Microbial control of greenhouse gas flux and thermally carbonized biomass decomposition in upland temperate soils

Dissertation

To Fulfill the Requirements for the Degree of

„doctor rerum naturalium“ (Dr. rer. nat.)

Submitted to the Council of the Faculty of Biology and Pharmacy

of the Friedrich Schiller University Jena

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Date of defense: 23-03-2015

ACKNOWLEDGMENT

I am thankful from the core of my heart to my research supervisor, Prof Susan Trumbore, for her admirable guidance, constructive suggestions, inspiring behavior and valuable knowledge. Her contributions throughout research period and especially during the preparation of this thesis are admirable. As a matter of fact, I have no appropriate words that fully express the immense indebtedness and deep gratitude I owe to her.

I am also thankful to my co-supervisor, Prof Gerd Gleixner. Whenever I seek guidance, he was there for me and always swayed me out of the dilemma. I must say the most memorable time during my stay in Germany was the one when we had long discussions on different scientific as well as social topics.

I will always appreciate friendly behavior of my both supervisors and their honest feedback on my progress in scientific knowledge. In future if I will get any chance to guide someone I look forward to them as my role models.

I must extend my thanks to Dr. Joseph von Fischer, assistant professor at Colorado state university, USA and Dr. Steffen Kolb, assistant professor at Friedrich Schiller University, Jena, for their guidance and discussion especially on my work regarding high affinity methanotrophy.

I am grateful to Prof, Ralf Conrad, Max Planck institute for terrestrial microbiology, Marburg, for providing me opportunity to work in his lab. Special thank goes to Dr. Andreas Reim for his guidance and supervision for all of the molecular work. I must extend my thanks to Dr. Amanda Barbosa for providing *USCα* plasmid and primers. I also want to pay my gratitude to Dr. Dheeraj Kanaparthi and Katrin Aschenbach for giving me company during the three month stay in Marburg.

I must thank to Dr. Carlos Sierra for his continuous support and help in data analysis, Dr. Elisabeth Jueschke for her sisterly love, supervision and contribution in hydrochar studies, Dr. Thomas Wutzler for providing R code to extract data from data-logger.

I am also thankful to all the technical staff in MPI-BGC especially Karl Kuebler for spending hours in preparation of air mixing system and finding leakages in it. I must thank to teams of Isolab, RoMA and Gas lab especially Dr. Willi Brand, Michael Rothe, Heike Geilmann, Petra Linke, Jürgen Richter, Ines Hilke, Birgist Frohlich Dr.Armin Jordan and Bert Steinberg**.** Special thank goes to Theresa Klötzing and Iris Kuhlman for their assistance in routine lab work and soil enzyme analysis. I also want to extend my gratitude to IT service group and workshop.

I would like to thank my lab mates and colleagues, particularly Steffany Theissen for constructing the automated incubation system, Julia Baumart for her help in pyrochar preparation and TGA analysis, Stefan Karlowsky and Enrico Weber for helping me in soil sampling, Martin Nowak for translating thesis summary in German, Zhang Haiyang for helping me in lab work, Andrea Scheibe, Andrej Thiele, Ashish Malik, Carolin Thoms, Emilly Solly, Perla Mellado, Vanessa Roth, Markus Lange, Roman Witt, Ronny Thoms, Vanessa Roth and many more for the great working environment and discussions.

Special thanks to Dr. Anna Goerner, Dr. Katrin Krause and John Schmidt for making my transition from China to Germany as smooth as possible.

My research was partly funded by Max Plank society (MBG), International Max-Planck research school for global biogeochemical cycles (IMPRS-gBGC) and German Research Foundation (DFG) in the framework of graduate school 1257: "Alteration and element mobility at microbial-mineral interface". I am thankful to all of them for providing me the opportunity.

At the last but not the least I want to thank my parents, family members and friends for their unconditional love and support throughout my life. And very special thanks and gratitude to Dr. Nivedita Chatterjee, for being the most beautiful part of my life.

Saadat Ullah Malghani

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‰	Per mill
°C	Degree celsius
^{13}C	Isotopic carbon
$^{2}H/D$	Isotopic hydrogen/deuterium
ANOVA	Analysis of variance
bp	Base pair
C	Carbon
C_3	Plants that start photosynthesis with Calvin-Benson cycle
C_4	Plants that start photosynthesis cycle by producing 4-carbon molecule in mesophyll cells followed by Calvin-Benson cycle in bundle sheet cells
CH_4	Methane
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
e.g	Example gratia (for example)
etc.	et cetera (and the rest)
g	Gram
GC-MS	Gas chromatography-mass spectroscopy
GC-C-IRMS	Gas chromatography-combustion-isotope ratio mass spectrometry
GHG	Greenhouse gas
HTC	Hydrothermal carbonization char/hydrochar
IPCC	Intergovernmental panel on climate change
Km	Michaelis constant
L	Litre
M	Molar
N_2	Nitrogen gas
N_2O	Nitrous oxide
O_2	Oxygen
PC	Pyrolysis char/pyrochar
PCR	Polymerase chain reaction
P.E	Priming effect
pMMO	Particulate methane monooxygenase
ppb	Part per billion
ppm	Part per million
SOM	Soil organic matter
SOC	Soil organic carbon
TC	Total carbon
TN	Total nitrogen
TOC	Total organic matter
T-RFLP	Terminal-restriction fragment polymorphism
VPDB	Vienna pee dee belemnite
WFPS	Water filled pore space

CHAPTER 1

General Introduction

Recent climatic changes are directly linked with increasing atmospheric concentrations of three greenhouse gases (GHG; CO_2, CH_4 and N_2O) that altogether amount 80% of the total radiative forcing (IPCC, 2007). Studies of the gases trapped in ice cores reveal that the current concentrations of these GHGs exceed levels in past 800,000 years (Flückiger *et al.*, 2002; Ahn and Brook, 2008; Luthi *et al.*, 2008; Pongratz *et al.*, 2009). Over the last decade, the average growth rates for CO_2, CH_4 and N_2O concentrations in the atmosphere were 0.5%, 0.4% and 0.25% per year respectively (IPCC, 2013). These continuing rates of increase are reflect the imbalance between sources and sinks, with human sources overwhelming the processes removing these gases from the atmosphere (Allison, 2009; Kirschbaum *et al.*, 2013).

Gas exchange between soils and the atmosphere is an important contributing factor to the global budgets of all three major greenhouse gases (CO_2, CH_4 and N_2O). The metabolic activities of soil microbes that are main controller of production and/or consumption of these gases in soils are regulated by a number of environmental variables (Conrad, 2009). In this Chapter, I review these processes briefly for the upland (unsaturated) soils that were the main object of study in this thesis.

1.1 Carbon Dioxide (CO_2)

Soils are of particular importance in the atmospheric CO_2 budget for a number of reasons. Soil organic matter contains one of the largest terrestrial reservoirs of carbon roughly 3 times greater than compared to C stored in the biosphere or atmosphere. Soils have a large capacity to sequester C, with an estimated capacity to store a further 800 GT of carbon globally (Batjes, 1998; Broecker, 2007; Schmidt *et al.*, 2011; Batjes, 2014). Approximately 50% of the atmospheric CO_2 that enters the biosphere and is made into plant material gets recycled through the biological 'funnel' of soil microbes (Lehmann, 2007). At present, options for mitigation of climate change have focused attention of the capacity of soil C storage to offset ongoing increases in atmospheric CO_2, as well as to prevent losses of natural soil C stores to the atmosphere. Current estimates of terrestrial carbon sink that accounts carbon cycling between atmosphere and biosphere, including soils, not only predict a decrease in capacity of this sink during this century but also evident for release of carbon which is already stored (Friedlingstein *et al.*, 2006; Bond-Lamberty and Thomson, 2010). Fundamental knowledge about the mechanisms of soil organic matter (SOM) stabilization and destabilization are required if we are to manage soil carbon stocks.

Carbon enters soils as plant litter and root biomass, and is decomposed largely by soil microorganisms, the biological 'funnel'. Microbial conversion of organic matter is a complicated process and its rate is influenced by a number of factors, including: the quality and quantity of

fresh plant biomass, soil physicochemical characteristics (soil mineralogy, structure), environmental controllers (temperature, moisture, O_2 availability) and microbial community composition (Schmidt *et al.*, 2011). Among the environmental factors that govern microbial activity in soils, temperature and moisture are the most documented factors. There is a current consensus that increases in temperature will lead enhanced degradation of soil carbon stocks and increase emission of CO_2 to the atmosphere (IPCC, 2013), although there is major uncertainty about the magnitude and duration of such effects. Soil moisture can play dual role. It regulates the exchange of gases between soils and atmosphere and thereby influences microbial activities by regulating supplies of substrates (e.g. like CH_4) as well as the major electron acceptor responsible for decomposition, oxygen (O_2).

It is experimentally evident that the soil matrix has strong gradients in O_2 and all sites where decomposition occurs are not equally aerated (Greenwood, 1961; Greenwood and Goodman, 1967; Zausig *et al.*, 1993). However there is a scarcity of literature that can more quantitatively link estimates of areas subject to low aeration and decomposition or trace gas emission rates. Molecular studies show the presence of functionally redundant but physiologically different type of microbes at relatively short distances (Morris and Schmidt, 2013). This complexity of processes at relatively short distances is one of the biggest challenges in studying emergent properties of soils at larger spatial scales.

Additionally the decomposition of soil organic matter, as reflected in emissions of CO_2, are influenced by soil variables that influence either microbial activity or microbial community size and structure, such as pH, mineral nitrogen and other nutrients which, in turn, are controlled by a combination of soil properties (soil moisture, texture, structure) and management practices.

