

**Methane Consumption Potential in Response to  
Different Gas Phase Mixing Ratio in Tropical Vertisol  
of Central India**

**THESIS**



***Submitted to the***

**Rajmata Vijayaraje Scindia Krishi Vishwa Vidyalaya  
In partial fulfilment of the requirement for the Degree  
of**

**MASTER OF SCIENCE**

***In***

**AGRICULTURE**

**SOIL SCIENCE AND AGRICULTURAL CHEMISTRY**

***By***

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**2021**

## CERTIFICATE- I

This is to certify that the thesis entitled “**Methane Consumption Potential in Response to Different Gas Phase Mixing Ratio in Tropical Vertisol of Central India**” submitted in partial fulfilment of the requirement for the degree of MASTER OF SCIENCE in AGRICULTURE (Soil Science and Agricultural Chemistry) of the Rajmata Vijayaraje Scindia Krishi Vishwa Vidhyalaya, Gwalior (M.P.) is a record of the bonafide research work carried out by **Ms. Kanchan Panthi, ID No. 19131306**, under my guidance and supervision. The subject of the thesis has been approved by the Student’s Advisory Committee and the Director of Instruction.

No part of the thesis has been submitted for any other degree or diploma or has been published. All the assistance and help received during the course of the investigation has been acknowledged by the scholar.

Place - Sehore

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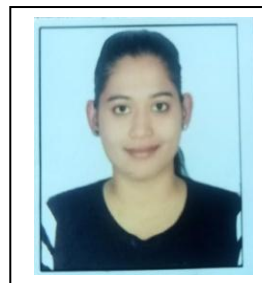
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**Date:** 21/12/2021

**KANCHANPANTHI**

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## LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviations	Full name
CH <sub>4</sub>	Methane
CO <sub>2</sub>	Carbon dioxide
g	Gram
Mg	Milligram
Cm	Centimetre
Ha	Hectare
ml	Millilitre
µg	Microgram
µl	Microlitre
Ng	Nanogram
Ppm	Parts per million
Ppb	Parts per billion
EC	Electrical conductivity
%	Percentage
d <sup>-1</sup>	Day
Nm	Nanometer
Kg	Kilogram
Ph	Potential of hydrogen
Tg	Tera gram
C	Carbon
N	Nitrogen
P	Phosphorus
K	Potassium

## CHAPTER - I

### INTRODUCTION

Methane (CH<sub>4</sub>) is one of the most important anthropogenic greenhouse gases (GHG) in the atmosphere. Current global atmospheric methane (CH<sub>4</sub>) concentration is 1.892 ppm, an increase of 6% since 2000 (Kozicka et al. 2021). CH<sub>4</sub> consumption is an important microbial process and about 80% of CH<sub>4</sub> produced from soil, gets oxidized in there itself (Conrad, 2009). It is estimated that at the global scale about 30 Tg CH<sub>4</sub> gets oxidized in soil in a year (Rice *et al.*, 2016). Methanotrophs are the group of organisms which carry out this process. Their diversity and activity has been well studied (Vanitchung *et al.*, 2014). Depending on CH<sub>4</sub> consumption potential methanotrophs can be different types. Those oxidize low concentration of CH<sub>4</sub> like atmospheric CH<sub>4</sub> (~1.8 ppm) are referred as 'high-affinity' methanotrophs (Cai et al., 2016) and others who oxidize high amount of CH<sub>4</sub> are referred as low affinity methanotrophs. The later types are commonly found in environment where CH<sub>4</sub> production occurs such as flooded rice soil or wetlands.

Methanotrophs use CH<sub>4</sub> as carbon source and O<sub>2</sub> as the final electron acceptor during respiration to oxidize CH<sub>4</sub>. In this process CO<sub>2</sub> is produced like any other aerobic metabolic process. Both reactants are available in soil CH<sub>4</sub> is present in soil as a product of methanogens which convert organic material to CH<sub>4</sub>. Therefore, all these gases are present in soil's air or gas phase. Generally, soil's air or gas phase is mainly constituted of CO<sub>2</sub>, O<sub>2</sub>, N<sub>2</sub>, and CH<sub>4</sub>. Concentration of CO<sub>2</sub> varies from 0 % to 25 % and O<sub>2</sub> 20 % to 6 %. The variation is due to multitude of factors including soil texture, moisture, nutrients etc those influence microbial processes. Concentration of both CO<sub>2</sub> and O<sub>2</sub> in agricultural soil vary depending on fertilizer and soil moisture (Russell and Appleyard, 1915). The CO<sub>2</sub> concentration in a silt loam soil found to occur at 8 % during

peak wheat growth. Soil moisture and soil temperature are responsible for > 50 % of CO<sub>2</sub> fluctuations (Buyanovsky and Wagner, 1983).

Soil is the major source of CH<sub>4</sub>, and its production is niches where anaerobiosis occurs (Maier *et al.*, 2017). In conservation agriculture, the content of CH<sub>4</sub> in the soil pore water remains significantly higher in the deep soil layer than the upper layer (Feng *et al.*, 2021). CH<sub>4</sub> concentration can remain up to 1000 ppm in upland soils and more than 10000 ppm in subsoil. CH<sub>4</sub> concentration can reach even higher in sediments and flooded rice soils (Mohanty *et al.*, 2006).

Based on the methanotrophic pathways, it is obvious that CH<sub>4</sub> consumption can be influenced by reactants and products. However, literature in this regard is feeble. We hypothesize that both the reactants molecules like CH<sub>4</sub> and O<sub>2</sub> would stimulate CH<sub>4</sub> oxidation, while CO<sub>2</sub> would inhibit the methanotrophs. Being a product of reaction, CO<sub>2</sub> would render the inhibitory effect to CH<sub>4</sub> consumption through feedback inhibition. To test the hypotheses, experiments were conducted with the following objectives

1. To estimate CH<sub>4</sub> consumption potential of soil under different gas phase mixing ratio.
2. Quantify the abundance of methanotrophs in soil to evaluate the relation among gas phase mixing ratio, CH<sub>4</sub> consumption potential and methanotrophs abundance.

## CHAPTER-II

### REVIEW OF LITERATURE

It is well known fact that methane is a greenhouse gas and contributes in global warming. Present study entitled “Methane Consumption Potential in Response to Different Gas Phase Mixing Ratio in Tropical Vertisols of Central India” focus on methane dynamics in response to gas phase mixing ratio. A brief review of research work done related to our investigation is scripted in this current chapter.

#### 2.1. Methane dynamics and its effect on environment

Yonemura *et al.*, (2014) reported that agricultural activities directly produce and release about 10-12 percent of the atmospheric greenhouse gases (GHGs), such as CO<sub>2</sub>, methane (CH<sub>4</sub>), nitrous oxide (N<sub>2</sub>O). Application of organic fertilizers potentially increase emissions by over 50% than non-organic fertilizers ( Denier van der Gon *et al.*, 1992; Liu *et al.*, 2007; Smith *et al.*, 2009; Yonemura *et al.*, 2014) CH<sub>4</sub> can be consumed (oxidized) by soil microorganisms which is sensitive to both temperature and soil water content (Adamsen and King, 1993; Dalal and Allen, 2008). Total CH<sub>4</sub> flux from soils is the difference between the production of CH<sub>4</sub> under anaerobic conditions and CH<sub>4</sub> consumption. Agricultural soils, particularly those that have been fertilized, have a significantly lower CH<sub>4</sub> oxidation rate compared to natural soils (Bronson and Mosier, 1994; Frank *et al.*, 2015) and higher oxidation rates are observed in temperate compared to tropical soils, Dalal *et al.*, (2008).

#### 2.2. Atmospheric CH<sub>4</sub> and global warming

In stratosphere, CH<sub>4</sub> influences ozone (O<sub>3</sub>) by secluding O<sub>3</sub> by destroying Cl atoms into HCl molecules which on reaction with –OH radicals releases O<sub>3</sub> deleting Cl and ClO radicals. It also undergoes photochemical oxidation and produces water vapour that reacts with O<sub>3</sub>

destroying NO and NO<sub>2</sub> to less reactive HNO<sub>3</sub> (Bouman 1990; Crutzen 1991). CH<sub>4</sub> contributes about 15-20% of the current increase in global warming (Lindau et al 1993). In addition to general climatological effects, global warming may affect the global carbon by greatly reducing the soil organic carbon content, which may be released as CO<sub>2</sub> and is likely to add to the current burden of CO<sub>2</sub> in the atmosphere (Kirschbaum,1995).

### **2.3. Methanogens**

Methanogens are strictly anaerobic Archaea (Woese,1987). These microorganism are involved in the last step of the electron transfer chain during sequential terminal electron accepting process and organic matter decomposition (Garcia,1990). Shen *et al.*, (2016) in a study reported that methanogens are strictly anaerobic *Archaea*. These microorganisms are involved in the last step of the electron transfer chain during sequential terminal electron accepting process and organic matter decomposition. Depending upon 16S rRNA sequence 20 genera of CH<sub>4</sub> producing bacteria have been described, but only four genera viz., *Methanobacterium*, *Methanosarcina*, *Methanobrevibacter* and *Methanocarpusulum* have been isolated from submerged soil ecosystem like rice soils (Alpana *et al.*, 2017). Methanogens such as *Methanospirillum* and *Methanocarpussulum* which had been isolated from fresh water sediments may also be present in the wetlands rice soils (Zhou *et al.*, 2014).

