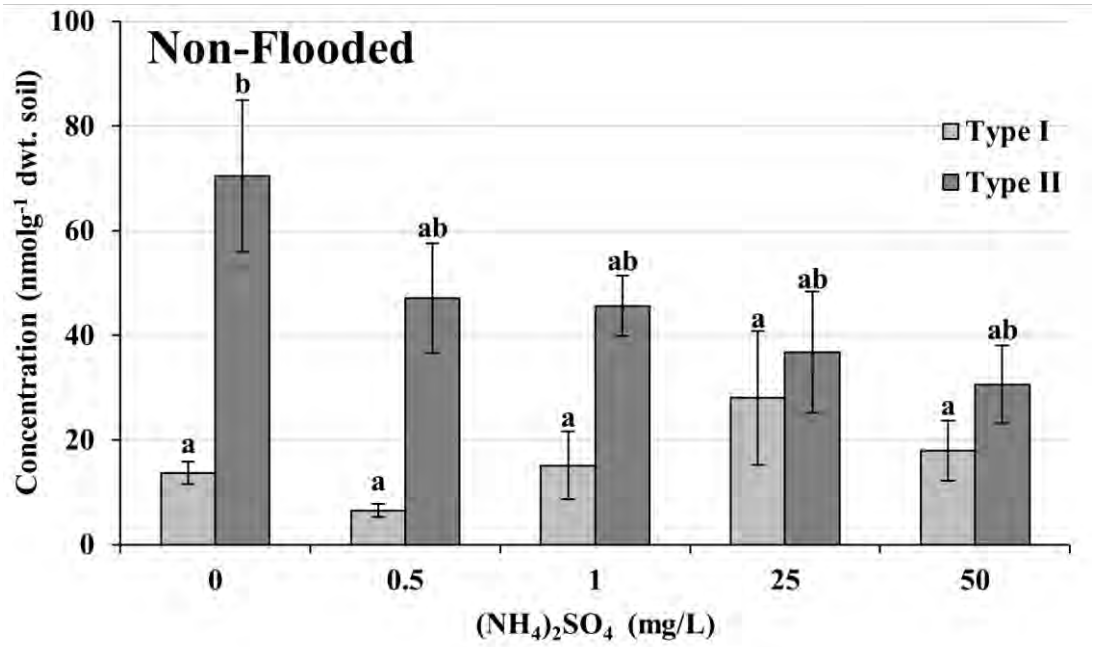

Figure 5.3: Relative abundance of type I and type II methanotrophs in non-flooded conditions at varying concentrations of NH_4-N. Different lowercase letters indicate ***significantly different means p<0.05 (Tukey's Post Hoc test).*** *Values represent means (n = 3).*

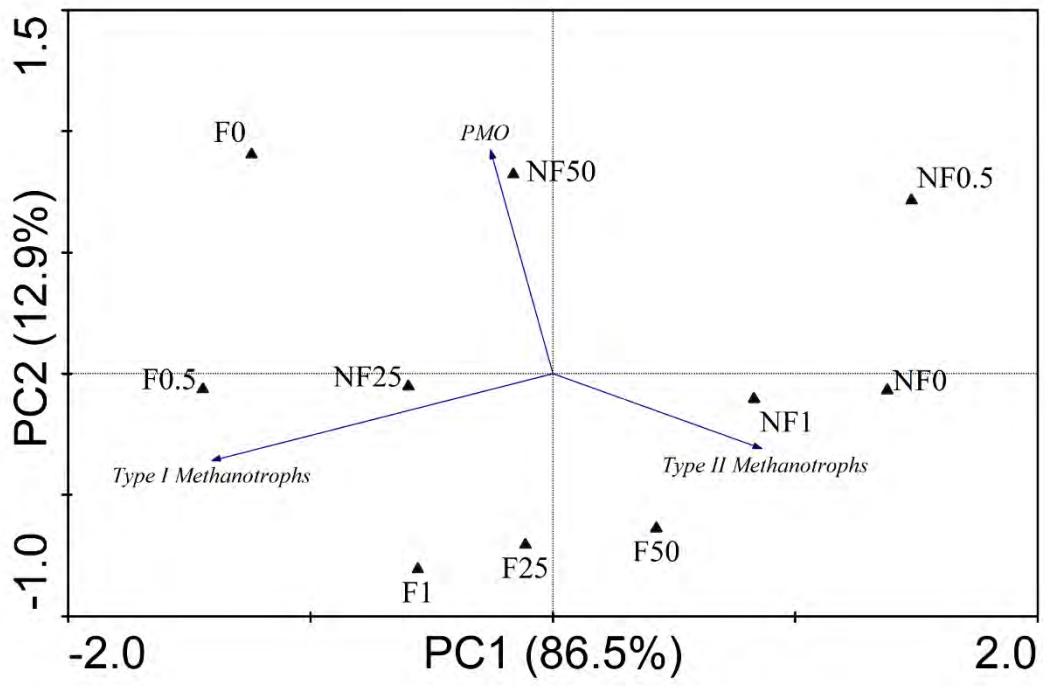

Figure 5.4: PCA biplot of PMO, type I, and type II methanotrophs against varying concentrations of NH4-N in flooded and non-flooded conditions.

5.4.3. PMO of Soil

PMO rates were significantly varied at different concentrations of NH_4-N under flooded and un-flooded conditions. In flooded treatment, an inverse trend was observed in the PMO rates with increasing concentration of NH_4-N in the treated soil samples where the maximum PMO value was 15.5 at 0.5 mg/L and the minimum was 7.6 at 50 mg/L of NH_4-N (Figure 5.5). For non-flooded conditions, the highest value was observed in un-amended soil, and it differs insignificantly with PMO rates at 0.5, 25 & 50 mg/L of NH_4-N. Correlation indexes showed a weak association between PMO rates and varying concentrations of NH_4-N in soil under flooded and un-flooded conditions. The RDA analysis of soil physicochemical properties with methanotrophs (type I and type II) and PMO showed a strong correlation between PMO and type II methanotrophs and

a weak correlation between type I and type II methanotrophs (Figure 5.6). The triplot showed 55.3 and 8.6% variance at RD1 and RD2 axes, respectively. The PMO rates were significantly associated with soil physicochemical properties (P = 0.02, F= 5.95).

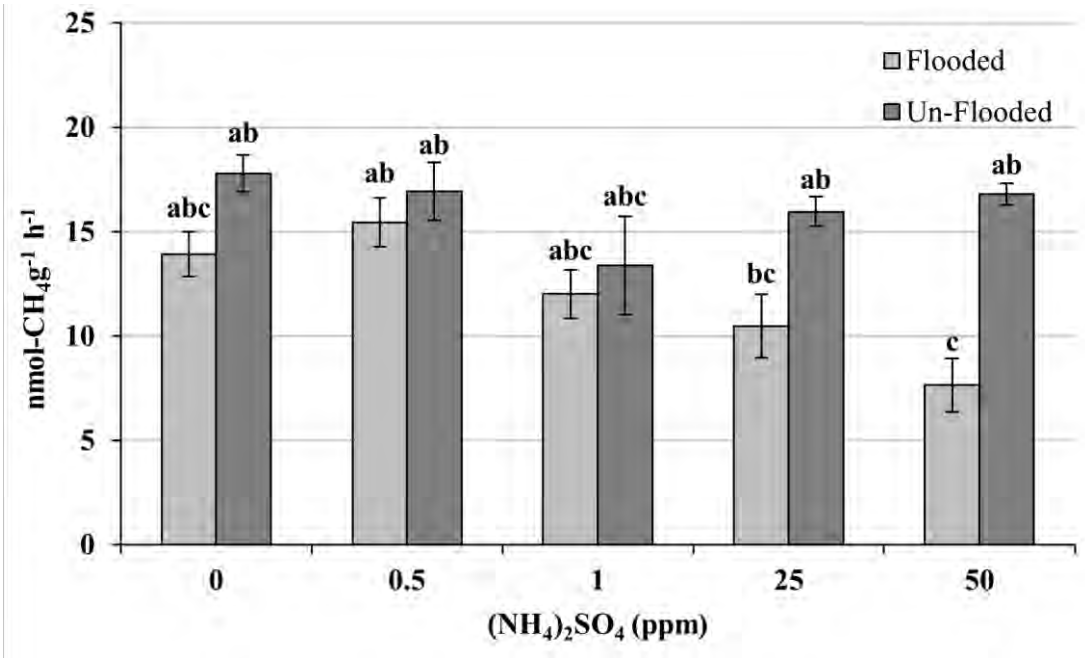

*Figure 5.5: PMO activities under flooded and non-flooded conditions in all treatments. Values represent means and error bars represent SE (n = 3). Different letters indicate significantly differe**nt means p<0.05 (Tukey's Post Hoc** test).*