1.2 Methane (CH_4)

Soils are important sources as well as sinks for atmospheric CH_4 and play crucial role in the global cycling of this greenhouse gas with a global warming potential ~ 23 times greater than CO_2 (IPCC, 2007). The capability of a soil to act as sink or source for atmospheric CH_4 depends on soil conditions, largely due to the fact that the consumption and production of CH_4 are carried out by two physiologically distinct groups of microorganisms (Conrad, 1996). Methanogens, the methane producing microbes, are extremely sensitive to oxygen and to oxygen reactive species and produce CH_4 only under highly reducing conditions (Thauer *et al.*, 2008). Thus, anaerobic soils, such as rice paddies and wetlands, which are rich in organic matter, can be significant sources of methane. On other hand, methanotrophs, the methane consuming microbes, can consume CH_4 in the presence of O_2 and anaerobically. Different populations are responsible for methane consumption in regions of high and low methane supply. In well aerated oxic soils net consumption of CH_4 directly from the atmosphere requires methanotrophs able to use very low CH_4 concentrations. In these soils, CH_4 production is either too low or completely absent to complete their cell specific activities methanotrophs depend on atmospheric CH_4.

1.2.1 Methanogenesis

Methanogens are phylogenetically diverse group of microbes within the domain of *Archea*, including some of the most ancient microbes, as some extreme thermophile and halophiles are also members of this group (Topp and Pattey, 1997). Because methanogens are obligate anaerobes, it was assumed that methanogens may not play active role in CH_4 fluxes of upland soils. However methanogens are not only present in upland soils but they actively produce CH_4 under wet and anoxic conditions (Angel *et al.*, 2012). Such favorable conditions may available in clay rich aggregates and at deeper in soil profiles. Methanogens depend on an extremely narrow range of substrates including hydrogen + carbon dioxide, acetate, formate, methanol and some alcohols. In soils, CH_4 is produced using two main substrates, the acetoclastic ($CH_3COOH \rightarrow CH_4+CO_2$) and hydrogenotrophic ($CO_2+4H_2 \rightarrow CH_4+2H_2O$) using acetate and hydrogen respectively. Studies on active methanogens in rice fields and one recent report on biologically active crust in desert soils found different niches for the activity of acetoclastic and hydrogenotrophic methanogens O_2 (Conrad, 2009; Angel, 2010). The acetoclastic group of methanogens, *Methanosarcina*, was active only under anoxic conditions whereas, hydrogenotrophic were the dominant substrate in the presence of O_2 (Angel, 2010). However, there is no experimental evidence about the threshold level of O_2 at which rates of CH_4 production exceed CH_4 oxidation rates for soils. Field studies measured active production of CH_4 at different moisture levels but the rate of CH_4 production was mostly lower than the rate of CH_4 oxidation in well-drained soils (von Fischer, 2002). Although presence of methanogenesis in upland soil system is evident the contribution of the balance of methane production to consumption and its overall potential role in the global CH_4 cycle are require further investigation.

1.2.2 Methanotrophy

CH_4 is the most reduced form of carbon and its transformation into any other form requires an electron acceptor. In soils, this process is carried out by ubiquitous group of microorganisms known as methanotrophs. It is evident that the exchange rates of CH_4 between soil and atmosphere are governed by CH_4 oxidation process as approximately 20-90% of CH_4 produced in saturated soils is consumed close to the production site by CH_4 oxidizers. In addition, unsaturated soils also exhibit CH_4 oxidation and under natural conditions the source is atmospheric CH_4. Estimates showed that globally unsaturated or upland soils uptake about 20 to 45 Tg CH_4 per year which accounts 10% or less of the total capacity of global sink for atmospheric CH_4 (Dutaur and Verchot, 2007).

Oxidation of CH_4 is a purely enzymatic process through which methane is converted to CO_2 via methanol, formaldehyde, and formate. Enzymes, including methane monooxygenase (MMO), methanol dehydrogenase (MD), formaldehyde dehydrogenase (FLD) and formate dehydrogenase (FMD), catalyze all these sequential steps of oxidation (Hanson and Hanson, 1996). All known aerobic methanotrophs posses the monooxygenase enzyme that facilitates conversion of CH_4 to methanol which is the first step of CH_4 oxidation. The preferd substrates for this enzyme are CH_4 and other simple compounds (Semrau *et al.*, 2010). Methane monooxygenase comes in two forms: soluble (sMMO) and membrane bound (pMMO) and both forms differ in their mechanism, origin (Holmes *et al.*, 1999) and sensitivity towards Cu^{+2}. Almost all type of methanotrophs except *Methylocella palustris* contain pMMO enzyme and therefore the gene

"*pmoA*", responsible for encoding the active-site subunit of pMMO, is extensively used as biomarker for methanotrophs identification (Knief *et al.*, 2003). Based on the C-assimilation pathway, the recognized genera of methanotrophs are divided into two groups, *Type I* (further divided into *Types I* and *X*) affiliated to *γ-proteobacteria*, and *Type II* which have phylogenetic affiliation to *α-proteobacteria*. However, this classification does not include phylum *Verrucomicrobia* and the phylum level candidate division *NC10,* relatively new groups of methane oxidizing bacteria (Nauer *et al* 2013). As the newly discovered methanotrophs are physiological so distinct from *proteobacteria*, the old classification still serves well for aerobic methanotrophs.

The sMMO is found only in *Type I* and *Type X* methanotrophs. Moreover, some methanotrophs are discovered only in upland soils and are mainly grouped into two clades, the upland soil cluster α and γ (*USCα* and *USCγ*) (Holmes *et al.*, 1999; Bull *et al.*, 2000; Knief *et al.*, 2005; Kolb *et al.*, 2005; Judd, 2011). Both types of methanotrophs, *Type I* and *II*, follow same steps under catabolic pathway of CH_4 oxidation. Conversely, differences appear in anabolic pathway, CH_4-C is assimilated at the level of formaldehyde. The anabolic pathway are further divided into two sub-pathways, ribulose monophosphate pathway and serine pathway which are carried out by *Type I* and *Type II* methanotrophs, respectively (Hanson and Hanson, 1996). Besides, some methanotrophs, phylum *Verrucomicrobia,* can also assimilate carbon via Calvin Benson-Bassham cycle (Trotsenko and Murrell, 2008).

The enzymetic reactions of methane oxidation could be best explained by Michaelis-Menten (M-M) kinetics as initial process rate increases linearliy with increase in CH_4 and at higher concentrations it reaches to maximum (Vmax) (Bender and Conrad, 1993; Benstead and King, 1997; Bull *et al.*, 2000). Enzymatic parameters such as maximum oxidation rates (V_{max}), apparent half saturation constant (K_m) and threshold mixing ratio (T_h) have been extesively studied in soils from different environments and the capacity of the enzyme to perform at certain substrate concentration is best defined by the K_m; lower K_m values indicate higher affinity for the low substrate levels. Bender and Conrad (1994) reported enzymetic kinetics of different saturated and unsaturated soils and found that K_m ranged from 0.01 to 0.28 µM CH_4 in unsaturated soils. While K_m values in saturated soils as well as in unsaturated soils exposed to high CH_4 contents (50,000 ppm) were higher than 1 µM CH_4. K_m (2–12 µMCH_4) values of saturated soils were similar to those exhibited by pure culture of methanotrophs. These studies highlight the presence of either physiologically different organisms or similar organisms but having enzymes capable to perform the reaction with diffrential affinities.

Untill now no pure culture of methantrophs demonstrated the ability to grow on low CH_4 concentrations and due to this fact, several physiological, biochemical and molecular techniques are extensively used to find link between CH_4 oxidation activities and presence and activity of certain methantrophic groups. Among several techniques, the use of stable or radioactive isotope probing methods, fluorescent probe-based approaches, or detection of biomarkers or fingerprinting of genetic markers are very common (Roslev and Iversen, 1999; Knief *et al.*, 2003; Maxfield *et al.*, 2006; Singh *et al.*, 2007; Nazaries, 2011). These techniques are excellent alternatives to gather information on active groups of methanotrophs especially at atmospheric CH_4 levels. Results of many studies revealed that several methanotrophs may be present in both low and high CH_4 environments. Additionally, certain other groups of methanotrophs specific to upland soils containing *pmoA* gene were also detected and are known as upland soil cluster

(Kolb *et al.,* 2005; Kolb, 2009; Degelmann *et al.,* 2010). Among different groups of upland cluster, *USCα* is the most commonly found specifically in forest soils. Approximately 77% of all reported studies on forest ecosystems recorded presence of *USCα* (Kolb, 2009) and *pmoA* gene based phylogenetic analysis suggest that *USCα* is most closely related to *Methylocapsa acidiphila* (Family *Beijerinckiaceae*) (Ricke *et al.,* 2005). Other novel methanotrophs only reported in upland soils include *Cluster 1* or *USCγ* and are reported mostly in neutral pH soils (Knief *et al.,* 2003; Horz *et al.,* 2005). Presence of several representatives of *Type I, Methylobacter, Methylococcus* and *Type II* are also evident by previous studies (Horz *et al.,* 2005; Zheng *et al.,* 2012). However, it is not well understood whether *Type I* and *Type II* methanotrophs observed in upland soils are active participants in atmospheric methane oxidation. In addition, strategy of these organisms to survive at low concentration of CH_4 is also not clear and questions remain whether these organisms solely depend on atmospheric CH_4 or also co-oxidize some other compounds (Benstead *et al.,* 1998).