### **2.4. Mechanism of methanogenesis**

Dolfing, (1992) reported that a number of obligate hydrogen producing acetogenic or OHPA can metabolize into acetate, H<sub>2</sub>, and inorganic C. Homoacetogens are very versatile and can use sugars, alcohols, fatty acids, purines, aromatic compounds as well as methanol, formate, H<sub>2</sub>, and CO<sub>2</sub> to produce acetate as the sole fermentation product. The processes involved in methanogenesis are solubilization,

fermentation (or acidogenesis), and methanogenesis (Neue,1993). There are generally four types of bacteria needed : (a) hydrolytic and fermentative bacteria, (b)  $H^+$ - reducing bacteria, (c) homoacetogenic bacteria, and (d) methanogenic bacteria (Conrad, 1996). The first group hydrolyzes polymers and ferments the resulting monomers to smaller molecules such as alcohols, short chain fatty acids (propionate, n-and isobutyrate, n- and isovalerate),  $H_2$ , and  $CO_2$ . Methanogens immediately convert  $H_2/CO_2$ , formate, acetate, few other simple compounds including methanol, methylated amines and dimethyl sulphide to  $CH_4$  and  $CO_2$ . Several end product molecules in fermented step cannot serve as methanogenic substrates; these include volatile fatty acid anions with three or four carbon atoms, lactate, ethanol, aromatics, and long chain fatty acids. The other methanogenic i.e hydrogenotrophic methanogens oxidize  $H_2$ , and reduce  $CO_2$  to form  $CH_4$ ; methylotrophic methanogens use methyl compounds such as methanol, methyl amines, or dimethylsulphide and acetotrophic methanogens utilize acetate to produce  $CH_4$  (Garcia, 1990). The hydrogenotrophic methanogens reductively fix  $CO_2$  as C1 group on enzyme methanofuran (MFR) to produce formyl-MFR. This group is then transferred to another coenzyme, tetrahydromethanopterin and subsequently, the formyl group is dehydrated and reduced by two 2-electron reduction to methyl level, using the coenzyme  $F_{420}$ , as electron carrier. The methyl group is then transferred from methanopterin to the coenzyme M (2-mercaptoethanosulphonate, HS-CoM) to form  $CH_3-S-CoM$ , which is the substrate for methyl coenzyme- M reductase (MR). Finally  $CH_4$  is reductively cleaved from  $CH_3-S-CoM$ , by reaction with methanogen specific cofactor, 7 mercaptoheptanoylthreonine phosphate (HS-HTP). A total of four reduction steps are envisaged for reduction of  $CO_2$  to  $CH_4$ . Most of the  $CH_4$  produced in nature originates from the methyl group of acetate ( $CH_3COO$ ).

Ferry and Kastead, (2007) reported that the CO dehydrogenase is a key enzyme catalyzing the decarbonylation of acetyl-CoA, the resulting

methyl group is transferred to  $\text{CH}_3\text{-S-CoM}$ , followed by reduction of  $\text{CH}_4$  using electron derived from oxidation of the carbonyl group to  $\text{CO}_2$  by the CO dehydrogenase. Methylophilic methanogens transfer the methyl group of  $\text{CH}_3\text{OH}$  and methylamines to  $\text{CH}_3\text{-S-CoM}$  to  $\text{CH}_4$  are provided by the oxidation of methyl groups to  $\text{CO}_2$ .

## 2.5. Taxonomy and Physiology of Methanotrophic bacteria

Adamsen and King, (1993) reported that methanotrophs are strict aerobes and are divided into two groups viz Type I and Type II, based on their pathways of carbon assimilation, numerical taxonomic evaluation, DNA-DNA hybridization, phospholipid fatty acid (PLFA) composition analysis, genomic, physiochemical properties, and phylogenetic relationships. Type I methanotrophs include three broadly homologous clusters of species and the

family Methylococcaceae contains the genera, *Methylobacter*, *Methylomonas*, *Methylomicrobium*, and *Methylosinus*. Type I methanotrophs oxidize  $\text{CH}_4$  through ribulose monophosphate pathway (RuMP) for assimilation of formaldehyde as cell carbon, while Type II methanotrophs use serine pathways. A third group, Type X is also believed to exist which possesses enzyme that catalyze both serine and RuMP pathway (Whittenbury and Dalton, 1981). They grow at higher temperature than Type I and Type II groups are characterized by high GC content than that of Type I (Whittenbury and Krieg, 1984).

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Prior and Dalton, (1985) reported that the oxidation of CH<sub>4</sub> by aerobic methanotrophs is initiated by methane monooxygenase (MMOs), that utilize two reducing equivalents to split the O-O bond of O<sub>2</sub>. One oxygen atom is reduced to form H<sub>2</sub>O and the other is incorporated into CH<sub>4</sub> to form CH<sub>3</sub>OH. Two forms of MMO have been found in methanotrophs, soluble CH<sub>4</sub> monooxygenase (sMMO) and particulate CH<sub>4</sub> monooxygenase (pMMO). sMMO catalyzes the oxidation of CH<sub>4</sub> to CH<sub>3</sub>OH which is subsequently oxidized to HCHO and eventually CO<sub>2</sub>, as the end product. All methanotrophs are believed to be capable of expressing pMMO when grown in the presence of copper (Dalton, 1992). The sMMO has a broader substrate specificity than the pMMO and other oxygenases (Burrows *et al.*, 1984). Methanotrophic bacteria are unique in their ability to utilize CH<sub>4</sub> as the sole carbon and energy source (Hanson and Hanson, 1996). These bacteria also utilize a variety of different one carbon compounds including CH<sub>4</sub>, methanol, methylated amines, halomethanes, and methylated compounds. Cells containing sMMO rapidly oxidize naphthalene to 1-and 2-naphthols, which can be detected by addition of tetrazotized O-dianisidine resulting the formation of purple diazo dyes with large molar adsorivities (Brusseau *et al.*, 1994).

Bedard and Knowles, (1989) reported that chemoautotrophic ammonium oxidizers belonging to the family Nitrobacteriaceae are also implicated in the oxidation of CH<sub>4</sub> in certain environments. The common habitat of methanotrophs and ammonium oxidizers and similarity between CH<sub>4</sub> monooxygenase and NH<sub>4</sub> monooxygenase, probably make ammonium oxidizers capable of CH<sub>4</sub> oxidation. It is apparent that methanotrophs are ubiquitously distributed and play a significant role in the global CH<sub>4</sub> budget and therefore in moderating the impact of CH<sub>4</sub> on global warming.

Nisbet *et al.*, (2019) studied the growth of methane in environment in recent years and found that growth of methane was so rapid in recent 4 years and such growth rate was not since observed since the 1980s. It

was  $12.7 \pm 0.5$  ppb/year (in 2014),  $10.1 \pm 0.7$  ppb/year (in 2015),  $7.0 \pm 0.7$  ppb/year (in 2016), and  $7.7 \pm 0.7$  ppb/year (in 2017), abrupt increase in methane concentration started in 2007 and it resulted from 1,775 ppb in 2006 to 1,850 ppb in 2017.

## **2.6. Microbiology of methane dynamics.**

Chan and Parkin, (2001) reported that two microbial processes, methanogenesis and CH<sub>4</sub> oxidation play crucial role in methane (CH<sub>4</sub>) flux from soil to the atmosphere and activities of three different microbial populations: the methanotrophs, the ammonia oxidizing nitrifiers, and the methanogens reflects its dynamics.

Mohanty *et al.*, (2007) explored the effect of temperature on CH<sub>4</sub> consumption and methanotrophs population by terminal restriction fragment length polymorphism (T-RFLP) targeting particulate methane monooxygenase (pmoA) genes, individual T-RFs were tentatively assigned to different methanotrophic populations (e.g. Methylococcus/Methylocaldum, Methylomicrobium, Methylobacter, Methylocystis/Methylosinus) results clearly represented that temperature affected the relative abundance of most T-RFs so temperature can be an important factor regulating the community composition of methanotrophs in soil.

Kollah *et al.*, (2015) studied the effectiveness of biochar (BC) on influencing methane (CH<sub>4</sub>) consumption in a tropical clayey vertisol and through the findings revealed that BC enhanced CH<sub>4</sub> consumption potential in agricultural land on a tropical vertisol, particularly using the smaller size (<0.25 mm), and could be an effective strategy to mitigate atmospheric CH<sub>4</sub>.

Hofmann *et al.*, (2016) reported the abundance of Methanogens and methanotrophs along an altitudinal gradient (2700–3500 m) in the Austrian Alps and found that methanogens seem to be capable of

persisting despite a highly oxic low-temperature environment. Methanogenic and methanotropic.

Kollah *et al.*, (2017) estimated methane (CH<sub>4</sub>) consumption in different soil (vertisol) aggregates under elevated carbon dioxide (eCO<sub>2</sub>) and temperature and found that mesoaggregates of 0.25–1.00 mm are hotspots for CH<sub>4</sub> consumption and that rising atmospheric CO<sub>2</sub> and temperature may inhibit CH<sub>4</sub> consumption significantly in a tropical vertisol.

## CHAPTER III

### MATERIAL AND METHODS

The present investigation entitled “**Methane Consumption Potential in response to different gas phase mixing ratio in tropical vertisol of central india**” was under taken in an existing long term Methane consumption experiment which was initiated during 2010 at the research form of ICAR-Indian Institute of Soil Science, Bhopal. The materials used and methods employed during the present investigation have been described in this chapter in details.

#### **3.1 Experimental layout ,Soil sampling and physico chemical properties**

Soil samples were collected from the experimental fields (Mohanty et al., 2015) located at the Indian institute of soil science, Bhopal, Madhya Pradesh, India (23°18'N / 77°24'E, 485 m above sea level). These fields are continuously planted with rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) during the summer and winter seasons, respectively. The location has a humid subtropical climate, with a hot summer and a humid monsoon season. It experiences southwestern monsoon rains between July and September. Mean annual temperature remains about 25 °C. Highest temperature reaches near 45°C during the mid-summer (May-June) and lowest about 5°C in January. The average precipitation is 1200 mm and humidity 65 %. A composite sample representing no inputs control soybean field will be collected. Three randomly collected individual samples will be mixed to make a composite sample. Soils will be then hand processed by removing plant material, stones, and large organisms. Subsequently, soils will be stored in plastic bags at 4°C to prevent moisture loss and will be used within 7 d of collection.