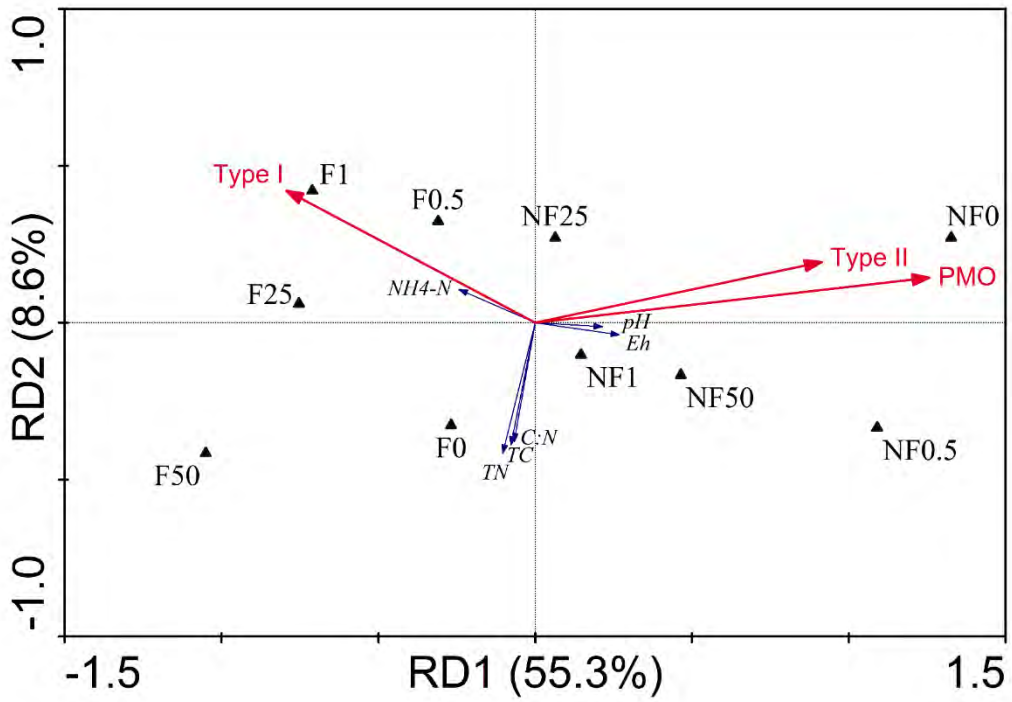

Figure 5.6: Triplot ordination of Redundancy analysis (RDA) of the correlations soil physicochemical properties with PMO, type I, and type II methanotrophs.

5.4.4. Controls on the relative abundance of methanotrophs and PMO

The influence of soil physicochemical characterization on the relative concentration of methanotrophs (type I and II) in flooded and non-flooded conditions are depicted by CFA (Figure 5.7 and 5.8). In flooded conditions, the strongest factors influencing both types were TN and C/N. pH and Eh positively influenced type II methanotrophs only. TC on the other hand negatively influenced both types. Type I and type II were positively and negatively related to PMO respectively. In non-flooded conditions, TN and C/N had a strong negative impact on type II methanotrophs while a positive impact on type I methanotrophs. pH and Eh did not impact significantly to any type of methanotroph while TC had a positive impact on type II while the negative impact on

type I. The PMO rates were insignificant for both types of methanotrophs in non-flooded conditions.

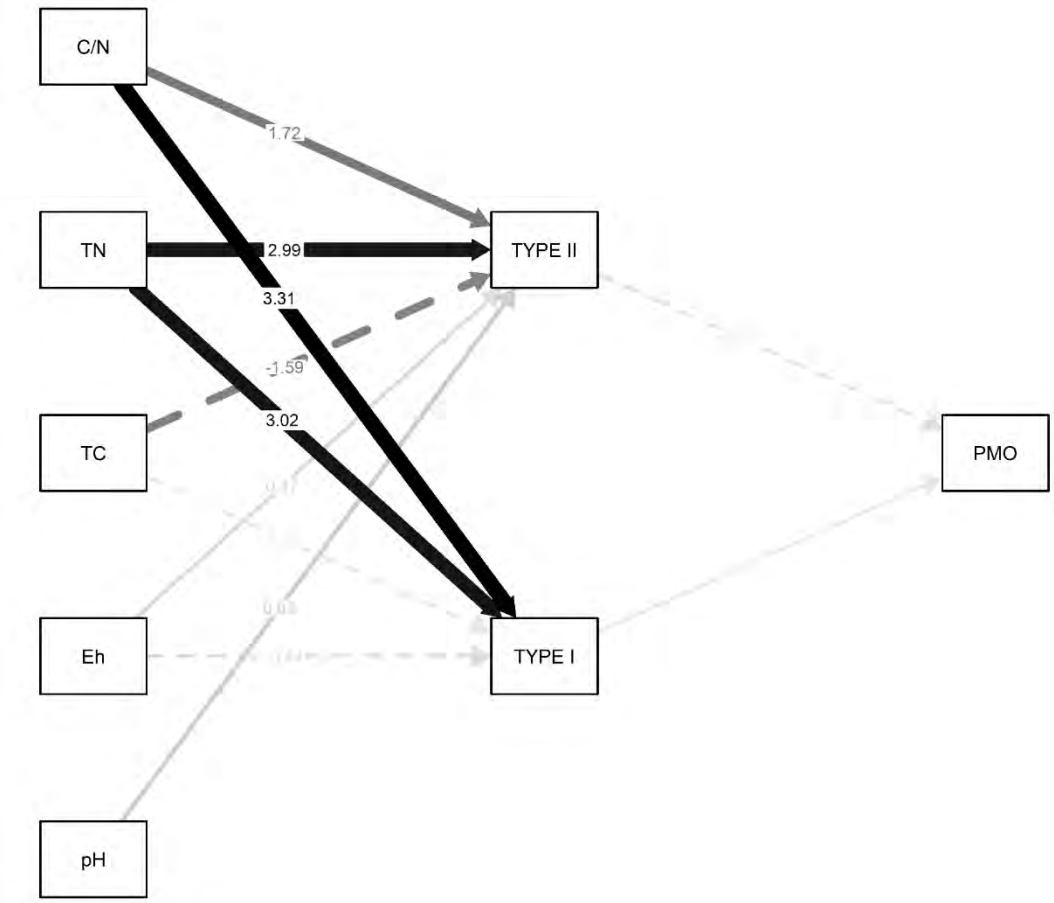

Figure 5.7: CFA of soil physicochemical properties, PMO, and type I and II methanotrophs in flooded conditions at different concentrations of $(NH_4)_2SO_4$.

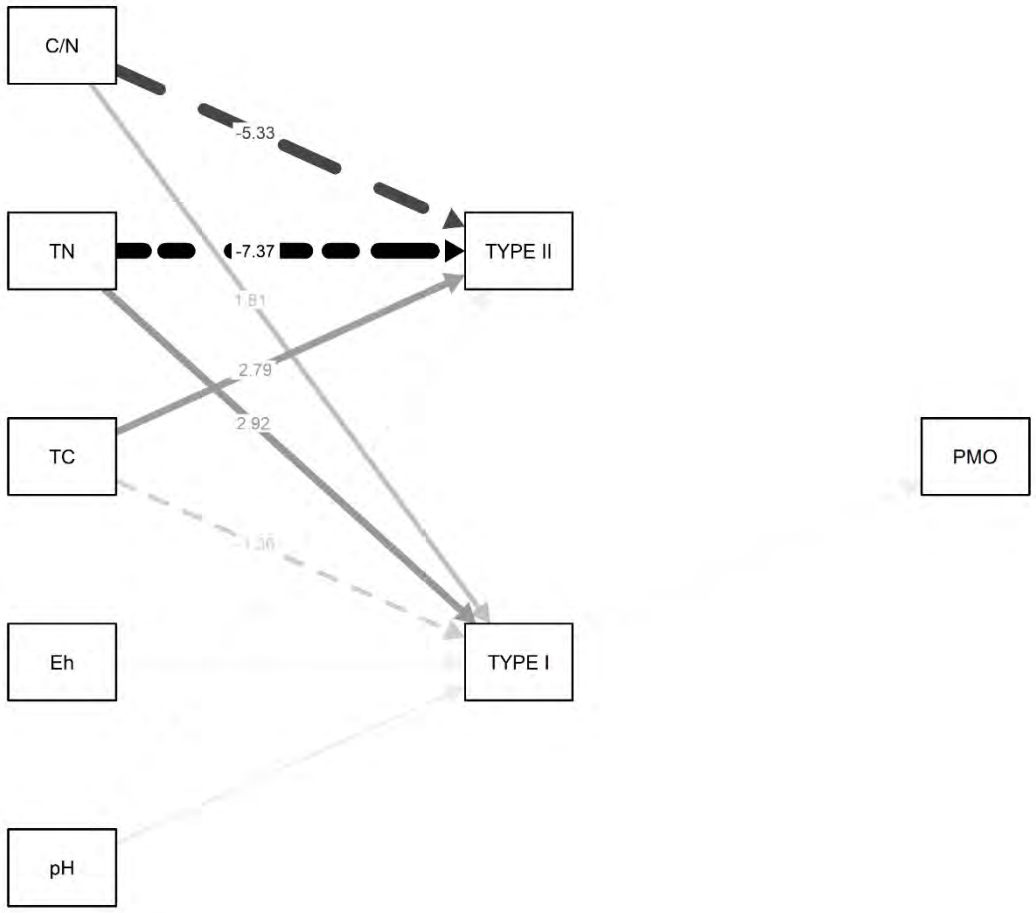

Figure 5.8: CFA of soil physicochemical properties, PMO, and type I and II methanotrophs in non-flooded conditions at different concentrations of (NH4)2SO4.