1.2.2.1 Factors regulating rates of methanotrophy in upland soils

Two primary variables are responsible for the uptake rates of atmospheric CH_4 exhibited by upland soils; air diffusion and methanotrophic activity. The scale of the impact of these two variables on CH_4 oxidation rates depend on environmental conditions. Soil air diffusivity controls availability of CH_4 and O_2 to methanotrophic communities. However, it is not clear between CH_4 and O_2 which factor is exhausted first and thereby controls methane oxidation rates. The threshold level of CH_4, below which methane oxidation may halted completely, was reported as <0.3 ppm in most of oxic soils (Bender et al 1992). On other hand, contrasting results have been reported on the influence of O_2 concentration on CH_4 oxidation rates. Bender et al (1992) found that CH_4 oxidation rates were insensitive to change in O_2 concentrations up to 2% and below 2% O_2, CH_4 oxidation rates were considerably decreased. In contrast, one recent study observed enhanced CH_4 oxidation rates when O_2 concentrations were decreased to 5% (Brzeziska *et al.,* 2012). These contrasting results highlight the complexity of high affinity methanotrophy and advocate further investigations.

Moisture is one factor that controls the rate of diffusivity of gases in soils, and thus is considered one of the most important regulators for CH_4 fluxes in different ecosystems. The optimum moisture range for methane oxidation varies for soils vary but are mostly reported between 18-51% by volume (Czepiel, *et al.,* 1995). At optimum conditions soil moisture may have a limiting direct effect on methanotrophs activity but desiccation cause serious damage to methanotrophic activity (von Fischer *et al.,* 2009). However upon rewetting the methanotrophic community may recover quickly (Judd, 2011).

Environmental factors other than soil moisture that influence the activity of methanotrophs include temperature, soil carbon, dissolved organic carbon, salt concentration specially NH_4^+ and soil pH (Amaral *et al.,* 1998; Saari *et al.,* 2004; Maljanen *et al.,* 2006). Temperature has a relatively minor effect on CH_4 uptake rates and reported Q_{10} values are usually lower than 1.5 (He *et al.,* 2012). The effect of NH_4^+ on methane uptake rates is attributed to the possible competition of NH_4^+ for binding to the methane monooxygenase (MMO) enzyme (Gulledge *et al.,* 1997). However, several ecosystems especially in temperate regions do not show any inhibitory effect of NH_4^+ on CH_4 oxidation rates. In this context, the role of methanotroph

abundance is not fully exploited largely due to the lack of information about the specific methanotrophs responsible for CH_4 oxidation and how they respond to environmental variables.

Soils under different land uses also considerably vary in their CH_4 oxidation rates and these differences are largely related with soil carbon and composition of methanotroph communities. Based on published literature, the highest CH_4 oxidation rates are observed in forests, followed by grassland and cultivated soils (Le Mer & Roger, 2001). Cluster analysis based on the DGGE banding patterns indicated that the MB communities in a forest site differed from those at a farmland site (Knief *et al.*, 2005). The role of soil pH is also linked with its major effects on methanotrophic community composition, such as, *USCα* is measured in abundance only at acidic pH (Amaral *et al.*, 1998).

1.3 Nitrous dioxide (N_2O)

According to estimates both natural and anthropogenic sources emit approximately 8.5–27.7 Tg N_2O-N $year^{-1}$ with the largest anthropogenic contribution from agriculture, which contributes 1.7–4.8 Tg N_2O-N $year^{-1}$(IPCC, 2013). In comparison, global emissions for soils under natural vegetation are estimated to be 6.6 Tg N_2O-N $year^{-1}$ with large uncertainty (3.3–9.0 Tg N_2O-N $year^{-1}$) (Stehfest and Bouwman, 2006; Denman, 2007). The large uncertainties reflect poor understanding of the processes that govern N_2O emissions soils. Among natural vegetation, tropical forests are considered to be largest source of N_2O emissions contributing 1.34 Tg N_2O-N $year^{-1}$. The contribution of temperate forest system is relatively small and estimated about 0.15 Tg N_2O-N $year^{-1}$ (Stehfest and Bouwman, 2006).

Two main processes are considered as the main drivers for N_2O production in soils: nitrification and denitrification. Nitrification is an aerobic process and is carried out by chemoautotrophs that gain energy through oxidation of NH_4^+ and NO_2^-. Nitrification is carried out by relatively small group of soil microorganism, two different genera take part in each step. *Nitrosomonas* convert NH_4^+ into NO_2^- while *Nitrobacter* oxidize NO_2^- into NO_3^- (Gee *et al.*, 1990).

Denitrification occurs only at very low to zero level of O_2 by a vast group of facultative anaerobes, candidates from the phyla *Firmicutes, Actinomycetes, Bacteroides, Aquifaceae and α, β, γ and ε-Proteobacteria* have been found as active denitrifiers in soils (Philippot, 2002; Khalil *et al.*, 2004). In addition to bacteria, some fungi also reported to be capable of producing N_2O in soils (Shoun *et al.*, 1992). Broadly denitrifiers are heterotrophs which decompose organic matter and use oxidized form of nitrogen, NO_3^- and NO_2^-, as an electron acceptor.

Denitrification is considered more important for having a large potential for production of N_2O, which is one of the main products in denitrification and a byproduct in nitrification. Enzymatic reactions responsible for both processes are well documented including the factors responsible for regulating their rates. Absence of O_2 is pre-requisite for induction of denitrification process, though some studies also found active denitrification in the presence of molecular O_2 (Butterbach-Bahl *et al.*, 2013). However it is not clear whether denitrification can occur under sub-oxic condition or not. The persistence of anoxic conditions also leads to decrease in N_2O emissions as N_2O act as electron acceptor in absence of NO_3^- (Butterbach-Bahl *et al.*, 2013).

Several factors influence the rates of nitrification and denitrification in soils, including temperature, pH, texture, and the supply of labile organic C. By regulating the supply of O_2, soil moisture is the major driver of N_2O emissions. Field studies recorded maximum N_2O emission rates when moisture ranges between 70-80% WFPS depending on soil types, above 80% WFPS, Besides the direct roles of temperature on both processes by influencing microbial growth and increasing enzymatic activities, it also promotes denitrification by enhancing heterotrophic activities that consume O_2 in soil which is major the driver of the processes. Diverse groups of nitrifying and denitrifying microbial communities can survive at broad range of soil pH but process rates are found to be highly sensitive to change in pH (Stevens *et al.*, 1998; Chapuis-Lardy *et al.*, 2007). In general higher N_2O production due to active nitrification has been reported in acidic conditions (Ste-Marie and Paré, 1999). Soil carbon, regulates rates of denitrification in soils and is considered as important factor after soil moisture. Recently, it has been reported that presence of several labile carbon compounds results into reduced emissions of N_2O due to enhanced rate of N_2O reduction (Morley and Baggs, 2010).

As mentioned agricultural soils are largest single source for anthropogentic N_2O emissions and because of this the main focus of research in the past has been on agricultural ecosystems, with fewer studies highlighting the role of temperate forest ecosystems for N_2O emissions. In forests, low levels of available NH_4^+ and NO_3^- due to rapid microbial immobilization and uptake by plants are associated with low levels of N_2O production. However, increased atmospheric N deposition or disturbances like logging can alter the balance among nitrification, plant uptake, and microbial immobilization which in turn results in enhanced N_2O emissions (Thomson et al., 2012; Ussiri, 2013). Therefore, the additional focus on forest ecosystems will not only improve our knowledge on factors regulating N_2O production but also possibly will help to reduce uncertainties in the estimation of N_2O emissions.

1.4 Soil Carbon sequestration via thermally altered biomass

Soil carbon sequestration is a process in which CO_2 is removed from the atmosphere and stored in the soil carbon pool. Several agricultural management techniques, including cover crops (Kaspar *et al.*, 2011), mulches (Kahlon *et al.*, 2013), compost or manure additions (Luo *et al.*, 2014), have potential to sequester carbon in soils with positive impacts on crop productions. However, the benefits of such amendments are short-lived since decomposition rates are high and the added organic matter is usually mineralized to CO_2 within only a few cropping seasons (Tiessen *et al.*, 1994). In addition, concentrating amendments in a single location must be offset by the loss of inputs (e.g. for manure) in other locations – the overall net effect on soils at larger spatial scales may be negligible.

In this context, the application of thermally modified biomass (bio-char) has been suggested not only to improve crop production by increasing nutrient supplies but it also to enhance soil carbon storage due to the inert nature of its large portion (Lehmann *et al.*, 2006; Lehmann, 2007). The concept of biochar addition to agricultural soils is derived from pre-Columbian soil managed by indigenous people living in the Amazon basin between 600 and 8700 years ago (Glaser *et al.*, 2000). These 'anthropogenic' *Terra Preta* soils are characterized by higher C content and greater microbial diversity compared to unamended adjacent soils with similar mineralogy (O'Neill *et*

al., 2009; Khodadad *et al.*, 2011). It has been proposed that *Terra Preta* soils were modified via charcoal application along with manures, (Glaser *et al.*, 2000), and since after hundreds of years of agricultural practices these soils successfully maintained their high soil C contents. Several field studies on nutrient poor soils reported increases in crop yield from 220 to 800 % (Glaser *et al.*, 2000; Lehmann *et al.*, 2003) after char application but with the recommendation to add char along with compost or manure, to reduce potential negative impacts.

The production of biochar is derived from methods for charcoal production; dry biomass is treated at moderate to high temperature (500-800 °C) for a few seconds to a few hours (Bruun *et al.*, 2011). Generally, heating biomass such as corn silage at 500°C for 2 hour leads to 40-45% C yield as biochar (Malghani *et al.*, 2013). Apart from fast growing crops, large amounts of bio-waste produced globally are recommended feedstock for pyrolysis. In this context, Lehmann et al (2006) presented an extensive review of the amount of potential feedstock for biochar production on global scale and how much offset it can provide for global CO_2 emissions. Numbers of unconventional sources, such as bio-solid, the solid organic matter recovered from a sewage treatment process includes excreta, faecal matter, and seepage (Kalderis *et al.*, 2014) were ignored by Lehmann *et al* (2006). A major drawback of these sources is the large fraction of water contents that otherwise consume a major proportion of energy added during pyrolysis only for drying. In 2007, Titirici *et al* came up with the idea of using low temperature techniques with the same principle of biomass conversion but under high water conditions to produce 'hydrochar'.