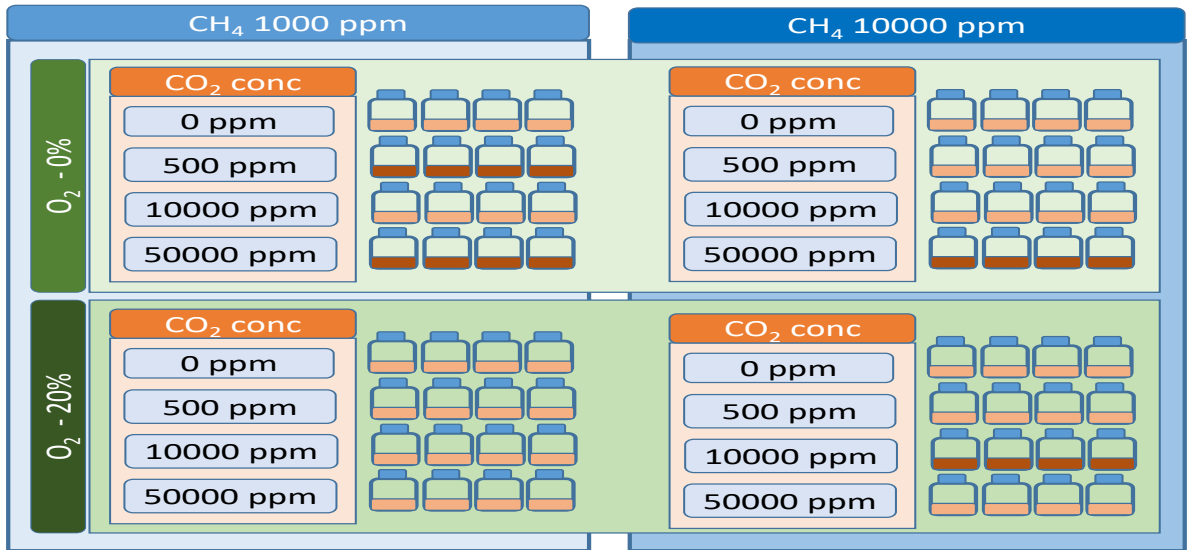
### **3.2. Physico-chemical properties of soil**

Soil samples were analyzed to estimate physical and chemical properties. The soil is a heavy clayey Vertisol (Typic Haplustert). The electrical conductivity (EC) was  $0.38 \text{ dSm}^{-1}$  and the pH was 7.78 (1:2.5 of soil and water in w:v) (Smith and Doran, 1996). The textural composition of soil was: sand 15.2 %, silt 30.3 %, clay 54. Soil organic C was determined by wet digestion method (Walkley and Black, 1934). Available N was determined by alkaline  $\text{KMnO}_4$  method (Subbiah and Asija, 1956). Available P was extracted by 0.5 N  $\text{NaHCO}_3$  solution buffer at pH 8.5 (Olsen, 1954) and P in the extract was determined by ascorbic acid method (Watanabe and Olsen, 1965). Available K was extracted by shaking with neutral normal ammonium acetate for 5 minutes (Hanway and Heidel, 1952) and then K in the extract was determined by flame photometer (Lindsay and Norvell, 1978). Soil organic carbon was 0.82%, available N was  $263 \text{ kg ha}^{-1}$ , available P was  $21 \text{ kg ha}^{-1}$  and available K was  $320 \text{ kg ha}^{-1}$ .

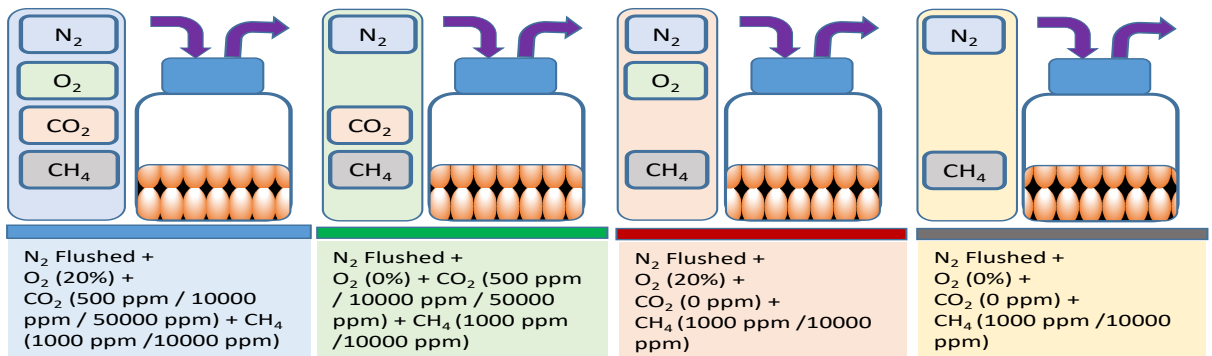
### **3.3. Set up to evaluate effect of gas phase composition on $\text{CH}_4$ consumption**

Experiments were carried out in completely randomized design with 16 treatments and 4 replicates. The treatments represented different gas mixing compositions as 2  $\text{CH}_4$  concentrations (1000 ppm, 10000 ppm), 2  $\text{O}_2$  concentrations (0 %, 20 %) and 4  $\text{CO}_2$  concentrations (0 ppm, 500 ppm, 10000 ppm, 50000 ppm). Total 64 serum bottles of 130 ml volume were used in this study (Fig 1). Sets of 16 vials were used for  $\text{CH}_4$  1000 ppm- $\text{O}_2$  0 % - 4  $\text{CO}_2$  concentrations, 16 vials for  $\text{CH}_4$  1000 ppm- $\text{O}_2$  20 %-4  $\text{CO}_2$  concentrations, 16 vials for  $\text{CH}_4$  10000 ppm-  $\text{O}_2$  0 % - 4  $\text{CH}_4$  concentration, and 16 vials for  $\text{CH}_4$  10000 ppm- $\text{O}_2$  20 %-4  $\text{CH}_4$  concentrations. Incubation was carried out following methods as described elsewhere (Mohanty et al., 2015). Briefly, a portion of 20g soil placed into 130 ml sterilized serum bottles. Soils were moistened with 5

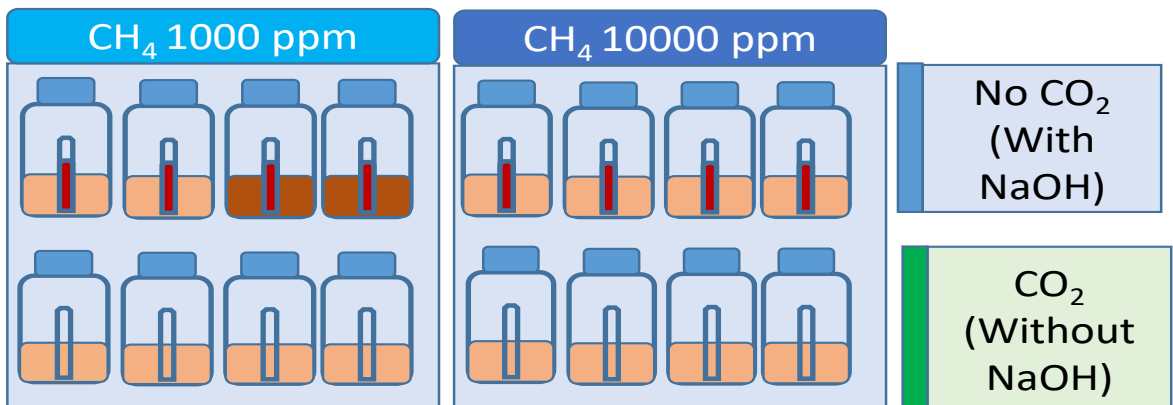
ml sterile distilled water to attain 40 % moisture holding capacity. The contents of the vials were mixed thoroughly, capped with rubber septa and sealed using aluminum crimp seal. Gas phase of the vials were reconstituted as per the treatments (Fig 1). Vials were flushed with N<sub>2</sub> for 30 minutes to remove all head space air. Set of vials representing O<sub>2</sub> 0 % - no O<sub>2</sub> was injected, while for O<sub>2</sub> 20 %, a required volume of pure O<sub>2</sub> was injected. Similarly, for CO<sub>2</sub> of 0 ppm – no CO<sub>2</sub> injected, while for all others required volume of pure CO<sub>2</sub> was injected. For CH<sub>4</sub> of 1000 ppm or 10000 ppm required volume of pure CH<sub>4</sub> was injected into each vials headspace. The gas composition of headspace represented initial condition of the vials. Subsequently, vials were incubated at 28±2°C in a biological oxygen demand (BOD) incubator (Metrex scientific instruments pvt ltd, N Delhi, India). At regular intervals (~1day), 0.1 ml of headspace gas was analyzed for CH<sub>4</sub>. After each sampling, the headspace was replaced with an equivalent amount of high purity helium (He) to maintain atmospheric pressure. The gas helium (He) was used because of its inert chemical nature. Vials were incubated for 15 days. The rate constant of CH<sub>4</sub> consumption (k) was determined from the slope of values of CH<sub>4</sub> versus time during the rapid decline phase.



### A - Experimental layout and treatments



### B - Reconstitution of gas phase of microcosms



### C- Set up to evaluate the role of CO<sub>2</sub> on CH<sub>4</sub> consumption

Fig.1 Experimental layout to evaluate the effect of soil's gas phase composition on CH<sub>4</sub> consumption. A – Outline of the experiment with different components comprising treatments of CO<sub>2</sub> (0ppm, 500 ppm, 10000 ppm, 50000 ppm), O<sub>2</sub> (0 %, 20 %), and CH<sub>4</sub> (1000 ppm, 10000 ppm). Experiment was carried out with four replicates. B – Procedure to maintain gas phase composition. Each vial had 20g soil, capped with rubber septum and sealed with aluminium crimp seal. Vials were flushed with N<sub>2</sub> to remove all head space air. For O<sub>2</sub> 0 % - no O<sub>2</sub> was injected, while for O<sub>2</sub> – 20 % required volume of pure O<sub>2</sub> was injected. For CO<sub>2</sub> of 0 ppm – no CO<sub>2</sub> injected, while for all others required volume of pure CO<sub>2</sub> was injected. For CH<sub>4</sub> of 1000 ppm or 10000 ppm required volume of pure CH<sub>4</sub> were injected into each vials headspace. C- Set up to evaluate the role of CO<sub>2</sub> on CH<sub>4</sub> consumption. Each vial contain 20g soil and a tube containing either 1 ml water (without NaOH) or 1 ml of 1 N NaOH. Solution of NaOH is used to trap CO<sub>2</sub>, making the headspace CO<sub>2</sub> free. Vials without NaOH (only water) had CO<sub>2</sub> in the headspace. CH<sub>4</sub> injected into vials to maintain 1000 ppm or 10000 ppm concentration. Gas samples were monitored to estimate temporal changes in CH<sub>4</sub> concentration. CO<sub>2</sub> concentration estimated after end of incubation (15 days).