5.5. Discussion

Methane emission in the atmosphere is a global issue and threat to our environment. It has greater Global warming potential than CO_2 thus trapping 25 times more heat in the atmosphere (Bernstein et al., 2008). Its normal concentration in the atmosphere is necessary for maintaining the Earth's temperature . However, its concentration in the atmosphere is increasing drastically due to anthropogenic activities. Rice fields are one of the most important source of methane emission and accounts for 15-20% anthropogenic methane emission globally. Due to increase in population, there is a significant increase in rice production which leads to the greater use of organic and inorganic fertilizers. These fertilizers turn the soil into the source of methane from the sink. These fertilizers actualy affect different types of methanogens and methanotroph present in the paddy fields . Some fertilizers can enhance the methanogenesis and others can inhibit it. Similarly, some fertilizers can enhance the methnotrophy while other can decrease it. Different combinations of fertilizers when applied to the paddy fields may have different effect on the methanogens and methanotrophic community.

Different concentrations of ammonium sulfate had a varying effect on soil physicochemical properties, methane oxidation rates, and relative abundance of methanotrophic bacteria in flooded and non-flooded microcosms. The relative abundance of type I and type II methanotrophs showed significant variation in flooded and non-flooded conditions. Because most of the previous studies are focused on flooded conditions which necessitate understanding its comparative effects in the non-flooded state.

Generally, agricultural or wetland soils that have been regularly exposed to flooding events and varying N species are prone to large physicochemical and biogeochemical

changes (Hamilton 2010, Laanbroek 2010). These intermittent changes lead to varying methane fluxes and eventually to promoting or negative responses to the ecosystems (Singh *et al.* 2010). N supplementation such as $(NH_4)_2SO_4$ is proven to increase methanotrophic PLFA by 2-3 fold in planted microcosms as compared to non-fertilized or non-planted controls (Bodelier *et al.* 2000, Walkiewicz *et al.* 2018) which is in accordance with our results. These results were also supported by CFA (Figure 5.7) which indicated TN and C/N to be the most influential environmental variables to controlling type I and type II methanotrophs in flooded conditions. In non-flooded conditions, however, the same variable had a strong negative influence on type II methanotrophs while a slightly positive influence on type I methanotrophs. pH is another crucial factor with respect to microbial community ecology in flooded conditions (Zhao *et al.* 2020) which is evident in our case for type II methanotrophs (Figure 5.7).

The soil methanotrophic community was greatly dominated by type II which is more pronounced in non-flooded conditions (Wang *et al.* 2020). Their dominance is well reported in high levels of CH_4, limiting concentrations of N, and growth-restricted environments (Hanson and Hanson 1996, Shrestha *et al.* 2010, Shukla *et al.* 2013). No significant variation in type I methanotrophs was observed in any concentration of $(NH_4)_2SO_4$ in flooded or non-flooded treatments. Compared to controls i.e., F0 and NF0, type II methanotrophs significantly increased in flooded conditions (Mayumi *et al.* 2010, Ma and Lu 2011) and significantly decreased in non-flooded conditions (Reay *et al.* 2001, Malyan *et al.* 2021). Despite being significantly reduced in different concentrations of $(NH_4)_2SO_4$, type II methanotrophs were highest in NF0 probably due to the absence of inhibitory or interactive processes.

PLFA concentration showed a strong positive correlation between the potential CH_4 oxidation rates and the relative abundance of methanotrophs. Among methanotrophs, Type II appears to be the driver of CH_4 oxidation under favorable conditions of high CH_4 concentration and oxygen availability (Shukla *et al.* 2013). One of the most important results of this study is the strong positive correlation between the potential CH_4 oxidation rates and the type II methanotrophs as reflected by its respective PLFA biomarker concentration (Fig 6). Evidence for this is the strong correlation between PMO rates and the type II methanotrophs and our results suggest that CH_4 concentration is a major controller of PMO and methanotrophs populations. In comparison to methane oxidation, methane production is a favorable process in permanently flooded soil. Thus, it seems higher CH_4 concentrations encourage methanotrophic activity. The transport of CH_4 from sources to sinks and mass transfer from gas to aqueous phase may be the limiting factor in CH_4 oxidation in wet, well-aggregated soils (Bogner *et al.* 1997). The application of N fertilization can be stimulatory or inhibitory regarding CH_4 oxidation competence depending on the predominant type I or type II methanotrophs, respectively (Alam and Jia 2012). Since our community had predominant type II methanotrophs, decrease PMO activity with a higher concentration of $(NH_4)_2SO_4$ is well justified.

The labeling of 18:1ω7c indicated that CH_4 consumption was carried out mostly by more active Type II methanotrophs. The 18:1ω7c PLFA has been found in all known Type II methanotrophs (Keller *et al.* 1993, Boeckx *et al.* 1996, Hamilton 2010, Willers *et al.* 2015). Labeling could also be detected in 18:1ω8c, the PLFA characteristic of *Methylocystis* in soils from PFS and IFS but not in the UPS soils (Keller *et al.* 1993). However, it cannot be conclusively determined which group was controlling CH_4 oxidation. The relatively low or no labeling of the *Methylocystis*-specific PLFA

18:1ω8c in soils incubated at the low CH_4 mixing ratio compared to the high CH_4 indicates that other Type II methanotrophs were also active at the low CH_4 mixing ratio. Another possibility is that other Type II methanotrophs like the *Methylocapsa* were active but could not be detected by PLFA analyses. Type II and type X methanotrophs commonly can fix N_2, whereas N_2 fixation is absent in known type I methanotrophs.

The urea or $(NH_4)_2SO_4$ application within 200-400 kg ha^{-1} range enhance methane oxidation rates in rhizosphere regions of rice paddies. The same results have been reported in case of supplemented and planted paddy soil microcosms whereas the controls (non-supplemented and non-planted) showed no activity within 12 hours of experiment. The controls well over 50 hours to initiate methane oxidation. The essential role of ammonium is demonstrated in methane oxidation since its addition led to immediate activation of methanotrophs. The ^{14}C-PLFAs analyses had indicated the primary role of type II methanotrophs in methane metabolism in non-vegetated and non-supplemented soils. In contrary, ^{14}C-PLFA fingerprints analysis from different soils inferred that the urea or $(NH_4)_2HPO_4$ supplementation for longer periods resulted in enhanced activity of methane oxidation in rhizosphere by type I and II methanotrophs.

5.6. Conclusion

Type II methanotrophs increased significantly with increased concentration of $(NH_4)_2SO_4$ flooded conditions while they decreased significantly with $(NH_4)_2SO_4$ in non-flooded conditions. For type I methanotrophs, however, the shift in abundance was insignificant. The PMO rates had a strong positive correlation with the type II methanotrophs while a strong negative correlation with the type I methanotrophs. In

flooded conditions, the PMO rates decreased significantly while they remained more or less substantially stable in the non-flooded state. According to CFA, TN and C/N influenced type I and type II methanotrophs positively in flooded conditions while pH and Eh influenced type II methanotrophs only. TC had a strong negative influence on type II methanotrophs. TN and C/N had a strong negative influence on type II methanotrophs only in the non-flooded state while pH and Eh had an insignificant influence on methanotrophs. Type II methanotrophs are the drivers of methane oxidation potential in saturated soils.

Conflicts of Interest

None

CONCLUSIONS

6. Conclusions

The current study comprehended the single and integrative use of conventional fertilizers on soil physicochemical properties, microbial community ecology by PLFA biomarkers and 16S rRNA HTS sequencing, functional predictions by a bioinformatic tool, enzymes involved in CNP transformations, and plant biomass. Following general conclusions can be drawn from this study.

1. Soil pH, GWC, SOC, C/N, and AK have a profound impact on soil quality and physicochemical characterization of paddy soil. Different treatments showed significant variations in these attributes. The RDA and CFA results confirmed the significant contribution of these attributes towards PLFA profiling of bacterial and fungal communities.
2. Overall, archaeal, and bacterial communities were found more responsive compared to fungi due to waterlogged conditions. Among archaea, methanogenic genera were the most dynamic in different treatments with their least relative abundance in controls, U, K, and UK treatments. Considering bacterial community, *Proteobacteria*, *Actinobacteria* and *Firmicutes* increased in all treatments while a negative trend was found for *Chloroflexi*, *Bacteroidetes,* and *Verrucomicrobia.*
3. In functional prediction at level 1, most of the gene predicted accounted for metabolism for which KP and UKRs showed the highest values while C_0, C_f, and P showed the least values. Considering metabolism, most of the functional predictions belong to an amino acid, carbohydrate, and energy metabolism category. At the energy metabolism level (third level), oxidative phosphorylation, carbon fixation pathways in prokaryotes, and methane

metabolism were the most predicted functions. Generally, C_0, C_f, and P were the treatments that showed the least gene/ process predictions for any functional level.

4. GLU was found to be the most influential enzyme in the bacterial and fungal community while C/N and F/B negatively impact GLU activities.
5. KCl possibly produces an inhibitory impact on plant biomass, microbial composition, and diversity.
6. Overall, UP and UPK treatments accounted for better plant growth without significantly altering bacterial and fungal communities and resulted in increased biomass and better yield. Comparing UP and UPK, UP should be the choice since KCl influenced negatively on paddy field soils.
7. Type II methanotrophs compounded significantly with increased concentration of $(NH_4)_2SO_4$ in flooded conditions while they decreased significantly with $(NH_4)_2SO_4$ concentrations in non-flooded conditions.
8. PMO rates and type II methanotrophs are strongly correlated signifies the importance of type II methanotrophs in methane oxidation.