1.4.1 Hydrothermal carbonization char (hydrochar)

Hydrothermal carbonization (HTC), also known as wet torrefaction, is low temperature technique with tunable processes conditions to produce a final product with variable characteristics. Historically, this process was discovered while simulating natural process of bituminous coal by Friedrich Bergius in 1913 and this discovery led him to achieve the Nobel Prize in chemistry in 1932 (Bergius, 1932). The main features of the hydrothermal process include low to medium temperature (220-450^{o}C) and high pressures (18-22 bar) that results series of chemical and physical changes in the feedstock including hydrolysis, dehydration, decarboxylation, aromatization, and condensation (Funke and Ziegler, 2010).

One of the major advantages of hydrothermal carbonization over dry pyrolysis is its low energy usage but under low temperature (<180°C) biomass may also not be completely carbonized and large proportion of biomass remain unchanged, specifically lignin which requires higher temperatures to react. Moreover, bio-wastes are usually rich in pathogens and pharmaceutical products and to get rid of them, the HTC temperature needs to be increased (e.g. T > 250 °C). Thus by increasing temperature of hydrothermal processes not only will enhance carbonization process but it will also be suitable for bio-wastes. Besides temperature, for a good reaction condition it is important that biomass should be submerged in water. Lu *et al*. (2011) reported that increasing the solid to liquid ratio (SLR) results in a decrease in hydrolysis efficiency, so by decreasing SLR char production increases. Several studies show possible use of alternative liquid sources, such as seawater, municipal and industrial wastewaters, as fresh water itself is decreasing resource. "Using municipal and industrial wastewaters, with the exception of streams with high $CaCl_2$ concentrations, may impart little influence on final carbonization products/yields" (Lu *et al.*, 2014). It should also be mentioned that an acidic pH is always

necessary in order to promote the hydrolysis of lignocellulosic biomass or organic municipal waste, due to the strong resistance to hydrolysis, addition of catalysts promote an efficient reaction path via hydrolysis–dehydration (Eibisch *et al.*, 2013).

The final product of the hydrothermal carbonization process has numerous applications including fuel, gaseous adsorption on surface (Lee *et al.*, 2006; Yürüm *et al.*, 2009; Sevilla and Fuertes, 2011), soil enrichment (Qayyum *et al.*, 2012), catalysis (Titirici *et al.*, 2006), water purification, energy storage (Wang *et al.*, 2001), and CO_2 sequestration (Sevilla *et al.*, 2011). To suite the desired characteristics of the product for a specific use, the process conditions can be modified along with some additional treatments. As this process is recommended recently as tool to produce carbonized biomass for soil amendments, it is still unclear which process conditions will produce char with the greatest potential for CO_2 sequestration when added as a soil amendment.

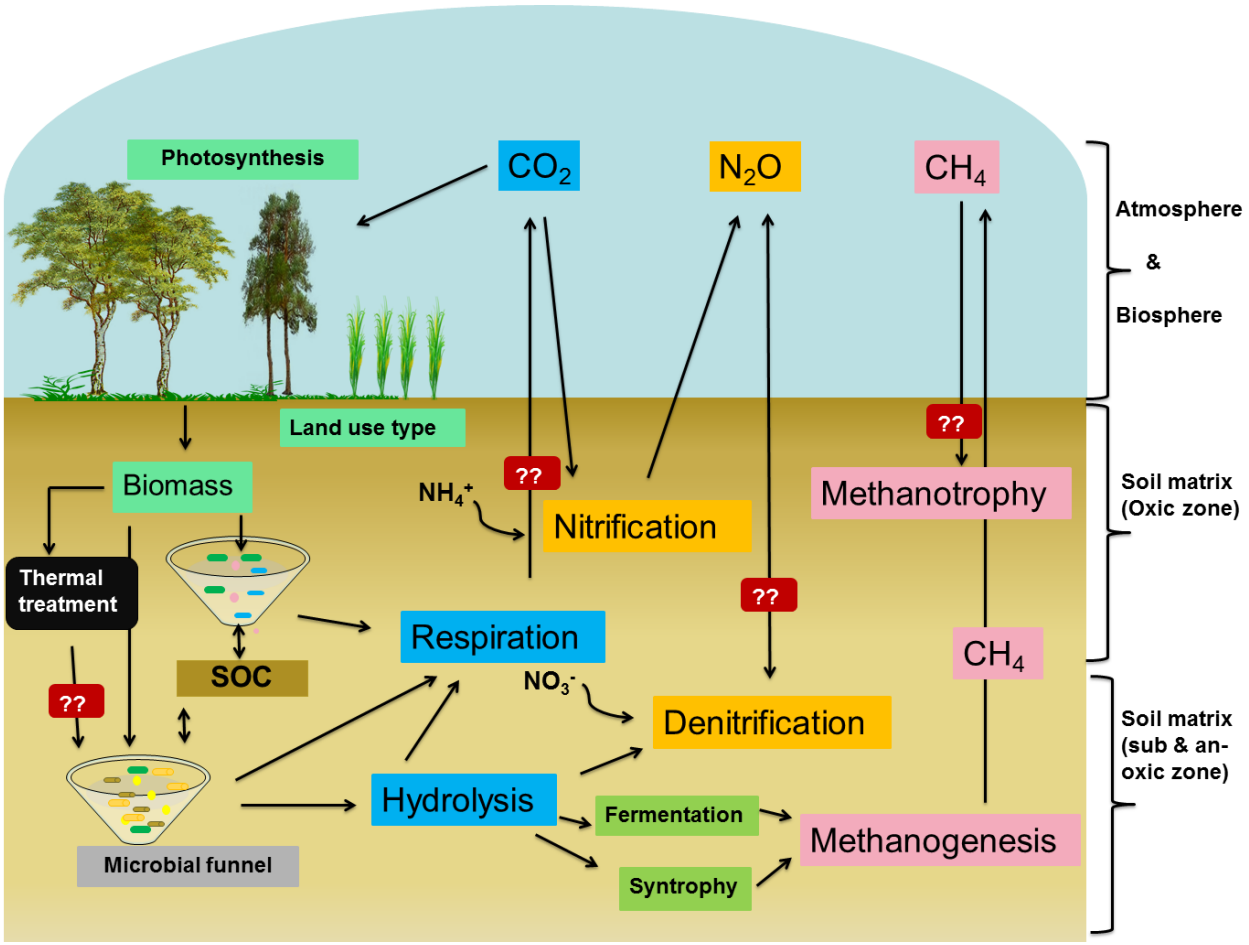

Figure 1.1: Schematic diagram of the biochemical process responsible for exchange of GHG (CO_2, CH_4 & N_2O) exchange between soil and atmosphere. Thermal treatment prior to plant biomass entering into soil matrix is recently proposed method for carbon sequestration. The boxes with question marks indicate some direction of this study.

1.5 Aims of this study

Upland soils play a crucial role in global cycling of atmospheric trace gases, acting as a sink and/or source for CO_2, CH_4 and N_2O. To cope with increasing growth rates of these gases in atmosphere, it is important to increase the terrestrial sink capacity by increasing uptake or reducing sources. Chapters 2 and 3 explore the influence of some environmental factors that play direct role in biochemical reactions responsible for production and/or consumption of CO_2, CH_4 and N_2O. Taking the advantage of flow through incubation system which enabled us to minimize role of diffusion as regulating factor for trace gases fluxes, impact of O_2, soil moisture and CH_4 concentrations was determined in different soils representing range of land uses. In Chapters 4 and 5, this work highlights several aspects of hydrochar application in agricultural soils including its stability, priming effect and impact on non-CO_2 gases. Specifically, the following questions were addressed:

Chapter 2: Variable responses of atmospheric traces gases (CO_2, CH_4, N_2O) production and consumption processes in upland soils to sequential shifts in O_2 concentrations

This was a preliminary study targeting production and consumption of greenhouse gas from a range of upland soils. The main idea behind this study was to determine the sensitivity of microbial metabolic processes to O_2 concentrations. Specifically following questions were pursued;

1. At what O_2 threshold can an upland soil switch from sink to source of atmospheric CH_4?
2. Do changes in O_2 concentrations results alter CO_2 emission rates?
3. At what O_2 concentration, will denitrification process dominate as the principal source of N_2O in contrasting soils?
4. Do soils representing contrasting land uses vary in their response to sequential shifts in O_2 concentrations?

Chapter 3: Abundance of high affinity methanotrophs may not be substrate driven: laboratory based incubation study using soil from contrasting land use types

Differences in CH_4 oxidation rates among land use type are generally linked with soil physical and biological parameters. In this study we reduced the effect of physical constrains on CH_4 supply to determine direct influence of CH_4 concentrations on oxidation process. Furthermore, the analysis was extended to determine differences in methanotrophic community size and composition among land use type and treatments. Specific objectives of this study were to answer the following questions:

1. Why do soils of contrasting land use type differ in intrinsic CH_4 uptake capacity even after minimizing the effect of diffusion?
2. Between methanotrophic population size and community structure, which factor particularly responsible for high CH_4 oxidation rates in forest soils?
3. What factors limit population of methanotrophic community?

4. Similar to low CH_4 environments i.e. paddy soils, does increase in CH_4 availability enhance methanotrophic efficiency and abundance in upland soils?

Chapter 4: Carbon sequestration potential of hydrothermal carbonization char (Hydrochar) in two contrasting soils; results of a one-year field study

Use of hydrothermal carbonization as a tool for carbon sequestration by amendment in agricultural soils is in its infancy. Previously published literature on the fate of hydrochar in soils and its impact on plant growth are limited to laboratory incubation or mesocosms studies. This study was especially designed to fill this gap in knowledge. Exploiting the differences in natural abundance of stable isotopes (^{13}C) between C3 and C4 vegetation, hydrochar carbon was tracked in all carbon pools including soil leachate, gaseous form (CO_2) and bulk soil carbon. This study was designed mainly to answer two main questions:

1. What fraction of the carbon associated with hydrochar amendment can persist in soil under field conditions, and how long can it sequester C?
2. What is the impact of hydrochar amendment on native soil carbon?