### **3.4. Quantification of CH<sub>4</sub> and CO<sub>2</sub>**

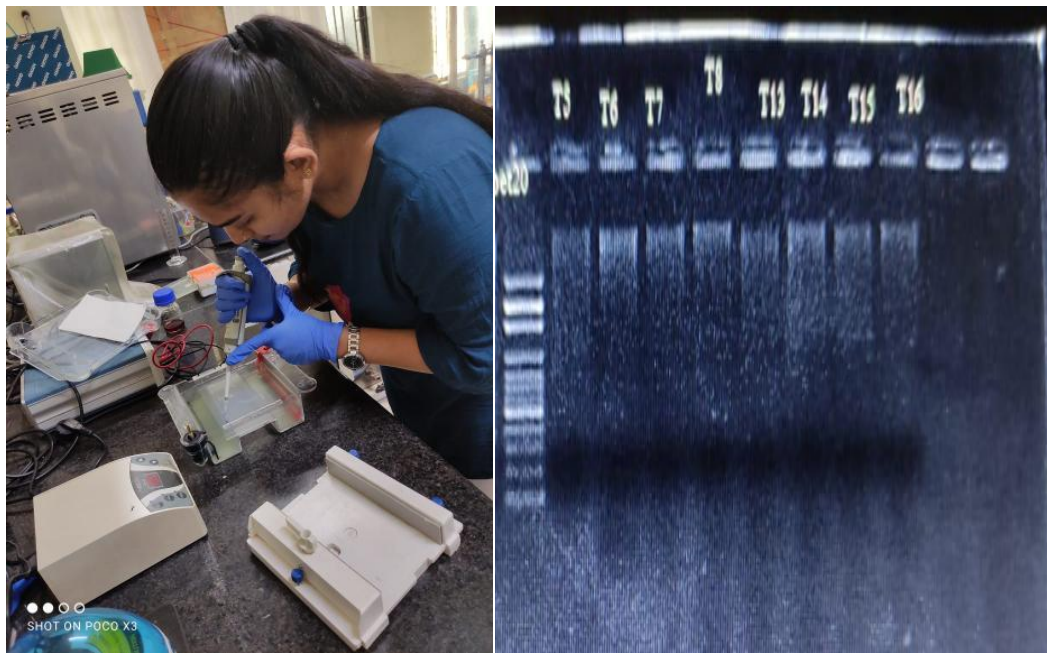
The CH<sub>4</sub> and CO<sub>2</sub> concentration in the headspace of vials were analyzed in a CIC gas chromatograph equipped with FID and a Porapak Q column. The column and detector were maintained at 60°C and 300°C respectively. The gas samples were injected through injection port of an on-column injector. The GC was calibrated before and after each set of measurement using different concentration of CH<sub>4</sub> and CO<sub>2</sub> in N<sub>2</sub> (Inox air, India) as primary standard. CO<sub>2</sub> was quantified with the attached methanizer module. Temperature of methanizer was 330°C. The minimum detectable limit for CH<sub>4</sub> was 0.5 micro litre ml<sup>-1</sup> and 300 micro litre ml<sup>-1</sup> for CO<sub>2</sub>. Under these conditions, the retention time of CH<sub>4</sub> and CO<sub>2</sub> was 1.30 and 2.5 minutes.



**Gas Chromatograph**

### 3.5. DNA extraction

After CH<sub>4</sub> consumption, about 0.5 g soil samples were taken out from bottles to extract DNA using the ultraclean DNA extraction kit (MoBio, USA) according to the manufacturer's instructions. The DNA concentrations were determined in a biophotometer (Eppendorf, Germany) by measuring absorbance at 260 nm (A<sub>260</sub>), assuming that 1 A<sub>260</sub> unit corresponds to 50 ng of DNA per µl. DNA extraction was further confirmed by electrophoresis on a 1 % agarose gel. The extracted DNA was dissolved in 50 µl TE buffer and stored at -20 °C until further analysis.



**DNA Extraction**

### 3.6. Set-up to demonstrate the effect of CO<sub>2</sub> on CH<sub>4</sub> consumption

Experiments were conducted to evaluate the role of CO<sub>2</sub> on CH<sub>4</sub> consumption. Treatments included 2 CO<sub>2</sub> concentration (no CO<sub>2</sub>, with CO<sub>2</sub>) with four replicates. Each vial contain 20g soil and moistened with 5 ml water to attain 40 % moisture. A small glass tube was inserted into vials as shown in Fig 1.

It contained either 1 ml water or 1 ml of 1 N NaOH. Solution of NaOH is used to trap CO<sub>2</sub>, making the headspace CO<sub>2</sub> free. Vials without NaOH (only water) had CO<sub>2</sub> in the headspace. CH<sub>4</sub> injected into vials to maintain 1000 ppm or 10000 ppm concentration. Gas samples were monitored to estimate temporal changes in CH<sub>4</sub> concentration. CO<sub>2</sub> concentration were estimated after the end of incubation (15 days).

### 3.7. Real time PCR quantification of methanotrophs *pmoA* genes

Real time PCR was performed on a Step one plus real time PCR (ABI, USA) to quantify the representative microbial species. Reaction mixture prepared with 2 µl of DNA template, 10 µl of 2X SYBR green master mix (Affymetrix, USA), 200 nM of primer (GCC Biotech, N Delhi). Final volume of PCR reaction mixture was made to 20 µl with PCR grade water (MP Bio, USA). Primers targeting *pmoA* gene (particulate methane monooxygenase) of methanotrophs were used to quantify their abundance. The primers for *pmoA* were A189F (5- GGN GAC TGG GAC TTCT GG-3) and mb661R (5- CCG GMG CAA CGT CYT TAC C-3)(Mohanty *et al.*, 2017). This primer set targets methanotrophs covering both type I and II including *Methylobacter* or *Methylosarcina*, *Methylococcus*, *Methylosinus* group, *Methylocapsa*, *Nitrosococcus*. *Quantification of microbial genes was carried out by real time PCR approach targeting the functional groups* (Kolb, 2009). Thermal cycling was carried out by an initial denaturizing step at 94 °C for 4 min, 40 cycles of 94 °C for 1 min, 52 °C for 30 sec, 72 °C for 45 sec; final extension carried out at 72 °C for 5 min. Fluorescence was measured during elongation step. Data analysis was carried out with Step one plus software (ABI, USA) as described in user's manual. The cycle at which the fluorescence of target molecule

number exceeded the background fluorescence (threshold cycle [ $C_T$ ]) was determined from dilution series of target DNA with defined target molecule amounts.  $C_T$  was proportional to the logarithm of the target molecule number. The quality of PCR amplification products were determined by melting curve analysis with temperature increase of 0.3 °C per cycle. Standard for the genes was made from series of 10 fold dilutions of purified amplified products and data presented as number of cells per gram of soil.



**Real time PCR quantification**

### **3.8. Statistical Analyses**

Results for the experiments were presented as arithmetic means and standard deviation of replicated observations. To define relation among CO<sub>2</sub> concentration, CH<sub>4</sub> consumption rate, and methanotrophs pmoA gene abundance linear models estimated. All analysis and XY graphs were prepared by MS Excel.

## CHAPTER- IV

### RESULTS

#### 4.1. Temporal variation in methane consumption

Soil exhibited  $\text{CH}_4$  consumption as the headspace  $\text{CH}_4$  concentration declined over incubation period. Temporal variation of  $\text{CH}_4$  consumption was monitored to evaluate how  $\text{CH}_4$  consumption progressed under the influence of different gas phase mixing ratio. The headspace gas contained  $\text{CH}_4$ ,  $\text{O}_2$ , and  $\text{CO}_2$  at different concentrations.  $\text{CH}_4$  consumption occurred only in vials containing 20 %  $\text{O}_2$  and no  $\text{CH}_4$  consumption occurred in vials of no  $\text{O}_2$  (0 %) (Fig 2). Under the condition of 20 %  $\text{O}_2$ , 0 ppm of  $\text{CO}_2$  and 1000 ppm of  $\text{CH}_4$ , lag phase was about 5 days. Similarly lag phase was about 4 days at 100000 ppm of  $\text{CH}_4$ . Lag phase decreased with  $\text{CO}_2$  concentration and it was 1d when  $\text{CO}_2$  concentration increased to 10000 ppm or 50000 ppm. Headspace  $\text{CH}_4$  was consumed within 15 days under ambient condition. However under higher  $\text{CO}_2$  concentration, head space  $\text{CH}_4$  completely consumed within 10 days under both 1000 ppm and 10000 ppm.