FUTURE PROSPECTS

7. Future Recommendations

1. The study should be conducted on plot-based mesocosms to have a deeper and wider insight concerning fertilizer usage and microbial community ecology.
2. Application of fertilizers with varying concentrations should be investigated with more focus on sustainable agriculture.
3. Integration of more organic fertilizers like farm manure, compost, biosolids, and crop residue should be investigated depending upon their regional availability.
4. The inhibitory impact of KCl should be investigated with multiple conventional inorganics and organic fertilizers in microbial ecology and crop yield.
5. The same study should be applied to plot-based planted mesocosms for a full cultivation period to have a better dynamic variation of CNPK mineralization, microbial community composition, and enzymatic activities.
6. Some important biological activities like nitrification, ammonium oxidation, methanogenesis, methanotrophy, and anaerobic methane oxidation must be monitored meanwhile.
7. The macro and micronutrient content of the crops also needs to be compared under growth in a range of soils in your country.

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9. Appendix

9.1. A1

Table A1: Alpha diversity indices i.e., Pielou's E and Shannon's for each sample. OTU = Operational Taxonomic Units, PD = phylogenetic diversity.

Treatment	***Pielou's E***	***Shannon's Index***
C_0	0.92	9.35
C_f	0.92	9.55
C_{neg}	0.92	9.96
U	0.92	10.02
P	0.92	9.56
K	0.90	9.74
Rs	0.91	10.01
UP	0.91	9.91
KP	0.91	10.25
RsP	0.92	10.15
UK	0.91	10.08
RsK	0.91	10.06
URs	0.92	9.90
UPK	0.91	9.99
UPRs	0.92	10.21
UKRs	0.91	10.14
PKRs	0.92	10.14
UPKRs	0.91	9.72

9.2. A2

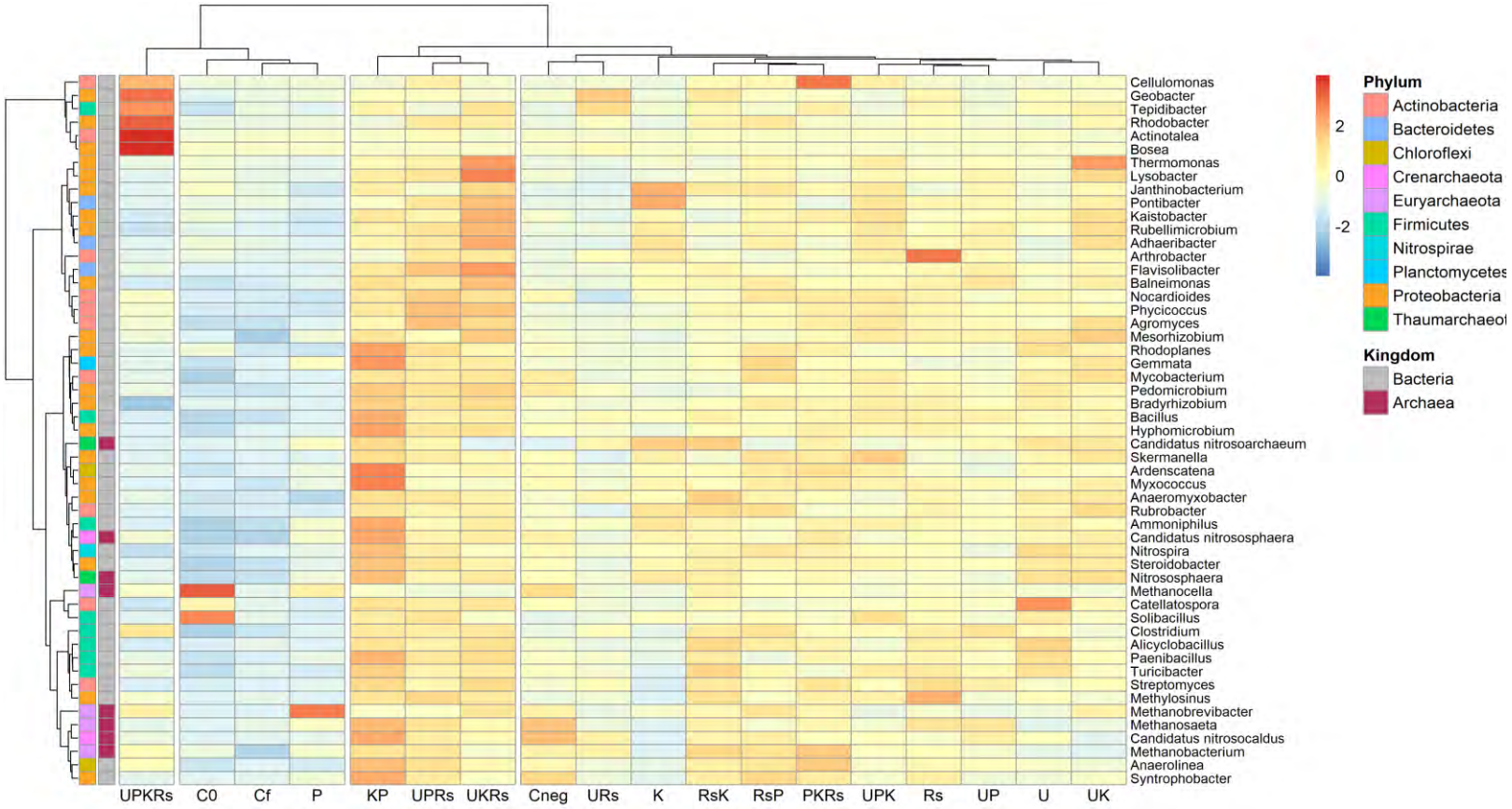


Figure A1: Heat map-based hierarchical clustering of identified archaeal and bacterial relative abundance in different treatments.

9.3. A3

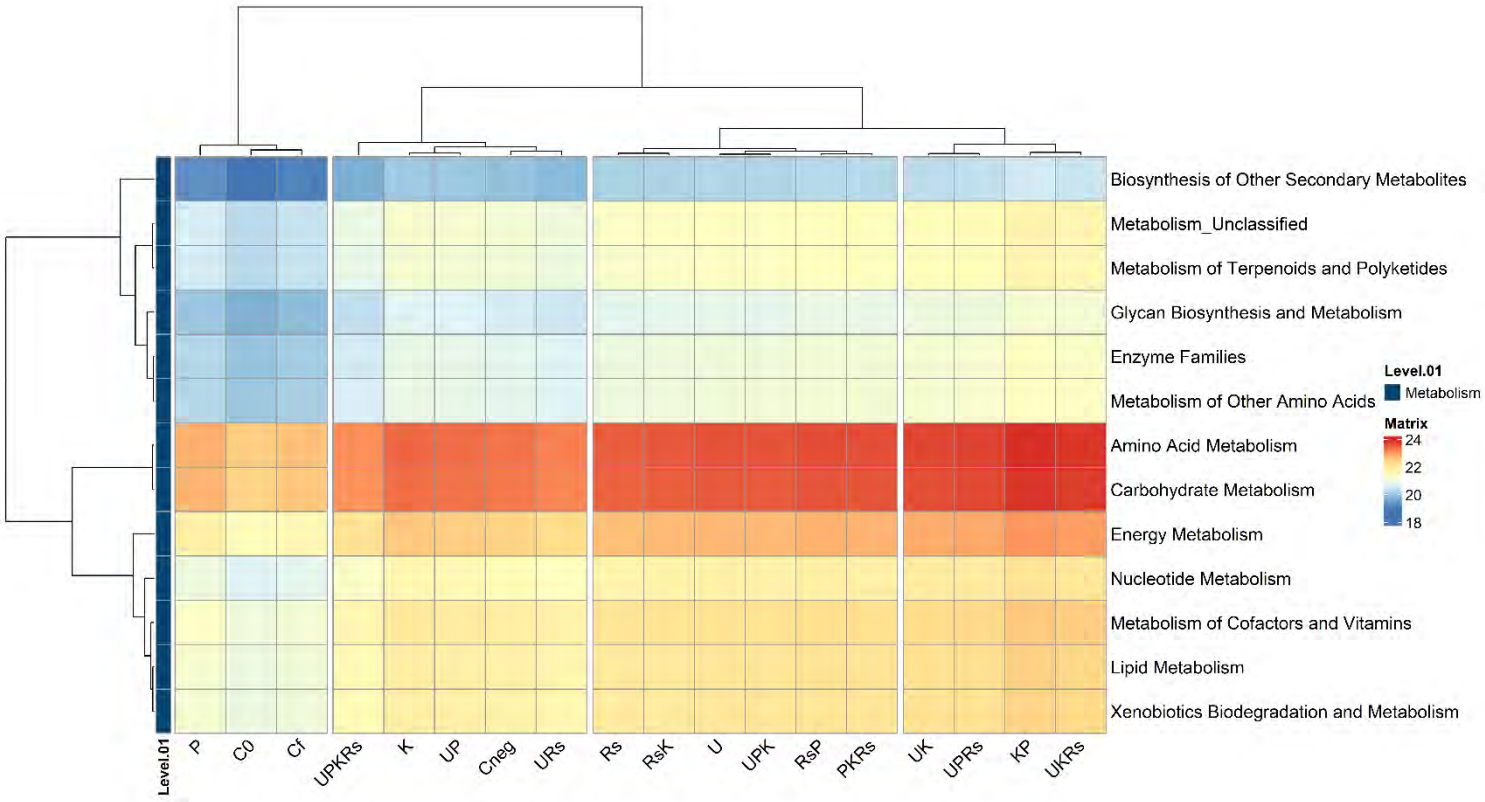


Figure A2: PICRUSt based functional prediction of metabolism (level 02) in different fertilizer treatments.