Chapter 5: Chars produced by slow pyrolysis and hydrothermal carbonization vary in carbon sequestration potential and greenhouse gases emissions

Two methods of thermal carbonization of biomass are the most promising based on their efficiency for carbon recovery. Pyrolysis char production is similar to conventional charcoal production and uses only dry biomass while hydrothermal carbonization char is produced at low temperature but higher pressure and suited to unconventional biomass, i.e sewage sludge, biowastes etc. The carbonized products of these two production methods vary in composition and other physical characteristics. In this study, the same feedstock - corn silage, was used to produce chars using slow pyrolysis and hydrothermal carbonization. These chars were added to soils and incubated in contrasting soil types to answer the following questions:

1. Does the carbon sequestration potential vary between char produced by slow pyrolysis and hydrothermal carbonization?
2. Do the impacts of the two char types on soil physicochemical characteristics differ in various types of soils?
3. What is the influence of pyrochar and hydrochar on emissions of greenhouse gases (methane and nitrous oxide) from the different soils?

CHAPTER 2

Variable response of atmospheric traces gases (CO_2, CH_4, N_2O) production and/or consumption processes in upland soils to sequential shifts in O_2 concentrations

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Abstract

Biogenic gas production and consumption in soils is influenced by the amount of oxygen, the electron acceptor yielding the most energy during the breakdown of organic matter. Although previous studies have documented variations in greenhouse gas fluxes with O_2 supply, most of them relate this to moisture, which regulates the supply of O_2 to soil organisms by slowing diffusion. The experiment described in this chapter was designed to determine the direct impact of O_2 concentration on soil CO_2, CH_4 and N_2O fluxes by controlling O_2 supply in airstream passing through soil columns. Five soils (DF, deciduous forest soil; SF, spruce forest soil; AG1, agricultural soil 1; AG2, agricultural soil 2; GR, grassland soil) that differ in land use and other properties were incubated at constant temperature and moisture in an automated soil incubation system. The system continuously flushed the chambers holding soil samples with an inlet air (30 ml/min), oxygen levels in inlet air were switched weekly, started from 20.5% followed by 10, 5, 2.5, 1, 0%, and all levels were repeated in reverse fashion (from 1 to 20.5%). The results showed significant ($p<0.05$) decrease in soil respiration rates in four of the five soils with shift in O_2 levels from higher to lower concentrations. The activities of extracellular enzymes (four hydrolytic-(BG, AG, NAC, CBH) and two oxidative (PhOx, POX)) were highest at 1% O_2. Non-CO_2 greenhouse gas fluxes were insensitive to the shift in O_2 concentrations above 2.5%, but N_2O production increased dramatically under anoxic conditions. CH_4 oxidation rates declined at 1% O_2 but were reduced to zero under anoxic conditions, with no CH_4 production observed. Altogether, the most dominant changes in greenhouse gas emissions from upland soils were recorded either at sub-oxic conditions (1% O_2) or under anoxia; sub-oxic conditions could significantly lower soil respiration and CH_4 oxidation rates of upland soils, while short term anoxic conditions could result in many fold increases in N_2O emission.

Key words: Trace gases production and consumption, O_2 levels, Land use, Stable isotopes, soil air

2.1 Introduction

Soils play crucial role in global climate change because they represent major sources or sink of the greenhouse gases, CO_2, CH_4 and N_2O and the exchange between soils and the atmosphere is an important contributing factor to their increasing rates (Conrad, 1996; Thomson *et al.*, 2012). Approximately half of the CO_2 that enters the biosphere is recycled through soils (Lehmann, 2007). Soils contribute about 2/3 of global N_2O emissions, which increases due to the intensification of N cycling through the addition of fertilizers. Wetland soils are major sources of CH_4 while upland soils consume CH_4 from the atmosphere (Thomson *et al.*, 2012; Nazaries *et al.*, 2013). Apart from being major contributor to greenhouse gas (GHG) emissions, soils are also potential sinks for these gases. Globally, soils are not only the largest reservoir for organic carbon (Lal, 2008), thus sequestering CO_2. They also act as the 2nd largest sink for atmospheric CH_4, oxidizing about 25-45 Tg-CH_4 per year (Dutaur and Verchot, 2007). Thus it is important to understand the biogeochemical processes, specifically the biological activities that are responsible for the production or consumption of these GHGs in soils.

Oxygen is the second most abundant gas in the Earth's atmosphere, but in the soil environment, its concentration could fluctuate depending on soil physical, chemical and biological properties and it may reduce to low or even undetectable levels (Brzezińska *et al.*, 2011). The rate of exchange of soil air and the overlying free atmosphere is strongly influenced by soil structure, soil moisture or depth of water table, soil compaction and the presence of an organic layer or organic crust (Hanslin *et al.*, 2005; Angel, 2010; Angel *et al.*, 2011). Low O_2 or anaerobic zones are found, even in rather well-aerated soils (Bhattarai and Midmore, 2009; Chen *et al.*, 2011). Large gradients in O_2 concentration have been observed over very short distances within soil aggregates (Greenwood, 1961; Greenwood and Goodman, 1967; Sexstone *et al.*, 1985; Silver *et al.*, 1999). In addition, the concentration of O_2 often decreases with soil depth (and increasing CO_2 pore space concentrations) (Strojny *et al.*, 1998).

Major greenhouse gas (CO_2, CH_4 and N_2O) are produced or consumed through microbial processes in soils (Conrad, 1996) where the microbial habitat, in turn, is regulated by physical and chemical constraints that affect the supply of substrate and nutrients (Or *et al.*, 2007). CO_2 is the end product of soil organic matter decomposition by heterotrophic microbial communities, with O_2, nitrate (NO_3^-), manganese (Mn IV), iron (FeIII) and sulphate (SO_4^{2-}), serving as electron acceptors in order of decreasing energy yield for oxidation of reduced organic C (Thauer *et al.*, 2008). CO_2 production thus is accompanied by changes in soil gases. For example, O_2 concentrations can be reduced in soil pore space when the rate of O_2 supply by diffusion from the free atmosphere is exceeded by the rate of O_2 consumption. Once O_2 is used up and the soil air is anaerobic, N_2O can be produced during the reduction of nitrate (NO_3^-) while, CH_4 is produced once the other electron acceptors have all been exhausted (Conrad, 1996; Khalil *et al.*, 2004). Thus the balance of supply of O_2 from the atmosphere with consumption in soils is one of a major factor regulating processes responsible for GHGs emission from soils (Silver *et al.*, 1999).

Although it is clear that O_2 levels are critical for the decomposition process, very few systematic studies have focused on how the rates of decomposition and production of atmospheric trace gases vary with the availability of O_2 supply. While, it is often assumed that the decomposition

of organic matter is intrinsically slower under anoxic than oxic conditions (Lee, 1992). In soils, O_2 supply generally controls the rates of heterotrophic respiration according to Michaelis-Menton relationships (Greenwood 1961), with rates strongly affected only once very low O_2 levels are reached (Lee, 1992). In soils, response of microbial community to shift in O_2 concentration can be determined by measuring ecophysiological indices, such as the metabolic quotient (qCO_2), which is an excellent indicator for shift in microbial activity (Anderson, 2003).

The feedback of upland methanotroph community to shift in O_2 concentrations is not well known. Phylogenetically distinct methanotrophs present in low O_2 environments exhibit different response to shifts in O_2 levels. For example, Henckle *et al* (2000) observed an increase in CH_4 uptake rates when O_2 concentrations were decreased to 1% (Henckel *et al.*, 2000; Le Mer and Roger, 2001). Similarly, Reim *et al* (2012) found distinct groups of methanotrophs exploiting niches with variable O_2 and CH_4 concentrations (Reim *et al.*, 2012). In addition, studies conducted on common soil nitrifying, denitrifying and nitrate respiring bacteria also found positive relationships between the production of N_2O and O_2 partial pressure (Bollmann and Conrad, 1998; Butterbach-Bahl *et al.*, 2013; Blagodatskaya *et al.*, 2014). Thus, quantifying the effect of O_2 supply on microbial process can be inferred from the observed fluxes of GHGs (CO_2, CH_4, and N_2O) for different soils.

Extracellular enzymes, the drivers of soil biogeochemical cycles, provide useful insights into the mechanisms of microbial sensitivity to environment changes (Sinsabaugh *et al.*, 2008; You *et al.*, 2014). Through excretion or lysis, these enzymes enter into the environment where their cumulative activities mediate key ecosystem functions (Burns, 2013). Based on type of reactions catalyzed by the extracellular enzymes, soil enzymes belong to two major groups; hydrolytic and oxidative. Compound specific hydrolytic enzymes catalyze reactions that cleave bonds between monomers while oxidative enzymes act on broader classes of substrates and use either O_2 or hydrogen peroxide (H_2O_2) as electron acceptors (Dick, 2011). Oxidative enzymes use molecular O_2 as electron acceptor, and therefore should be the most affected under low O_2 conditions. The mechanism of such reactions is not straight forward and may involve in the transfer of one or two H atoms (or electrons) to the O_2 (Freeman *et al.*, 2001). A limited activity of phenol oxidase and peroxidase in the absence of O_2 results into increase in phenolic compounds that in turn inhibit activities of hydrolytic enzymes, if present in high quantity (Freeman *et al.*, 2001; Brockett *et al.*, 2012). Considerable decreases in extracellular enzyme activities have been reported by a number of studies when measured in deeper horizon where O_2 is also found in at trace levels (Aon and Colaneri, 2001; Burns, 2013; Herold, 2013).