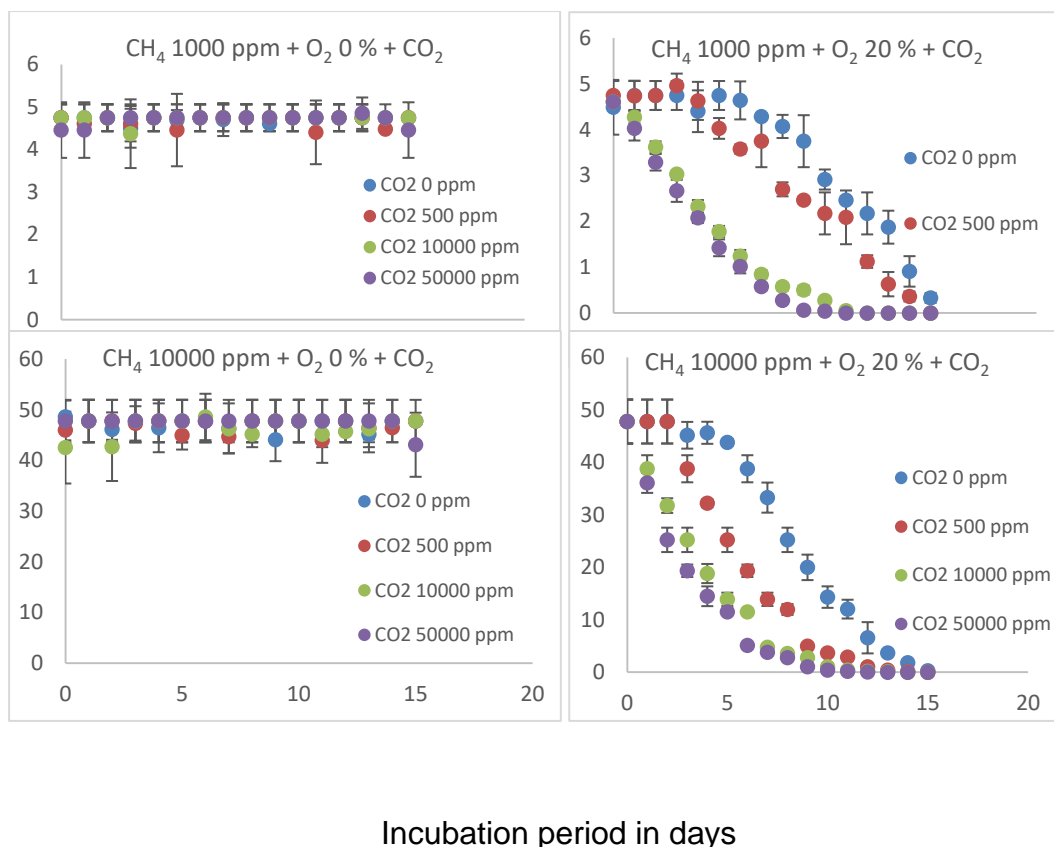


Fig 2. CH<sub>4</sub> consumption in soil under the influence of different gas phase mixing ratio. Soil of 20 g was incubated at different gasses. The headspace gasses were CH<sub>4</sub>, O<sub>2</sub> and CO<sub>2</sub>. CH<sub>4</sub> concentration was either 1000 ppm or 10000 ppm. O<sub>2</sub> concentration was either 0 % or 20 %. Different CO<sub>2</sub> concentration maintained initially was 0 ppm, 500 ppm, 10000 ppm or 50000 ppm. Headspace CH<sub>4</sub> concentration was measured at regular interval. Top left –CH<sub>4</sub> 1000 ppm, O<sub>2</sub> 0 % and different CO<sub>2</sub> concentration. Top right - CH<sub>4</sub> 1000 ppm, O<sub>2</sub> 20 % and different CO<sub>2</sub> concentration. Bottom left - CH<sub>4</sub> 10000 ppm, O<sub>2</sub> 0 % and different CO<sub>2</sub> concentration. Bottom right - CH<sub>4</sub> 10000 ppm, O<sub>2</sub> 20 % and different CO<sub>2</sub> concentration. Each data point represents arithmetic mean with error bar as standard deviation of four replicated observations.

#### 4.2. Methane consumption rate

To differentiate the effect of gas phase constituents on CH<sub>4</sub> consumption, rates of methane consumption were estimated and expressed as ng CH<sub>4</sub> consumed g<sup>-1</sup> soil d<sup>-1</sup> (Table 1). In the treatments of no O<sub>2</sub>, rate was

nil as no CH<sub>4</sub> consumption occurred. CH<sub>4</sub> consumption rate estimated from the vials of 20 % O<sub>2</sub>. It varied from 0.283 to 0.481 in 1000 ppm of CH<sub>4</sub> and 2.958 to 4.99 in case of 10000 ppm of CH<sub>4</sub>. Rate of CH<sub>4</sub> consumption increased with CH<sub>4</sub> concentration. CO<sub>2</sub> enhanced the rate of CH<sub>4</sub> consumption and the trend followed as 0 ppm < 500 ppm < 10000 ppm < 50000 ppm.

Table 1. CH<sub>4</sub> consumption rate of soil under the influence of different gas phase mixing ratio. The headspace gasses were CH<sub>4</sub>, O<sub>2</sub> and CO<sub>2</sub>. CH<sub>4</sub> concentration was either 1000 ppm or 10000 ppm. O<sub>2</sub> concentration was either 0 % or 20 %. Different CO<sub>2</sub> concentration maintained initially was 0 ppm, 500 ppm, 10000 ppm or 50000 ppm. Each data represents arithmetic mean ± standard deviation of four replicated observations.

CH <sub>4</sub> (ppm)	O <sub>2</sub> (%)	CO <sub>2</sub> (ppm)	CH <sub>4</sub> consumption rate (ng CH <sub>4</sub> consumed g <sup>-1</sup> soil day <sup>-1</sup> )
1000	0	0	-0±0.004
		500	0.002 ± 0.01
		10000	-0 ± 0.01
		50000	-0.01 ± 0.01
	20	0	0.283 ± 0.033
		500	0.352 ± 0.01
		10000	0.407 ± 0.007
		50000	0.481 ± 0.013
10000	0	0	0.034 ± 0.083
		500	0.015 ± 0.105
		10000	-0.11 ± 0.236
		50000	0.103 ± 0.211
	20	0	2.958 ± 0.559
		500	3.758 ± 0.216
		10000	4.536 ± 0.17
		50000	4.994 ± 0.193

### 4.3. CO<sub>2</sub> production

During CH<sub>4</sub> consumption CO<sub>2</sub> is produced as the end product of microbial metabolism. CO<sub>2</sub> production was evaluated in terms of ng CO<sub>2</sub> produced g<sup>-1</sup> soil, after the complete consumption of CH<sub>4</sub> (Table 2). CO<sub>2</sub> production varied from 194 to 331 ng g<sup>-1</sup> soil at 1000 ppm of CH<sub>4</sub>. Similarly, it varied from 139 to 272 ng g<sup>-1</sup> soil at 10000 ppm of CH<sub>4</sub>. Lowest CO<sub>2</sub> was from 0 ppm of CO<sub>2</sub> and highest was from 50000 ppm of CO<sub>2</sub>. CO<sub>2</sub> production per unit of CH<sub>4</sub> consumed was evaluated and presented in terms of ng CO<sub>2</sub> produced per ng CH<sub>4</sub> consumed. The values were highest in 0 ppm of CO<sub>2</sub> and lowest in 50000 ppm of CO<sub>2</sub>. The values were higher in 1000 ppm CH<sub>4</sub> than 10000 ppm CH<sub>4</sub> in all treatments. Secondly, it was observed that by increasing the concentration of CO<sub>2</sub>, the values of ng CO<sub>2</sub> produced per ng CH<sub>4</sub> consumed decreased. For example, at 1000 ppm of CH<sub>4</sub>, the value of CO<sub>2</sub> produced per unit of CH<sub>4</sub> consumed was 0.05 in 500 ppm CO<sub>2</sub> and -0.06 in 50000 ppm CH<sub>4</sub>. Similarly, at 10000 ppm CH<sub>4</sub>, the value was highest (0.004) in 500 ppm CO<sub>2</sub> and lowest (-0.008) in 50000 ppm CO<sub>2</sub>.

Table 2. Production of CO<sub>2</sub> from CH<sub>4</sub> consumption under the influence of different gas phase mixing ratio. Soils were incubated with a headspace of different concentration of CH<sub>4</sub>, O<sub>2</sub> and CO<sub>2</sub>. CH<sub>4</sub> concentration was either 1000 ppm or 10000 ppm. O<sub>2</sub> concentration was either 0 % or 20 %. Different CO<sub>2</sub> concentration maintained initially were 0 ppm, 500 ppm, 10000 ppm or 50000 ppm. Each data represents arithmetic mean ± standard deviation of four replicated observations.

CH <sub>4</sub> (ppm)	O <sub>2</sub> (%)	C (ppm)	O <sub>2</sub> ng soil	C ng	O <sub>2</sub> /g ng C oxidized	O <sub>2</sub> produced /ng CH <sub>4</sub>
1000	20	0	194.34		± 0.05	± 0.006
			24.54			
		500	197.28		± 0.05	± 0.007
			27.95			
		10000	208.90	±3.69	0.04	± 0.001
10000	20	0	139.09		± 0.0039	± 0.0005
			19.29			
		500	152.28		± 0.0042	± 0.0007
			25.38			
		10000	177.19		± 0.0030	± 0.0005
10000	20	0	17.14			
			272.02		± -0.0081	± 0.0005
		50000	28.44			

#### 4.4. Abundance of methanotrophs

Methanotrophs abundance was estimated after the end of incubation to define how the representative pmoA gene copies varied under the influence of different treatments. Methanotrophs abundance was estimated in terms of number of pmoA gene copies per g soil (Table 3). In case of 0 % O<sub>2</sub>, there was about 7 - 8 x 10<sup>5</sup> pmoA gene copies g<sup>-1</sup> soil irrespective of treatments. Abundance of methanotrophs increased by O<sub>2</sub> (20 %) irrespective of CO<sub>2</sub> concentration. Their abundance varied from 16.5 x 10<sup>5</sup> to 76 x 10<sup>5</sup> pmoA gene copies g<sup>-1</sup> soil at 1000 ppm CH<sub>4</sub> and 39 x 10<sup>5</sup> to 107 x 10<sup>5</sup> pmoA gene copies g<sup>-1</sup> soil in case of 10000 ppm CH<sub>4</sub>. Abundance of pmoA gene varied positively with CO<sub>2</sub> concentration, and followed the trend of CO<sub>2</sub> 0 ppm < 500 ppm < 10000 ppm < 50000 ppm.

Table 3. Abundance of methanotrophs (pmoA gene copies) in soil after CH<sub>4</sub> consumption under the influence of different gas phase mixing ratio. The headspace gasses were CH<sub>4</sub>, O<sub>2</sub> and CO<sub>2</sub>. CH<sub>4</sub> concentration was either 1000 ppm or 10000 ppm. O<sub>2</sub> concentration was either 0 % or 20%. Different CO<sub>2</sub> concentration maintained initially was 0 ppm, 500 ppm, 10000 ppm or 50000 ppm. Each data represents arithmetic mean ± standard deviation of four replicated observations.