9.4. A4

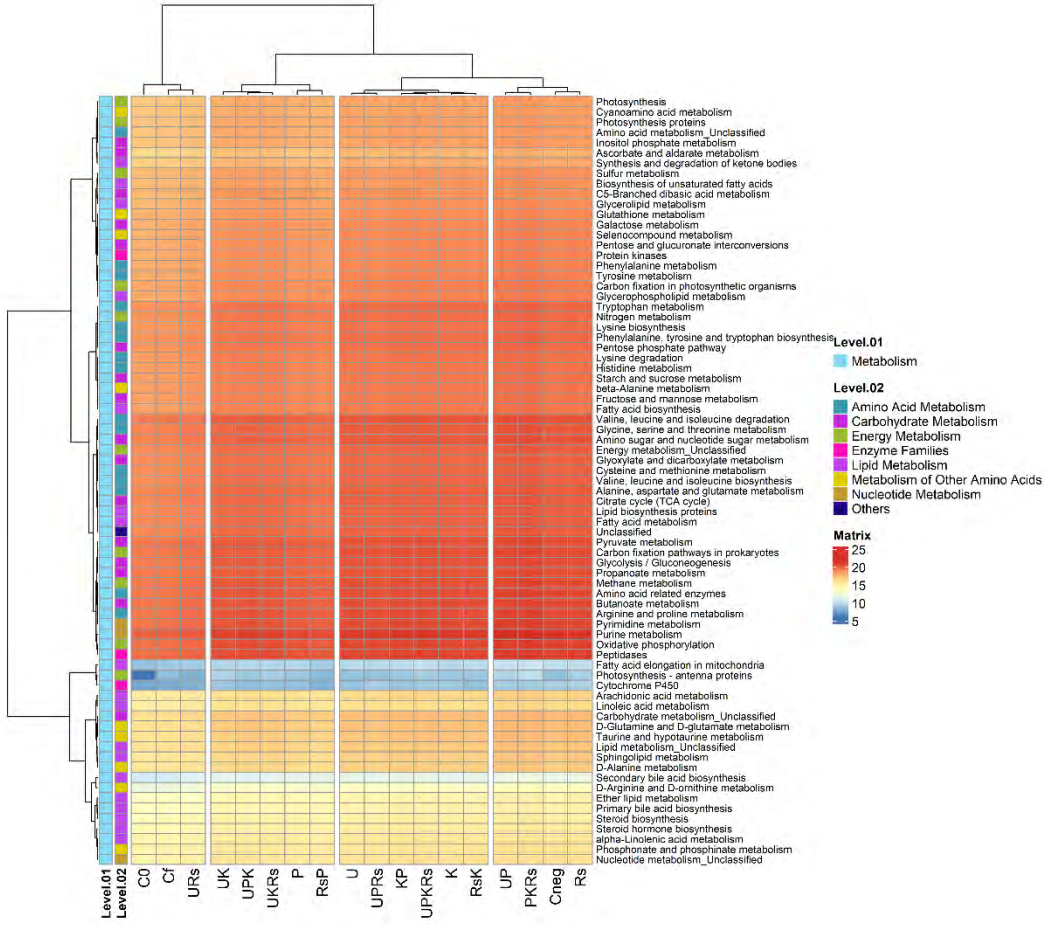


Figure A3: PICRUSt based functional prediction of metabolism (level 03) in different fertilizer treatments.

9.5. A5

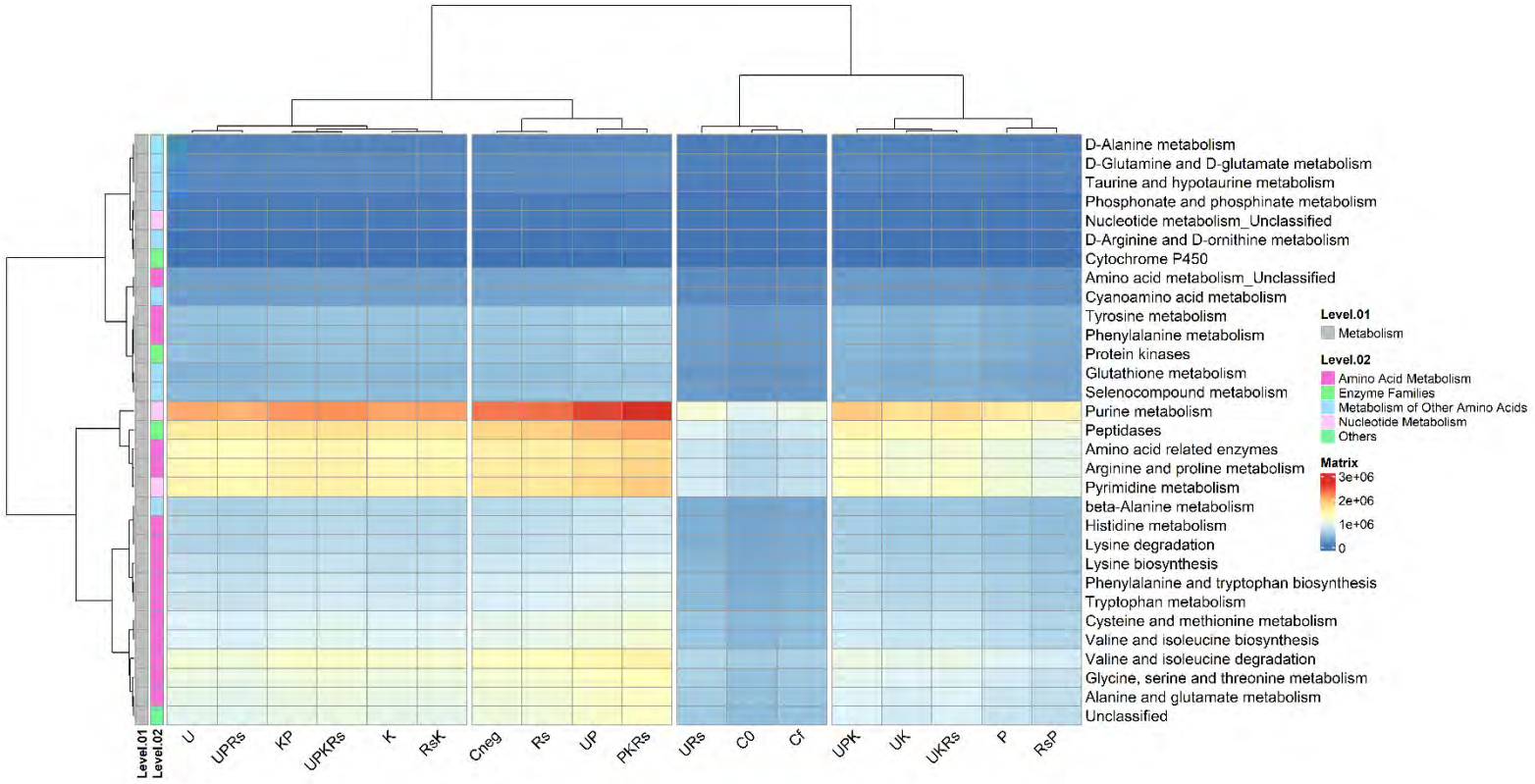


Figure A4: PICRUSt based functional prediction of enzyme families and metabolism of amino acids and nucleotide in different fertilizer treatments.