We used a unique soil incubation system that allowed us to continuously flush soils with air of differing O_2 content, thus eliminating the confounding effects of O_2 diffusion rate from the concentration effects of O_2 on decomposition rates. We used this system to compare the response of respiration rates, extracellular enzyme activity and fluxes of N_2O and CH_4 for five soils that differed in soil properties such as texture, pH, microbial biomass and N availability.

2.2 Material and Methods

2.2.1 Soil sampling

Soils used in this study were taken from well-established locations with a lot of available data on soils from sites in the state of Thüringen in Germany. We used soils that varied in land use (agriculture versus forests), and soil properties such as pH (4-7.3), organic C content (1.2-5.2%), and clay content (<10 to 41%); Table 1. Two cropland soils were sampled from the area near the city of Jena. One sample (AG1) was from the location of the 'Jena experiment' a well-known study site for plant biodiversity effects; we did not sample the experimental plots. The plot sampled is part of a C3-C4 vegetation shift experiment established in 2003 (Steinbeiss *et al.*, 2009). The second cropland sample (AG2) was selected to have lower pH and clay content, and is from a site established for a field greenhouse experiment in 2010. The other sites, from a deciduous forest (DF), a spruce forest (SF)) and a grassland (GR), are sites within the German biodiversity Exploratories experiment (Solly *et al.*, 2014) and are located near Hainich national park. The grassland site has been fertilized with approximately 80 kg N/ha and is mowed annually; further detail can be found in ((Herold *et al.*, 2014), under the name of HEG6 site).

At each site, an area of 20 m x 20 m was marked off and the 1-10 cm interval of mineral soil horizon was collected using a spade. For soil moisture and bulk density measurements, each soil was also sampled using small stainless steel cores. All soils were sampled approximately 2 months before the start of experiment except one cropland soil (AG1) which was sampled 1.5 years earlier and was stored in cold chamber (4°C). From the soil collected, approximately 12-16 subsamples were passed through 4 mm sieve to remove large plant material and roots. The sieved material was subsequently mixed together to get one composite sample.

Table 2.1: Basic characteristics of the soils used in present study

Soil	Type	Land use	SOC (%)	TN (%)	C:N ratio	pH	Clay	Silt (%)	Sand	δ^{13}C-SOC (‰VPDB)
AG1	**Eutric Fluvisol**	**Cropland**	**2.04^{a}**	**0.23 ^{a}**	**8.9^{a}**	**7.3^{a}**	**24**	**66**	**10**	**-23.9±1.2**
AG2	**Cambisol**	**Cropland**	**1.34^{b}**	**0.13 ^{b}**	**10.0^{b}**	**5.8^{b}**	**10**	**NA**	**NA**	**-26.9±0.1**
DF	**Luvisol**	**Deciduous forest**	**1.98^{c}**	**0.16 ^{c}**	**12.0^{c}**	**4.0^{c}**	**22**	**35**	**43**	**-26.1±0.1**
GR	**Luvisol**	**Grassland**	**1.20^{b}**	**0.14 ^{b}**	**8.7^{a}**	**5.9^{d}**	**26**	**29**	**45**	**-27.4±0.1**
SF	**Cambisol**	**Spruce forest**	**5.17^{d}**	**0.34 ^{d}**	**15.3^{d}**	**5.8^{bd}**	**41**	**34**	**26**	**-25.9±0.3**

Different letters in superscript represent statistical significance among soils (Tukey α=0.05)

2.2.2 Soil incubation

Homogenized soil samples (1 kg field moist) were placed in PVC columns (10 cm diameter and 20 cm height), and soil moisture was adjusted to 50% WFPS by sprinkling with distilled water. Details of the incubation system and soil chambers have been described elsewhere (Malghani *et al.*, 2013; Thiessen *et al.*, 2013). Briefly, columns with soil samples (12 replicate x 5 soils) were placed in a climate chamber, with constant temperature (20°C). The bottom of each column was

connected to an air inlet system that continuously flushed soil columns from the bottom (30±3 ml/min) with air of known composition. The headspace exiting each column (after passing through the soil) was connected to an automated multiport stream selection valve (Valco), then analyzed for CO_2, CH_4 and $^{13}CO_2$ concentrations using an infrared carbon dioxide analyzer ("LI-6262" LI-COR Biosciences Lincoln, USA) and cavity ring down spectrometer (PICARRO G2301,Santa Clara, USA) connected in series. Moisture loss (~1 g per day per chamber) due to continuous flushing of dry air was compensated by adding water to replace lost mass once every week.

An automated air mixing system was specially prepared for manipulation of inlet air composition. The central part of this system were four gas cylinders (20 L volume; internal pressure 1-4 bar) that were used produce gas mixtures with different O_2, N_2, CO_2 and CH_4 concentrations used to flush the gas containers. During the course of the experiment, we changed the mixtures so as to reduce the O_2 content of the inlet gas stepwise, first from 20.5%, then 5%, 1%, etc. then increased the levels again. We held the inlet gas composition constant at each level for a total of 7 days before switching to the next gas composition. Each cylinder then provided well-mixed inlet gas to a maximum of 20 chambers. The rate of flow through each chamber was adjusted manually, to be constant at 30 ml/min. The number of chambers was reduced during the experiment for destructive sampling to determine changes in soil microbial biomass, nutrients and extracellular enzymes activity during the course of experiment.

Table 2.2: Composition of inlet airstream

Gas	Mixing ratio (inlet air)	Isotopic ratio
CH_4	1.8 ppm	δ^{13}C,-35.9 ‰VPDB ;δD 161‰VMSOW
CO_2	350 ppm	δ^{13}C, -34.1 ‰VPDB
O_2	Variable (0-20.5%)	
N_2	Variable (100-O_2)	

VPDB, *Vienna Pee Dee Belemnite; VMSOW, Vienna Standard Mean Ocean Water*

Fluxes of gases from soils were calculated from the difference in composition between outlet and inlet air streams using flow rates, multiplied by the rate of air flow. Negative fluxes (e.g. for methane) indicate removal of a gas vial passing through the soil column; positive fluxes indicate net production of gas in soil.

2.2.3 Soil analysis

Soil samples were analyzed destructively after 20%, 5%, 1% O_2 treatments and at the end of experiment, following a return to 20% O_2. Each soil sample was homogenized and divided into three subsamples for measurement of enzyme activities, extraction for nutrients and microbial biomass, and elemental analysis, respectively. Elemental analysis was carried out on air dried (40°C) samples that were ground with a ball mill (4 mins, 70 rpm) and weighed into tin capsules for analysis on an elemental analyzer at 1150°C (vario Max CN, Elementar Analysensysteme GmbH, Hanau, Germany). Organic carbon concentrations were determined by calculating the difference between elemental analyses of the total carbon concentrations and soil inorganic carbon concentrations (Steinbeiss *et al.*, 2008). Extractable dissolved organic carbon (DOC) and soil microbial biomass (MB) were determined by extraction using 0.05 M K_2SO_4, with and

without chloroform fumigation (Brookers *et al.*, 2007; Karsten *et al.*, 2007) and analyzed with TOC analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). Extractable inorganic nitrogen contents (NH_4^+, NO_3^-) were extracted using 0.01M KCl and freezed until measured with flow injection system (Quikchem QC85S5, Hach Company, Loveland CO, USA)

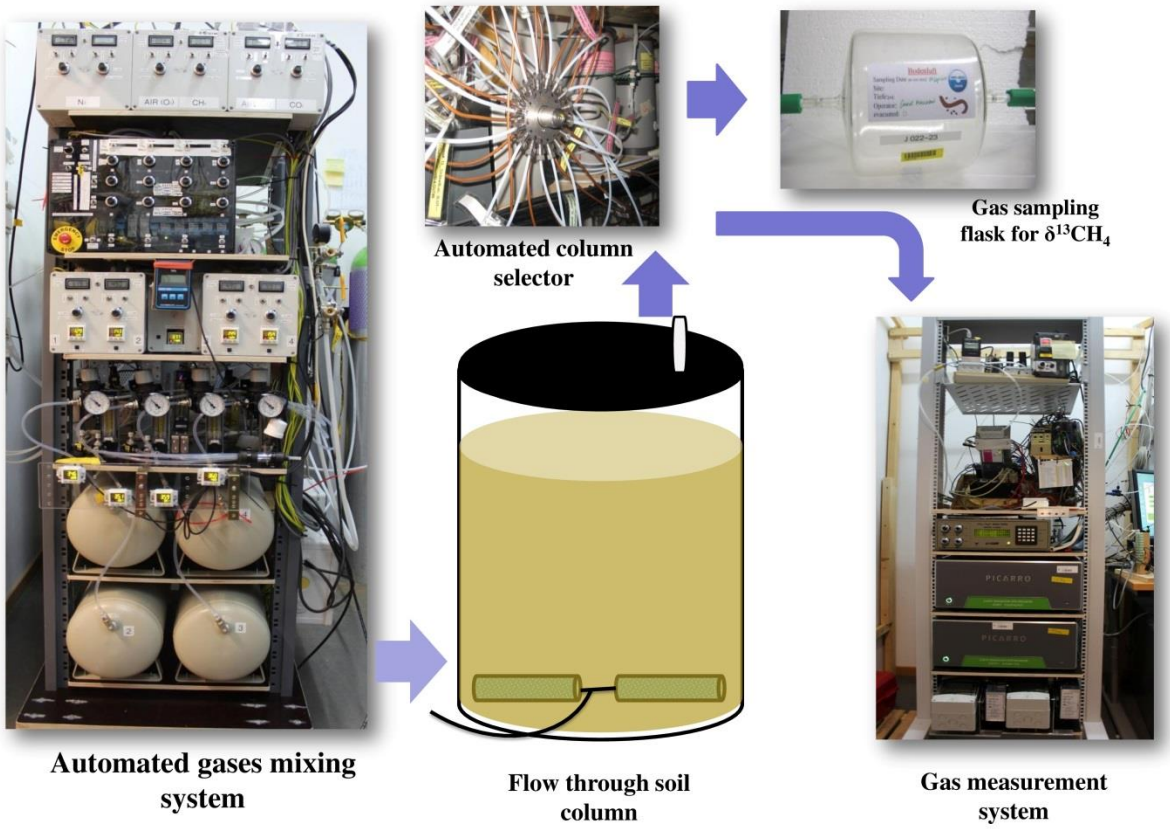

Figure 2.1: Systematic diagram of automated chamber measurement system with scheme of gas sampling and analysis. In total incubation system have capacity to run 64 soil columns.