CH <sub>4</sub> (ppm)	O <sub>2</sub> (%)	CO <sub>2</sub> (ppm)	Methanotrophs (pmoA gene copies x 10 <sup>5</sup> g <sup>-1</sup> soil)
1000	0	0	4.50 ± 0.58
		500	4.50 ± 0.58
		10000	4.50 ± 0.58
		50000	4.50 ± 0.58
	20	0	16.50 ± 3.00
		500	41.75 ± 3.30
		10000	60.50 ± 7.05
		50000	76.25 ± 4.27
10000	0	0	4.50 ± 0.58
		500	4.50 ± 0.58
		10000	4.50 ± 0.58
		50000	4.50 ± 0.58
	20	0	39.00 ± 3.16
		500	59.50 ± 4.65
		10000	75.50 ± 5.26
		50000	107.00 ± 7.26

#### 4.5. Linear regression models

The role of CO<sub>2</sub> on CH<sub>4</sub> consumption and methanotrophs pmoA gene abundance was evaluated by linear regression models (Fig 3). Concentration of CO<sub>2</sub> exhibited positive relation with rate of CH<sub>4</sub> consumption. CH<sub>4</sub> consumption can be derived from CO<sub>2</sub> concentration as  $Y = 3E-06x+0.333$  at 1000 ppm CH<sub>4</sub> and as  $Y = 2E-05x+4.005$  at 10000 ppm CH<sub>4</sub>. Linear

regression analysis between CO<sub>2</sub> produced per ng CH<sub>4</sub> consumed and CO<sub>2</sub> concentration indicated a significant negative correlation (r<sup>2</sup> 0.975). It can be predicted as  $y = -2E-06x + 0.0573$  at 1000 ppm CH<sub>4</sub> and  $y = -2E-07x + 0.0045$  at 10000 ppm CH<sub>4</sub>. CH<sub>4</sub> consumption was also correlated with pmoA gene copies (P < 0.05). Abundance of pmoA genes can be estimated from rate as  $Y = 280.45x - 58.078$  at 1000 ppm CH<sub>4</sub> and  $Y = 26.6x - 37.77$  at 10000 ppm CH<sub>4</sub>.

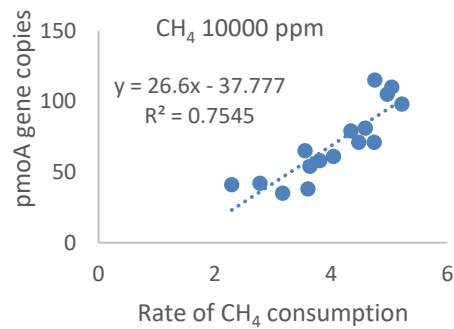
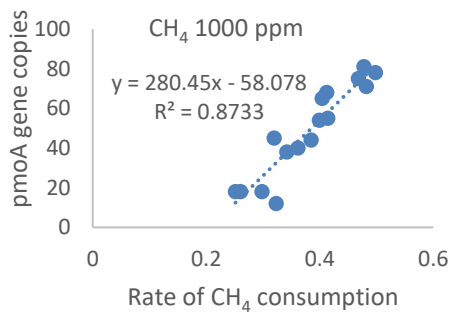
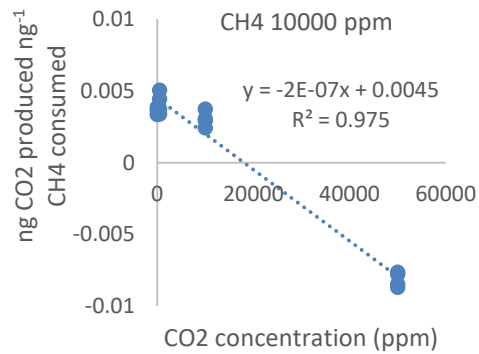
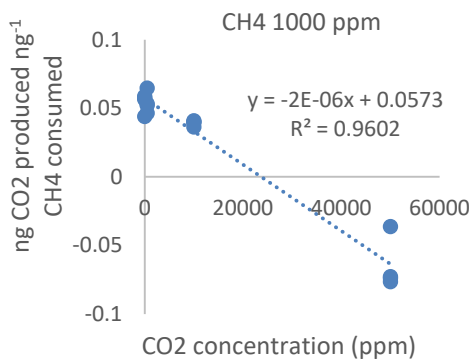
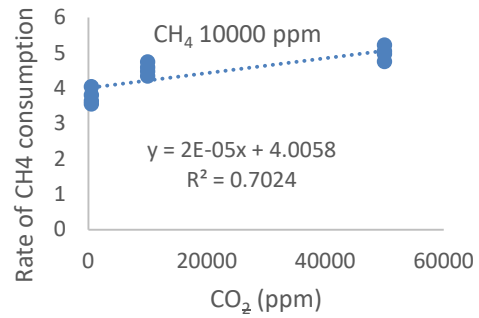
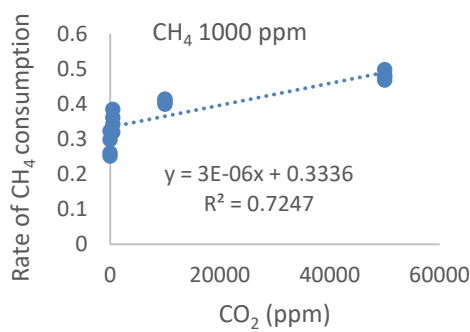
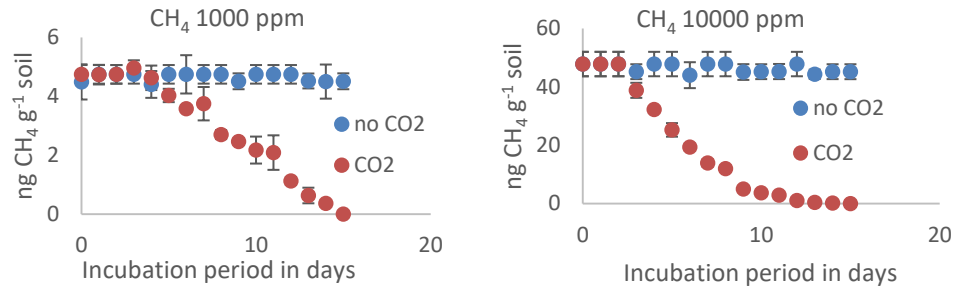


Fig 3. Linear models to predict relation among CO<sub>2</sub> concentration, CH<sub>4</sub> consumption and methanotrophs pmoA gene copy numbers. Top left- correlation between CO<sub>2</sub> concentration and rate of CH<sub>4</sub> consumption at 1000 ppm of CH<sub>4</sub>, Top right - correlation between CO<sub>2</sub> concentration and rate of CH<sub>4</sub> consumption at 10000 ppm of CH<sub>4</sub>, Middle left – Correlation between CO<sub>2</sub> produced per unit of CH<sub>4</sub> consumed and CO<sub>2</sub> concentration at 1000 ppm CH<sub>4</sub>. Middle right - Correlation between CO<sub>2</sub> produced per unit of CH<sub>4</sub> consumed and CO<sub>2</sub> concentration at 10000 ppm CH<sub>4</sub>. Bottom left – correlation between CH<sub>4</sub> consumption rate and pmoA gene at 1000 ppm, Bottom right - correlation between CH<sub>4</sub> consumption rate and pmoA gene at 10000 ppm CH<sub>4</sub>.

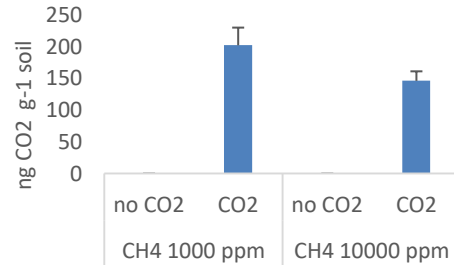
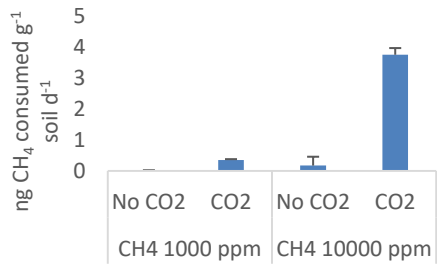
#### **4.6. CH<sub>4</sub> consumption in response to CO<sub>2</sub>**

Higher CO<sub>2</sub> concentration resulted higher CH<sub>4</sub> consumption irrespective of treatments, suggested that CO<sub>2</sub> plays crucial role in CH<sub>4</sub> consumption. To examine if CO<sub>2</sub> is essential for CH<sub>4</sub> consumption, experiments were conducted with CO<sub>2</sub> and without CO<sub>2</sub> by using NaOH as CO<sub>2</sub> trapping reagent. No CH<sub>4</sub> consumption observed in case of vials containing NaOH while CH<sub>4</sub> consumption proceeded normally in vials without NaOH (Fig 4). Vials contained NaOH had no CO<sub>2</sub> in their headspace as it was trapped by alkali (1N NaOH). Decline of CH<sub>4</sub> concentration was reflected by initial lag phase and log phase. However, in case of 10000 ppm CH<sub>4</sub>, log phase was faster than of at 1000 ppm of CH<sub>4</sub>. CH<sub>4</sub> consumption rate (ng CH<sub>4</sub> consumed g<sup>-1</sup> soil) was nil under no CO<sub>2</sub>. However, the rate was 0.352 in case of 1000 ppm of CH<sub>4</sub> and 3.758 in case of 10000 ppm of CH<sub>4</sub>. Production of CO<sub>2</sub> was monitored after 15 days of incubation. There was 202 ng of CO<sub>2</sub> produced g<sup>-1</sup> soil in 1000 ppm CH<sub>4</sub> and 146 ng of CO<sub>2</sub> produced g<sup>-1</sup> soil in 10000 ppm of CH<sub>4</sub>.



**A**

**B**



**C**

**D**

Fig 4. Effect of CO<sub>2</sub> on CH<sub>4</sub> consumption in soil. A- Temporal changes in the headspace CH<sub>4</sub> concentration in 1000 ppm of CH<sub>4</sub>. B- Temporal changes of CH<sub>4</sub> concentration under 10000 ppm CH<sub>4</sub>. C - Rate of CH<sub>4</sub> consumption in soils of different treatments. D –CO<sub>2</sub> produced after incubation period. Values in each analysis represent arithmetic mean and error bar as standard deviation of four replicated observations.