9.6. A6

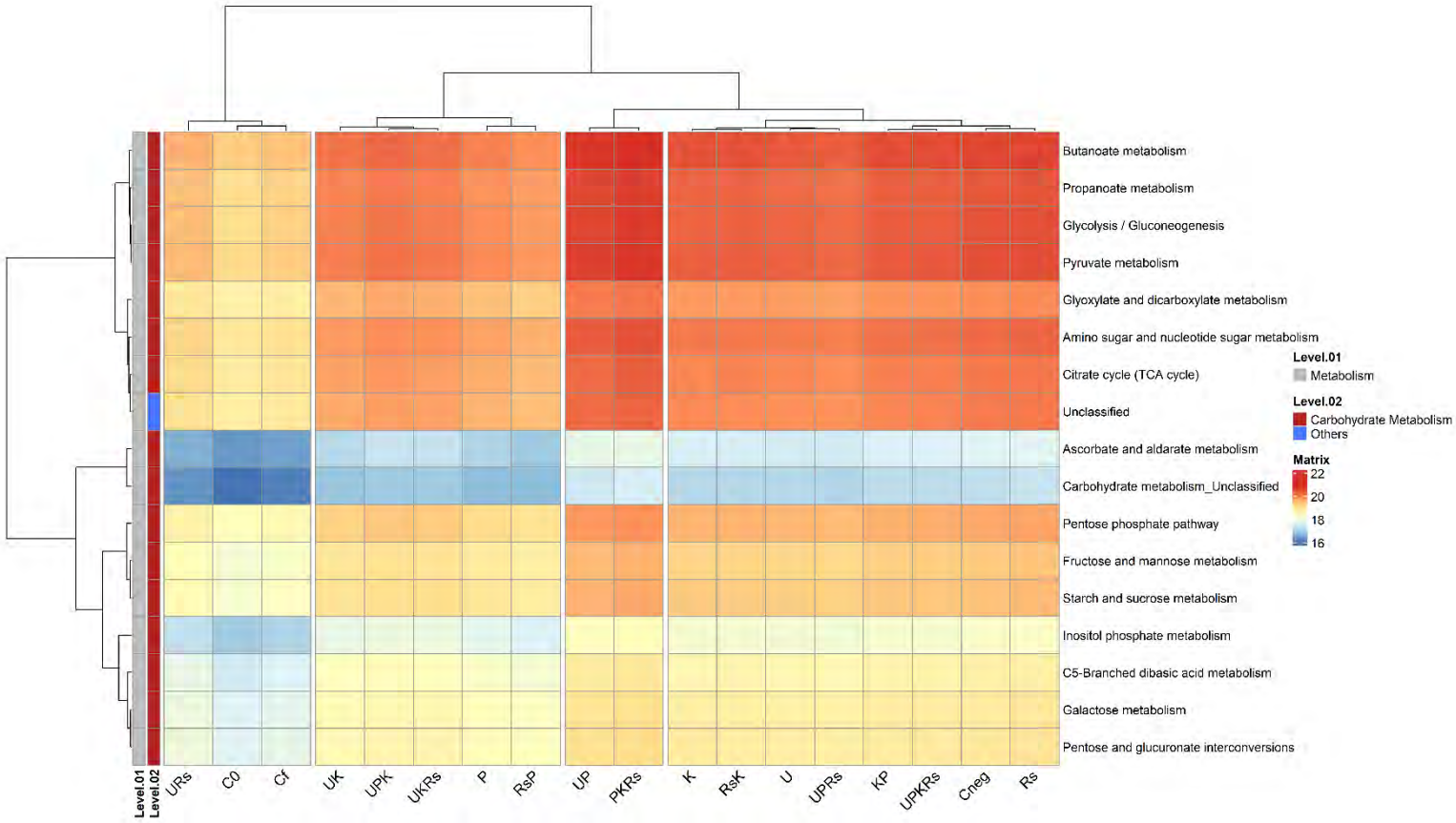


Figure A5: PICRUSt based functional prediction of metabolism of carbohydrates in different fertilizer treatments.

9.7. A7

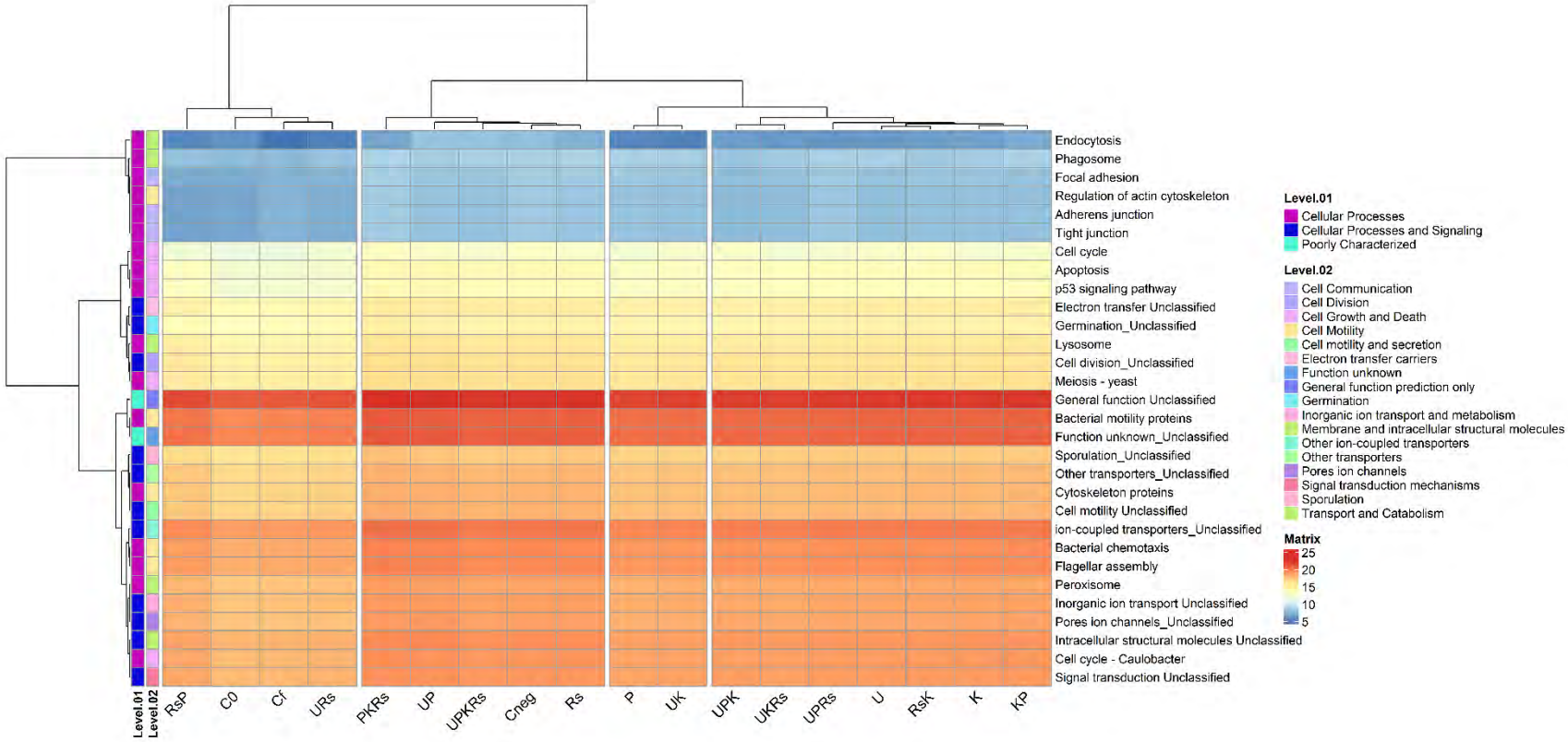


Figure A6: PICRUSt based functional prediction of cellular processes, cellular processes, and signaling and poorly characterized genes at levels 2 and 3 in different fertilizer treatments.

9.8. A8

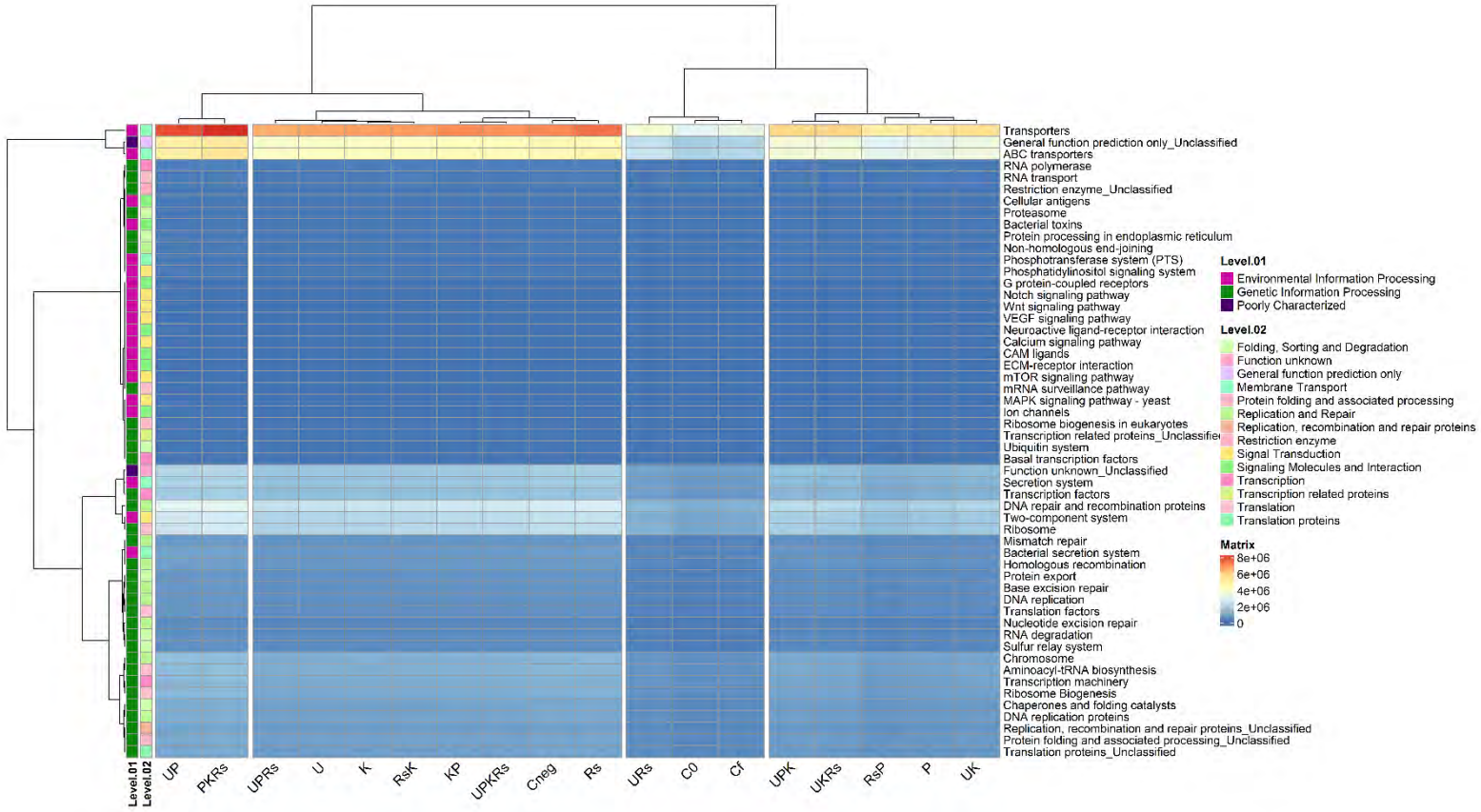


Figure A7: PICRUSt based functional prediction of environmental information processing, genetic information processing, and poorly characterized genes in different fertilizer treatments.