The ^{13}C content of soil organic carbon was measured only at the start of experiment by coupling an elemental analyzer (EA 1100, CE Instruments, Milano, Italy) to an isotope ratio mass spectrometer (Delta^{Plus}XL, Thermo Finnigan MAT Bremen, Germany). Values were reported as $\delta^{13}C$ in per mill (‰) calibrated relative to the VPDB reference standard using NBS19 (Werner and Brand, 2001), and represent repeated measurements with a standard deviation of less than 0.3‰. To avoid inorganic carbon impact on $\delta^{13}C$ values, samples were pre-treated with weak acid, 6% H_2SO_3 (Bisutti *et al.*, 2004; Steinbeiss *et al.*, 2009).

2.2.4 Enzyme assays

The hydrolase enzymes assays (6 replicates of each sample) were performed in black 96-well microplates according to the method as described by Herold et al, (Herold *et al.*, 2014). Briefly, a mixture of 1 g homogenized soil from samples taken after 20.5% (10 days), 5% (24 days) and 1% (34 days) O_2 treatments, and 50 ml of sterile deionized water was dispersed using an ultrasonic disaggregator with a low energy input (60 J ml^{-1}). The soil suspension was continuously stirred while an aliquot of 50 µl was transferred into black microplate. Approximately, 50 µl of autoclaved buffer and 100 µl of the respective substrate were added to the same wells. Standards were mixed with soil suspension and buffer to obtain final concentrations of 0, 100, 200, 500, 800 and 1200 *p*mol $well^{-1}$. The microplates were placed on shaker (30 rpm min-1) in the dark for 210 min at 20˚C. The Fluorescence intensity was then detected at an excitation wavelength of 360 nm and emission wavelength of 460 nm, with a Infite Tcan™ microplate reader (Infinite 200, Tecan, Crailsheim,Germany) at specific intervals of 0, 30, 60, 90, 150 and 210 min. The results of enzyme activities are expressed as nmol MUF/AMC g^{-1} dry weight soil (dw) h^{-1}.

The oxidative enzymes (phenol oxidase and peroxidase) were assayed in white 96- well microplates according to the method of Johnsen and Jacobsen (2008), modified by Herold (2013) (Johnsen and Jacobsen, 2008; Herold, 2013). Briefly, 0.4 g of moist soil was weighed and added to 50 ml acetate buffer and dispersed by an ultrasonic disaggregator (60 J ml^{-1}). Enzyme assays were carried out by combining 200 µl of soil suspension with 50 µl of substrate (Tetramethylbenzidine, TMB) solution. Controls included in each microplate were: substrate + buffer and soil suspension + buffer. For peroxidase assays, all wells received 25 µl of 0.3% hydrogen peroxide (H_2O_2), including controls. Microplates were incubated in the dark for 1 h at 25°C. Enzymes activities were determined by measuring absorbance at 630 nm with Infite Tcan™ microplate reader (Infinite 200, Tecan, Crailsheim, Germany) at 0, 15, 30, 45 and 60 min intervals. The Results of enzyme activities were expressed as excitation 630 nm (E630 nm) g^{-1} dry soil weight h^{-1}.

All enzymes assays were performed with freshly prepared solutions and six replicates for every soil sample were used for each assay

2.2.5 Gas analysis

Air exiting the soil chamber headspace was sampled at each O_2 treatment using 2.3 L glass flasks, the flasks were pre-flushed with synthetic air. For gas sampling, each flask was flushed with air stream coming out of soil columns or empty column for more than 18 hours to get representative samples by following the flask sampling method for continuous flow system as has been described elsewhere (Malghani *et al.*, 2013; Thiessen *et al.*, 2013). Gas samples were used to measure the concentrations of different gases (CO_2, CH_4, N_2O, CO, H_2) in gas chromatograph (Agilent technologies 6890, Santa Clara, USA) equipped with an electron capture detector (ECD), and flame ionization detector (FID). The isotopic values of CO_2 were determined by stable isotope ratio mass spectrometry (Finnigan MAT 252IRMS) (Jordan and Brand, 2001).

2.2.6 Statistics and calculations

Soil respiration (R) and soil microbial biomass (MB) ratio was used to calculate the metabolic quotient (qCO_2), which is the amount of CO_2– C produced per unit of MB carbon (Anderson and Domsch, 1986).

To determine $\delta^{13}CO_2$ respired from soils a two pool model was used:

$$\delta^{13}\mathrm{CO}_{2\,(outlet)} * CO_{2\,(outlet)} = (\delta^{13}CO_{2\,inlet\,air} \times \mathrm{CO}_{2\,inlet}) + (\delta^{13}CO_{2\,soil} \times \mathrm{CO}_{2\,soil}) \quad (1)$$

Significance of differences among/between treatments was determined using one way analysis of variance (ANOVA) followed by a post-hoc test (Tukey or LSD, $\alpha = 0.05$). All statistical analyses were carried out in SPSS (PASW statistics-18) and graphs were prepared in SigmaPlot (Version 12.5).

2.3 Results

2.3.1 Response of soil respiration to O_2 concentration of inlet air

The different soils incubated differed in the rates of CO_2 production, as well as the magnitude of response to changing O_2 concentration (Fig 2.2). At each shift in O_2 concentration, responses of soils via respiration were immediate and were positively correlated with O_2 concentration in inlet air stream. The only exception was DF soil that did not show any shift in CO_2 emissions during first 5 weeks of incubation, while the O_2 concentrations were sequentially lowered from 20.5% to 1% in inlet airstream. The most prominent response by all soils to shift in inlet air O_2 concentrations was during the switching on and off O_2 supply, CO_2 emissions rates were at their lowest levels when O_2 supply was switched off (Fig A1.1).

We performed linear regression between O_2 concentrations in inlet air stream and mean respiration rates at specific O_2 levels and found large differences in how soil respiration rates responded to decreasing versus increasing O_2 concentration (Fig 2.2). Initially, when O_2 concentrations were switched from higher levels to lower levels, respiration rates in all soils except DF decreased linearly ($p<0.001$) with decreasing O_2 concentrations (Fig 2.2). However, when O_2 levels were subsequently increased soil respiration rates increased in 3 of the 5 soils.

The metabolic quotient values for four of the soils decreased with O_2 levels, however, the scale of change was variable among land use (Fig 2.3). All soils exhibited lowest qCO_2 value at 1% O_2 levels except DF soil. Highest shift in microbial activity was observed in agricultural soils which showed 60% decrease in qCO_2 values at 1% compare to 20.5% O_2 levels. Interestingly, qCO_2 values, determined at the start and end of experiment when O_2 concentrations were 20.5%, were similar in all soils except SF. However, qCO_2 values measured at the end of experiment in SF soil were significantly ($p<0.05$) higher than value at low O_2 concentrations. Emission of CO_2 per unit MB (qCO_2) in DF soil were rather insensitive to O_2 levels as no differences in qCO_2 values were recorded among 4 measurement points.

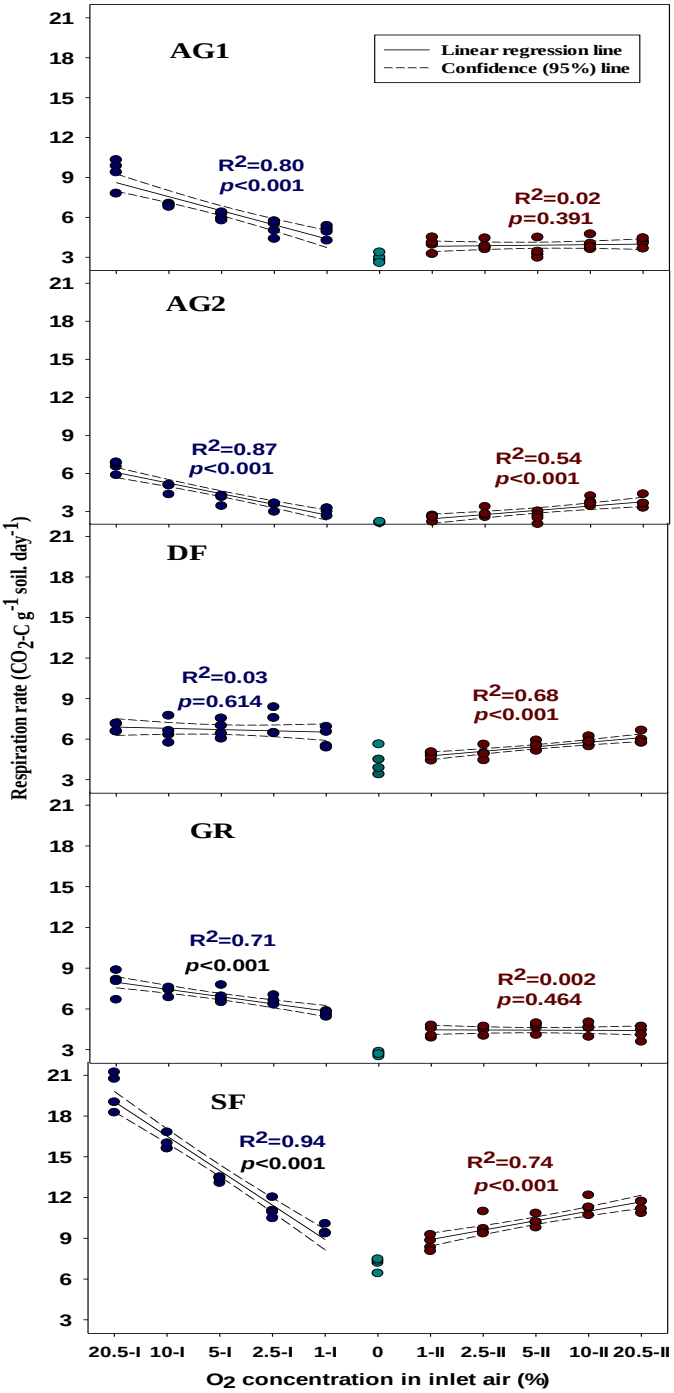

Figure 2.2: Impact of O_2 concentration on soil respiration rates (µg-CO_2-C/g soil. day). Each data point represent mean respiration rates during 6 days (n=6) and shift in respiration rates with increasing or decreasing O_2 level were correlated using linear regression. GR, grassland; DF, Deciduous forest; SF, spruce forest; AG1 & AG2, agricultural soils