## CHAPTER- V

### DISCUSSION

CH<sub>4</sub> consumption is a complex phenomenon and depends on many factors including soil properties and gas phase composition. The experiment was conducted to evaluate the effect of CH<sub>4</sub>, O<sub>2</sub>, and CO<sub>2</sub> on CH<sub>4</sub> consumption, because, during CH<sub>4</sub> consumption these three gasses are directly involved. For example, CH<sub>4</sub> is used as substrate, O<sub>2</sub> acts as terminal electron acceptor in aerobic respiration. CO<sub>2</sub> is the product of CH<sub>4</sub> consumption. Each gas was applied at different concentrations. CH<sub>4</sub> concentration was taken as either 1000 ppm or 10000 ppm, representing low and high CH<sub>4</sub> concentration. High affinity methanotrophs carry out CH<sub>4</sub> consumption, at lower CH<sub>4</sub> level (1000 ppm) while low affinity methanotrophs oxidize CH<sub>4</sub> at higher concentration. O<sub>2</sub> was taken as either 0% or 20%, because 20% O<sub>2</sub> represent upper soil which has gas phase similar to ambient air, while O<sub>2</sub> of 0% indicate anoxic condition or subsoil, where O<sub>2</sub> is limited. CO<sub>2</sub> was tested in 4 different concentrations : 0 ppm, 500 ppm, 10000 ppm and 50000 ppm. CO<sub>2</sub> concentrations can vary in soil's phase depending on conditions. For example, amendment of organic fertilizers (Yang et al, 2018), N fertilizers (Zamanian et al., 2018), biochar (Schlesinger and Amundson., 2019) to soil and tillage (De Araújo Santos et al., 2019) influences CO<sub>2</sub> production and its concentration can be more than 50000 ppm (Joun et al., 2019). Therefore, the composition of gas phase mimic natural soil ecosystems.

To evaluate CH<sub>4</sub> consumption, first the temporal variation of CH<sub>4</sub> concentration in the vials was monitored. There was an initial lag phase and a log phase which indicated that CH<sub>4</sub> consumption was a classical microbial metabolic process. Both phases varied depending on the methanotrophic activities. Low CH<sub>4</sub> consumption is exhibited as prolonged lag phase and log phase. Under normal condition (20 % O<sub>2</sub>, 500 ppm CO<sub>2</sub>), CH<sub>4</sub> consumption was completed within 2 weeks. Similar observation has been reported earlier (Mohanty et al., 2015).

CH<sub>4</sub> consumption was influenced by CH<sub>4</sub> concentration, for example CH<sub>4</sub> consumption was faster under higher CH<sub>4</sub> concentration. Therefore, at 10000 ppm CH<sub>4</sub>, both lag and log phases were shorter than 1000 ppm CH<sub>4</sub>. Probably, it was due to higher methanotrophic activity. Low affinity methanotrophs grow faster, while the high affinity methanotrophs are slow growers. Similar results has been reported in earlier studies (Mohanty *et al.*, 2006).

CO<sub>2</sub> concentration exhibited positive effect on CH<sub>4</sub> consumption. Lag phase was 5 days under ambient condition (O<sub>2</sub> 20 %, 500 ppm CO<sub>2</sub>), which decreased to 1 day at 10000 ppm or 50000 ppm of CO<sub>2</sub>. This indicated that CO<sub>2</sub> stimulated or activated methanotrophs. To differentiate the effect of different treatments, CH<sub>4</sub> consumption rates were estimated. CH<sub>4</sub> consumption rates were high in soil incubated with 10000 ppm CH<sub>4</sub> than 1000 ppm CH<sub>4</sub>. Similar results has been reported in different studies (Mohanty *et al.*, 2007; Ahirwar *et al.*, 2018). Higher CO<sub>2</sub> concentration stimulated CH<sub>4</sub> consumption rate. CH<sub>4</sub> consumption rate was lowest at 0 ppm CO<sub>2</sub>, which was enhanced by 1.24 times at 500 ppm CO<sub>2</sub>. Interestingly, the rate was further increased by 1.15 to 1.35 times by 10000 ppm CO<sub>2</sub> and 50000 ppm CO<sub>2</sub> than 500 ppm CO<sub>2</sub>. The effect of CO<sub>2</sub> followed similar trend in both of 1000 ppm CH<sub>4</sub> and 10000 ppm CH<sub>4</sub>. Results indicated that CO<sub>2</sub> is an essential part of CH<sub>4</sub> consumption.

CO<sub>2</sub> concentration was measured after complete oxidation of CH<sub>4</sub>. Total CO<sub>2</sub> production was higher at 1000 ppm CH<sub>4</sub> than 10000 ppm CH<sub>4</sub>. CO<sub>2</sub> produced per unit of CH<sub>4</sub> consumed was determined to define methanotrophs metabolism that shapes gas phase of CH<sub>4</sub> and CO<sub>2</sub>. Interestingly, the ratio of CO<sub>2</sub> produced per unit of CH<sub>4</sub> consumption decreased with increasing CO<sub>2</sub> concentration; which means CO<sub>2</sub> production declined with CH<sub>4</sub> consumption. It indicated that CO<sub>2</sub> was used by methanotrophs. For example, 0.05 ng CO<sub>2</sub> produced ng<sup>-1</sup>CH<sub>4</sub> consumed at 500 ppm CO<sub>2</sub>, while -0.06 ng CO<sub>2</sub> produced ng<sup>-1</sup>CH<sub>4</sub> consumed at 50000 ppm CO<sub>2</sub>. This effect could be due to two reasons : first, CO<sub>2</sub> production ceased due to inhibition of aerobic Heterotrophs, second, the produced CO<sub>2</sub> was mostly used up by

methanotrophs during consumption of CH<sub>4</sub>. However, the former process is less possible as we have observed that CO<sub>2</sub> concentration in soil's gas phase remains around 50000 ppm under normal condition. Therefore, it can be presumed that CO<sub>2</sub> was taken up by methanotrophs during CH<sub>4</sub> consumption.

Methanotrophs abundance increased in relation to CH<sub>4</sub> consumption rate. Their population increased by a factor of 2.5 to 4.6 times at 1000 ppm CH<sub>4</sub> and 1.52 to 2.74 in case of 10000 ppm CH<sub>4</sub>. Similar values of methanotrophs has been observed in other studies (Mohanty *et al.*, 2014). Data suggested that methanotrophs abundance was stimulated by CO<sub>2</sub>. Secondly, their abundance was stimulated by a factor of 1.24 to 2.36 times at 10000 ppm CH<sub>4</sub> than the lower CH<sub>4</sub> concentration.

The relation between CO<sub>2</sub>, CH<sub>4</sub> consumption potential and abundance of methanotrophs was evaluated by linear regression analysis. Positive correlation existed among CO<sub>2</sub> concentration, CH<sub>4</sub> consumption rate and pmoA abundance. Results indicated that CO<sub>2</sub> concentration positively influenced both CH<sub>4</sub> consumption rate and methanotrophs. Linear regression analysis revealed significant negative correlation between CO<sub>2</sub> concentration and CO<sub>2</sub> produced per unit CH<sub>4</sub> consumed.

We observed that CO<sub>2</sub> played a significant role in CH<sub>4</sub> consumption. The role of CO<sub>2</sub> was obvious at 10000 ppm and 50000 ppm than at lower concentrations. Higher CO<sub>2</sub> concentration enhanced methanotrophy through (1) short lag phase, log phase and (2) increased rate of CH<sub>4</sub> consumption. Based on the results, a question is raised; whether CO<sub>2</sub> is essential for CH<sub>4</sub> consumption. Generally, CO<sub>2</sub> is produced from soil through various microbial activities. So the treatments of different CO<sub>2</sub> concentrations represented only the initial headspace concentration, which is not suitable to answer the question. A follow up experiment was conducted with another set up to make headspace free of CO<sub>2</sub>. Vials were incubated with a tube containing NaOH, which trapped all CO<sub>2</sub> produced by microbial activities. Vials those were without NaOH had CO<sub>2</sub> in their headspace. Data highlighted that no CH<sub>4</sub> consumption was observed in the vials containing NaOH trap, while vials set without NaOH exhibited CH<sub>4</sub> consumption. Thus it is proved that CO<sub>2</sub> is an

essential component of methanotrophy. CH<sub>4</sub> consumption rates were higher in 10000 ppm CH<sub>4</sub> than 1000 ppm CH<sub>4</sub>. Results also highlighted that CO<sub>2</sub> concentration in the headspace was higher in the vials incubated with 1000 ppm CH<sub>4</sub> than 10000 ppm of CH<sub>4</sub>. This indicated that CO<sub>2</sub> was consumed by methanotrophs along with CH<sub>4</sub> consumption. It is intriguing to understand how CO<sub>2</sub> drives CH<sub>4</sub> consumption. There are two pathways through which CH<sub>4</sub> consumption occurs: ribulose monophosphate pathway and serine pathway. In the former case, CH<sub>4</sub> is oxidized to CO<sub>2</sub> and is assimilated for cellular biomass. In the later pathway CH<sub>4</sub> is oxidized to formaldehyde. Two molecules of formaldehyde react with 1 molecule of CO<sub>2</sub> to form a three-carbon intermediate, which is essential for synthesis of cellular biomass. Thus, CO<sub>2</sub> was used by this pathway for CH<sub>4</sub> consumption (Fig 5). However, it is not understood why CH<sub>4</sub> consumption did not proceed through RuMP pathway, when there was no CO<sub>2</sub>. Perhaps, both serine and RuMP pathway are interlinked. Further research in this aspect is necessary to understand CO<sub>2</sub> driven methanotrophy.