9.9. A9

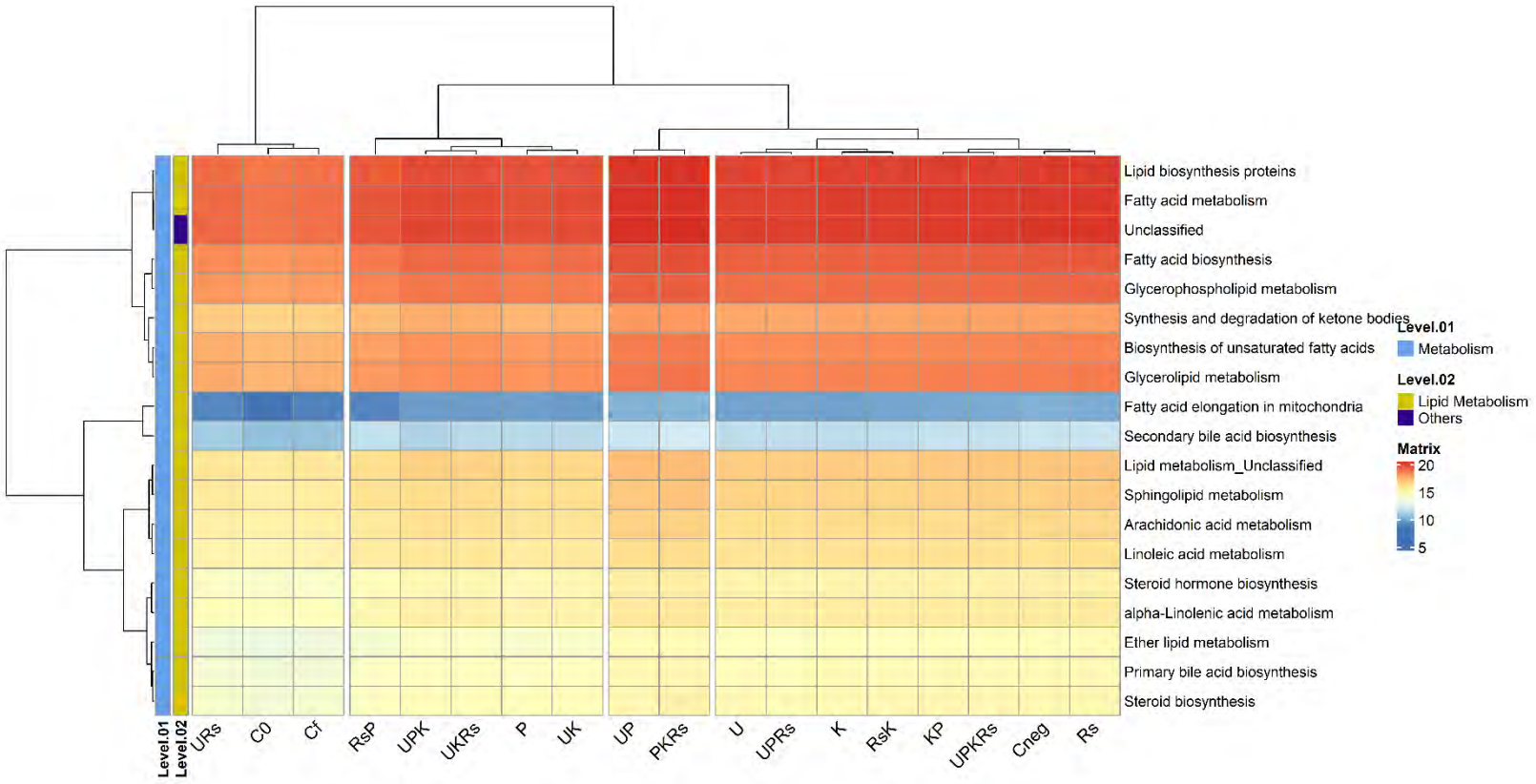


Figure A8: PICRUSt based functional prediction of lipid metabolism at level 03 in different fertilizer treatments.

9.10. A10

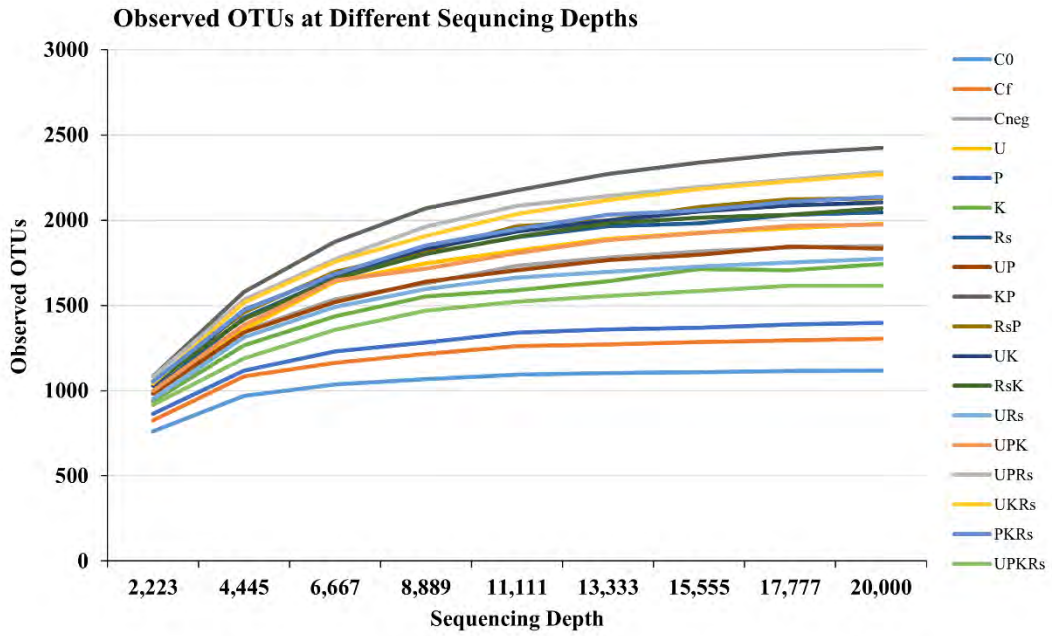


Figure A9: Observed OUT in all supplemented treatments at different sequencing depths.

9.11. A11

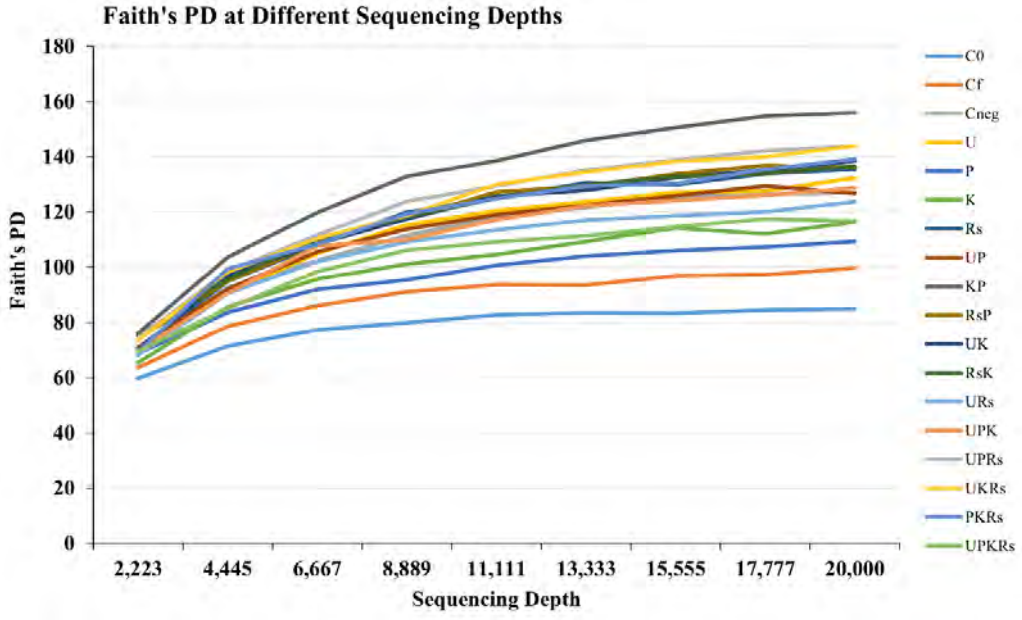


*Figure A10**: Values of Faith's phylogenetic diversity (PD) in all supplemented** treatments at different sequencing depths.*