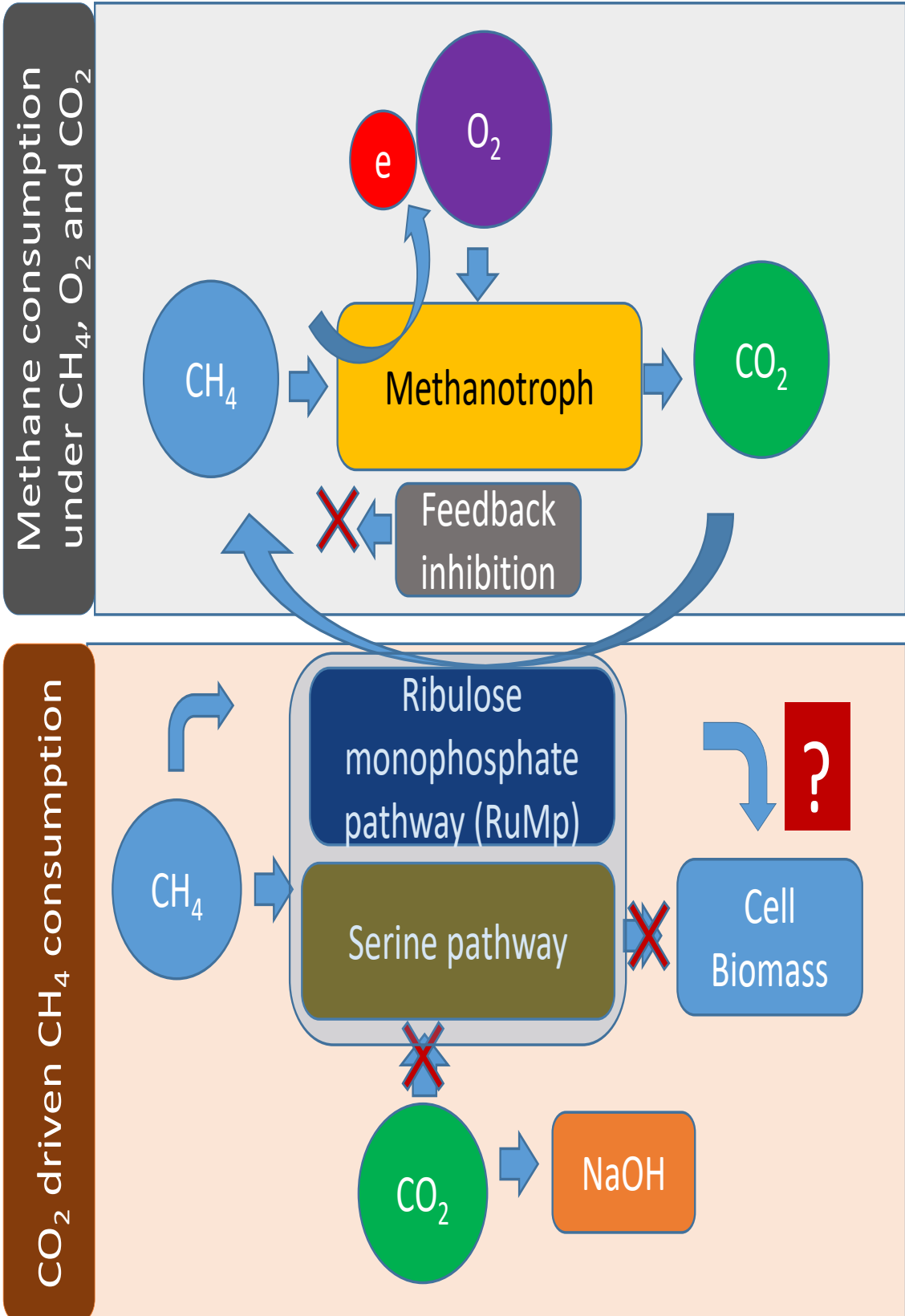


Fig 5. Methane consumption under the influence of different gasses. Top – CH<sub>4</sub> consumption or oxidation with CH<sub>4</sub> and O<sub>2</sub> as reactant. During CH<sub>4</sub> oxidation electron is transferred from CH<sub>4</sub> to O<sub>2</sub> and CO<sub>2</sub> is produced. CH<sub>4</sub> consumption occur only in presence of O<sub>2</sub>. Increasing the concentration of CH<sub>4</sub> stimulated CH<sub>4</sub> consumption. No feedback inhibition of CO<sub>2</sub> was observed. Down - Hypothetical mechanism of CO<sub>2</sub>drivenCH<sub>4</sub> consumption. Experiment indicated that CO<sub>2</sub> is essential for CH<sub>4</sub> consumption. CH<sub>4</sub> consumption occurs by both ribulose monophosphate pathway (RuMP) and serine pathway. Role of CO<sub>2</sub> in RuMP is not well understood. In serine pathway CO<sub>2</sub> is essential. However, in presence of NaOH,CO<sub>2</sub> is trapped resulting stoppage of CH<sub>4</sub> consumption.

## CHAPTER - IV

### SUMMARY, CONCLUSION AND SUGGESTION FOR FURTHER WORK

#### SUMMARY

CH<sub>4</sub> consumption is a complex phenomenon and it is not well understood, how different gasses present in soil regulates this process. Experiments were conducted to evaluate the effect of CH<sub>4</sub>, O<sub>2</sub>, and CO<sub>2</sub> at different mixing ratio on CH<sub>4</sub> consumption. The concentrations of CH<sub>4</sub> was either 1000 ppm or 10000 ppm, O<sub>2</sub> was at 0 % or 20 %, CO<sub>2</sub> concentrations were 0 ppm, 500 ppm, 10000 ppm or 50000 ppm. CH<sub>4</sub> consumption occurred only in the treatments of 20 % O<sub>2</sub>. Increasing the concentration of CH<sub>4</sub> also stimulated its consumption. The rate of CH<sub>4</sub> consumption estimated as ng CH<sub>4</sub> consumed g<sup>-1</sup> soil d<sup>-1</sup> varied from 0.283 to 0.481 at 1000 ppm of CH<sub>4</sub> and 2.958 to 4.994 at 10000 ppm of CH<sub>4</sub>. CO<sub>2</sub> production was estimated as ng CO<sub>2</sub> produced g<sup>-1</sup> soil, varied from 194 to 331 at 1000 ppm CH<sub>4</sub> and 139 to 272 in 10000 ppm of CH<sub>4</sub>. Production of CO<sub>2</sub> was high at lower CH<sub>4</sub> concentration. Abundance of methanotrophs enumerated as pmoA gene copies g<sup>-1</sup> soil and ranged from 4.5 x 10<sup>5</sup> to 107 x 10<sup>5</sup>g<sup>-1</sup> soil. Linear regression models predicted CH<sub>4</sub> consumption potential significantly (P 0.05) under the influence of different factors. CH<sub>4</sub> consumption rate and methanotrophs were stimulated with CO<sub>2</sub> irrespective of treatments. The role of CO<sub>2</sub> on CH<sub>4</sub> consumption evaluated by regulating headspace CO<sub>2</sub> using alkali trap, which indicated that CH<sub>4</sub> consumption occurred only in presence of CO<sub>2</sub>. Results highlighted that composition of soil's gas phase regulates CH<sub>4</sub> consumption. Gasses, particularly O<sub>2</sub> and CO<sub>2</sub> are essential for methanotrophs to oxidize CH<sub>4</sub> through serine pathway.

## CONCLUSION

CH<sub>4</sub> is a potent greenhouse gas and its concentration is increasing constantly, raising serious concern for global climate change. Agricultural soil is a major contributor of greenhouse gas, CH<sub>4</sub>. However, about 80 % of CH<sub>4</sub> gets oxidized in soil itself. Therefore, this process plays a greater role in mitigating atmospheric CH<sub>4</sub> budget. The gas phase of soil mainly contains O<sub>2</sub>, CO<sub>2</sub>, and CH<sub>4</sub>. Other major gas is N<sub>2</sub>. Depending on soil condition the composition of soil's gas phase vary. This study aims to highlight how the composition of different gasses regulates CH<sub>4</sub> consumption in soil. Experiments were conducted with three gasses comprising CH<sub>4</sub>, O<sub>2</sub>, and CO<sub>2</sub>. The concentrations of CH<sub>4</sub> was either 1000 ppm or 10000 ppm, O<sub>2</sub> was at 0 % or 20 %, CO<sub>2</sub> concentrations were 0 ppm, 500 ppm, 10000 ppm or 50000 ppm. Results indicated that CH<sub>4</sub> consumption occurred only in the treatments of 20 % O<sub>2</sub>. CO<sub>2</sub> stimulated CH<sub>4</sub> consumption. Generally it is assumed that CO<sub>2</sub> production is concomitant with CH<sub>4</sub> consumption, but it was reverse and CO<sub>2</sub> production was less under higher CH<sub>4</sub> consumption. For example, CO<sub>2</sub> was high in case of 1000 ppm of CH<sub>4</sub> than 10000 ppm of CH<sub>4</sub>. Abundance of methanotrophs pmoA gene increased with CH<sub>4</sub> consumption rates. To unravel the role of CO<sub>2</sub> on CH<sub>4</sub> consumption a follow up experiment was conducted, where headspace CO<sub>2</sub> concentration was regulated by NaOH. Interestingly no CH<sub>4</sub> consumption occurred in absence of CO<sub>2</sub>. Results highlighted that O<sub>2</sub> and CO<sub>2</sub> are essential for methanotrophy, which occurs through both ribulose monophosphate pathway (RuMP) and serine pathway. CO<sub>2</sub> is required to convert CH<sub>4</sub> to biomass through serine pathway. It was not understood why CH<sub>4</sub> consumption did not occur through RuMP, when there was no CO<sub>2</sub>. Results provided insightful information on CO<sub>2</sub> driven CH<sub>4</sub> consumption process and opened avenues for enhancing CH<sub>4</sub> uptake potential soil by managing soil resources. However, further research is required to (1) elucidate the differential role of RuMP and serine pathway on CH<sub>4</sub> consumption, (2) elucidate inter linkage between the pathways and (3) quantify the uptake of CO<sub>2</sub> in relation to CH<sub>4</sub> consumption to predict global warming potential of agriculture soil under future climate.

## **SUGGESTIONS FOR FURTHER WORK**

The current experiment identified two knowledge

1. Firstly we can change locations or different soil type and different soil conditions.
2. Secondly we can try it in another gases mixture or different ratio of gases.

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