9.12. A12

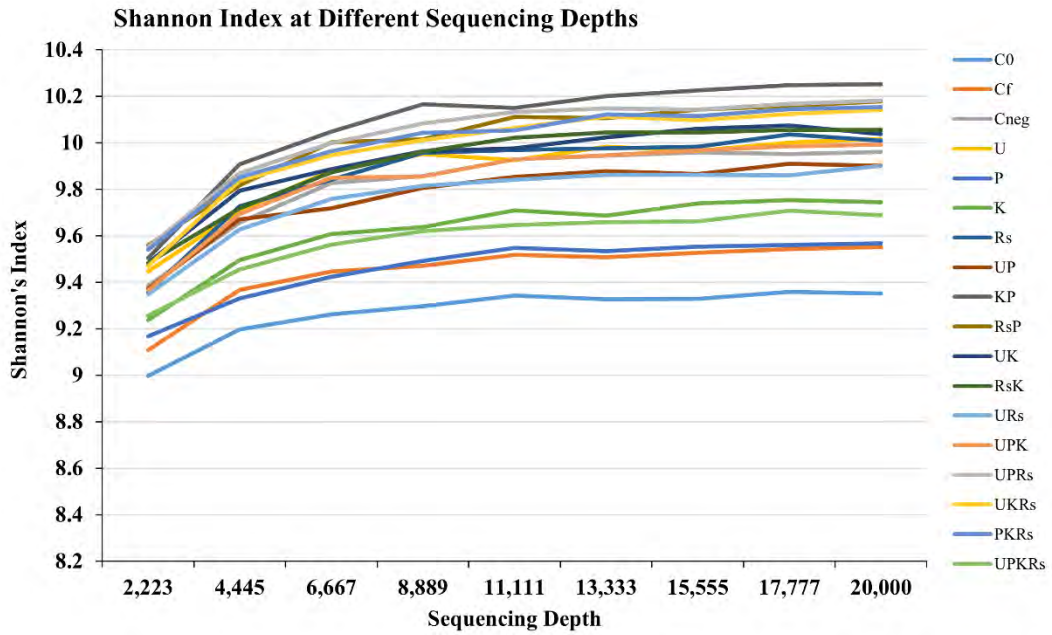
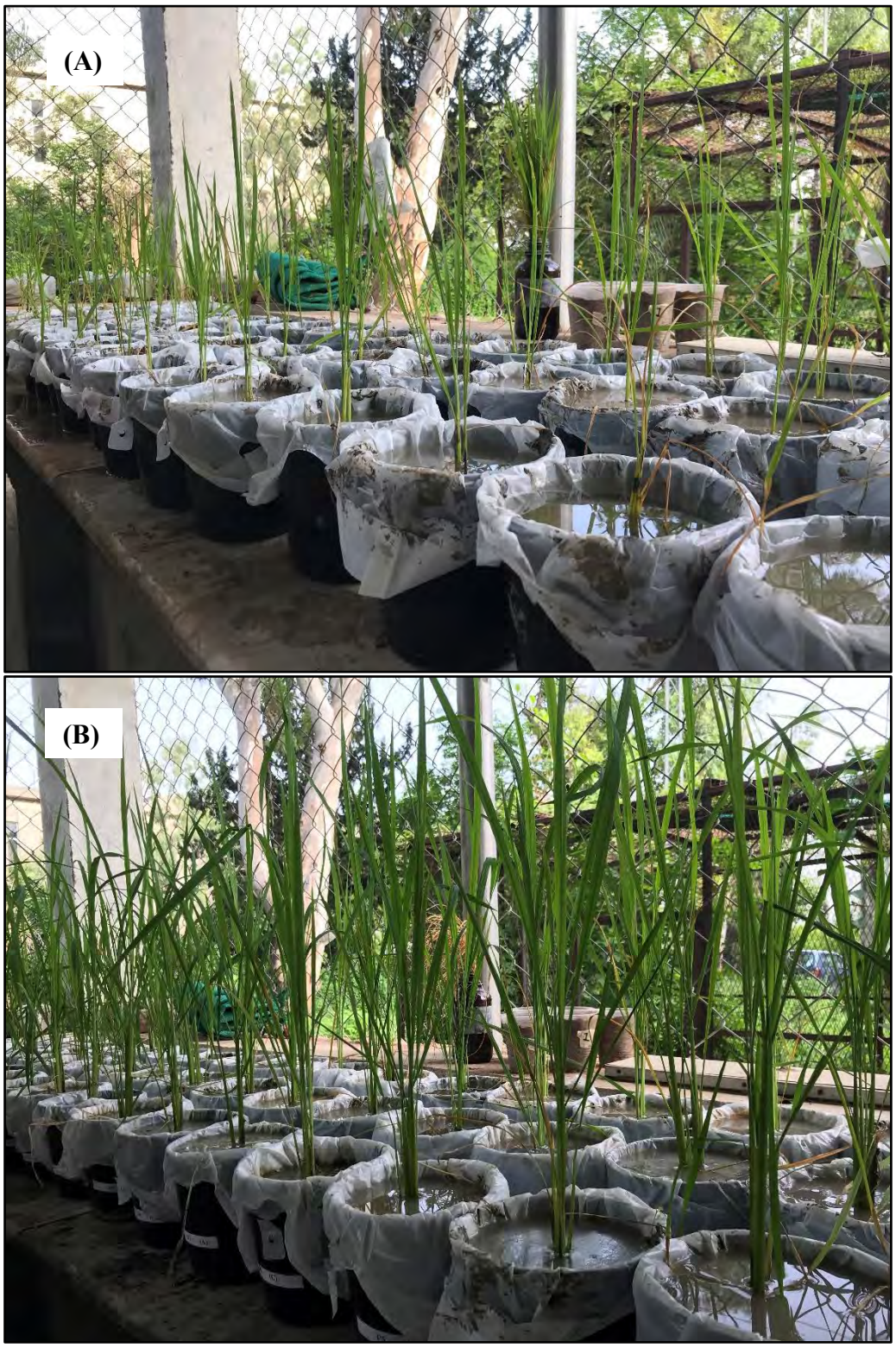


Figure A11: ***Shannon's index in all supplemented treatments at different*** *sequencing depths.*

9.13. A13



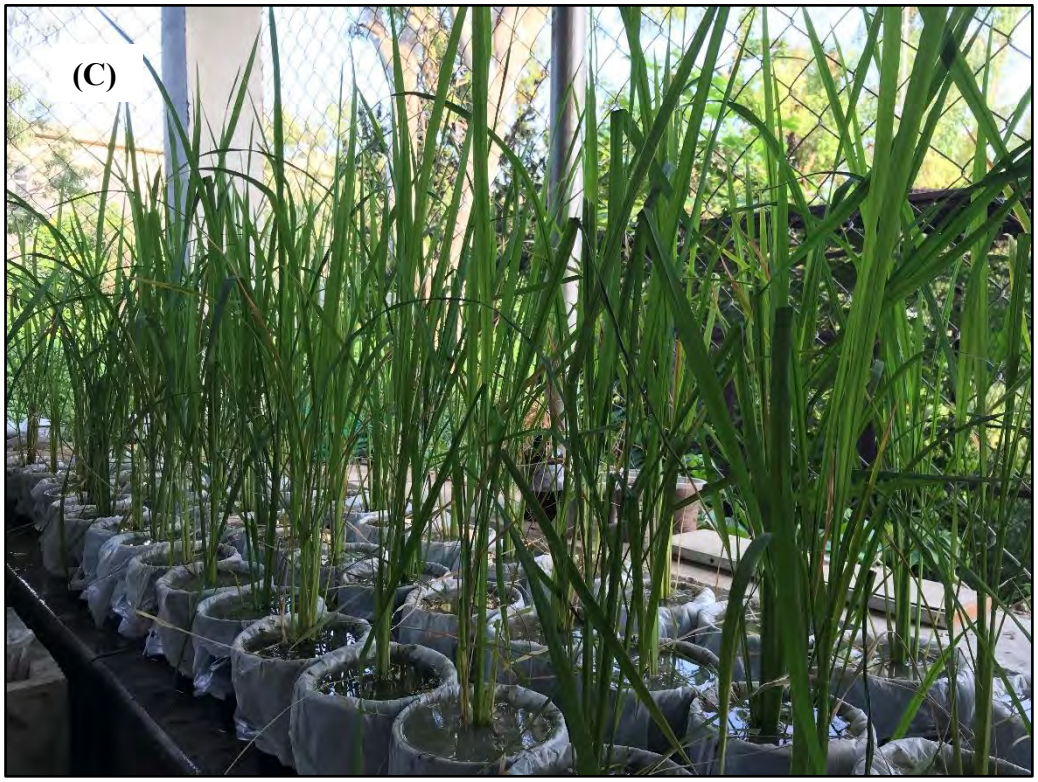


Figure A12: Greenhouse microcosms in indigenous environmental conditions, planted with Oryza sativa at day 0 (A), day 30 (B), and day 45 (C).

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