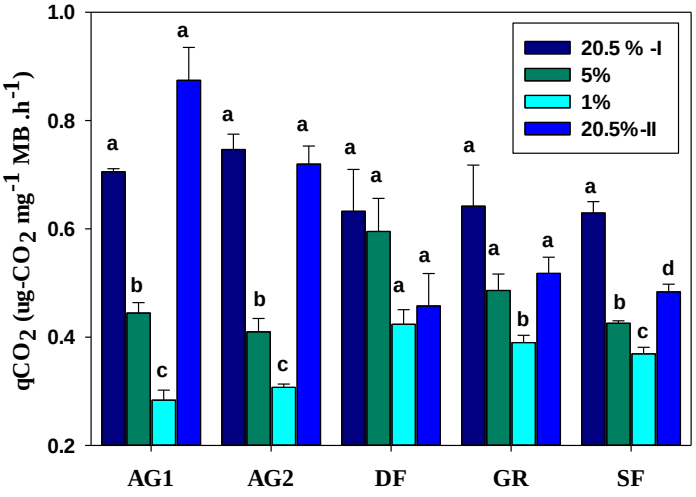

Figure 2.3: Response of heterotrophic activity (metabolic quotient, qCO_2) of five different soils under contrasting land use to shift in O_2 concentrations in inlet air stream. Each bar represent Mean±S.E (n=3). Different letters on top of the bar represent statistical significance among O_2 treatments in each land use type.

2.3.2 $\delta^{13}CO_2$ of respired carbon only shifted when O_2 supply was switched off

Stable isotope of respired CO_2 was determined using two pool model (Eq1) as CO_2 in inlet air had distinct isotopic ($\delta^{13}CO_2$) values, AG1 isotopic signature were not presented due to possible contribution of an inorganic carbon source (Table 2.1). All soils had a long history of C3 vegetation and the values of $\delta^{13}C$ of respired CO_2 were close to those of soil organic carbon (Fig 2.4). There were no significant differences $\delta^{13}CO_2$ values among air samples collected at different levels of O_2. However, $\delta^{13}CO_2$ values of air samples, from all soil types except DF soil collected during the week when soils were subjected to anoxic conditions were 3-7‰ higher than $\delta^{13}CO_2$ values determined before and after this period (Fig 2.4).

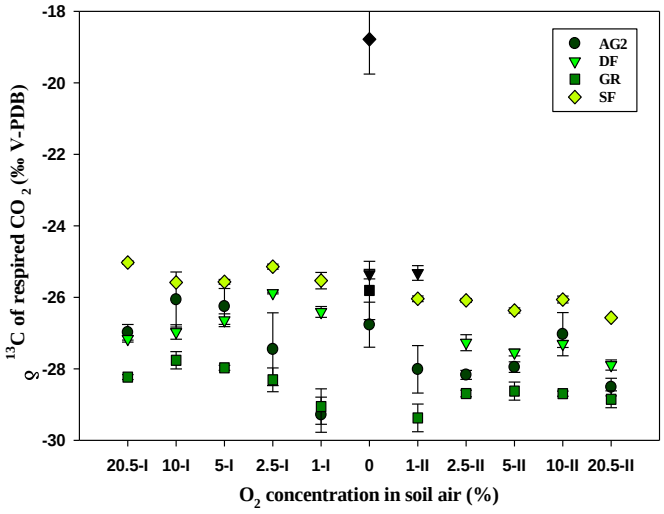

Figure 2.4: The $\delta^{13}C$ values of CO_2 emitted from different soil types at variable O_2 concentrations in soil air. Symbols filled with black color represent statistical significant differences in stable isotopes values of respired carbon

2.3.3 CH_4 oxidation rates were only sensitive to very low O_2 concentrations (1%)

Three of the five (AG1, DF & SF) soils had the capacity to oxidize CH_4 at atmospheric concentrations. The other two soils (AG2 & GR) were neither a sink nor a source for CH_4. CH_4 uptake rates were insensitive to O_2 concentration changes between 20.5% to 2.5% (Fig 2.5), but responded with decreased uptake rates when the O_2 level in the inlet airstream dropped to 1% or below. The response differed by soil, with decreased ($p<0.05$) CH_4 uptake rates in the DF soil, but increased CH_4 uptake rates in the SF soil. However, when O_2 concentration was increased from lower to higher levels, these changes were not reversed. Once agricultural soil (AG1) exhibited (tukey $\alpha=0.05$) lower CH_4 oxidation rates than the forest soils, but these rates did not respond to changes in O_2 concentrations (Fig 2.5). On the contrary to our assumption, only DF soil produced CH_4 under anoxia but production was started on 5th day of treatment. Fractionation factors calculated for $\delta^{13}C$ and δD (Eq2) for CH_4 oxidation are presented as box plot in appendix A1.4. We did not observe any correlation between CH_4 isotopic fractionation factor and O_2 level in the inlet airstream. The only differences observed in mean isotopic fractionation factor among soil types. The trend in α_{13C} was opposite with the trend in α_D among CH_4 consuming soils (Fig A1.4).

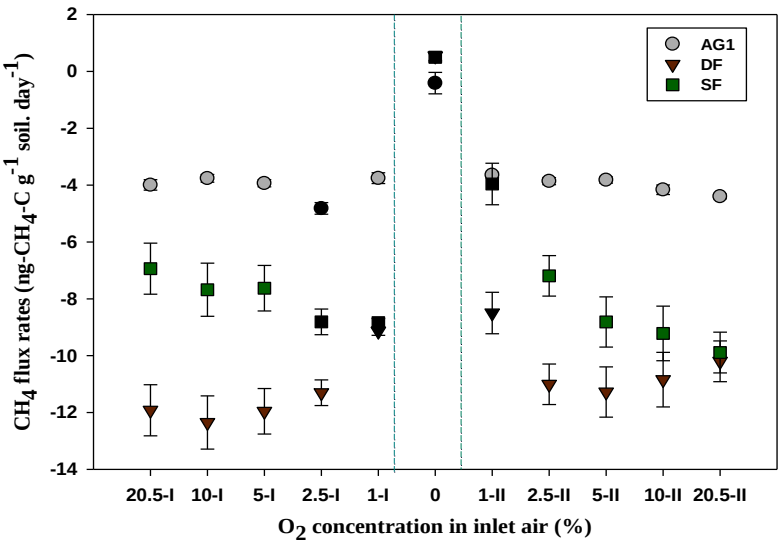

Figure 2.5: CH_4 fluxes from three different soils under different O_2 concentrations. Each symbol represents Mean±standard error (n=4). Black color symbols represent statistical significance (Tukey, $\alpha=0.05$).

2.3.4 Nitrous oxide emission only influenced in the presence and absence of O_2

While we measured CO_2 and CH_4 concentrations continuously, N_2O concentrations determined from flasks were measured only on the 5th day after the shift in O_2 concentrations. N_2O fluxes in anoxic conditions were 4-100 folds higher than in the presence of even low levels of O_2. The forest soils (SF and DF) had N_2O emission rates nearly twice those of the GR soil (Fig 2.6) with

lowest emission rates in agricultural soils (AG1 and AG2). In the presence of O_2, N_2O emissions were much lower and there were no significant differences among soil types or O_2 levels (Fig A1.5).

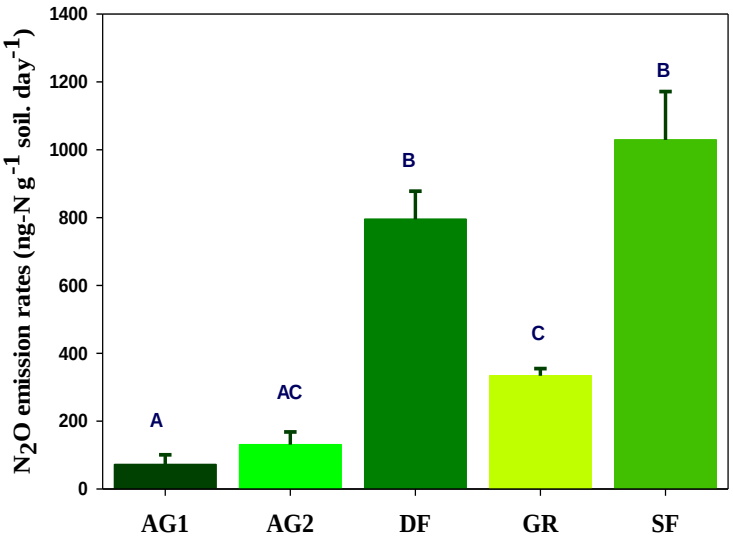

Figure 2.6: Nitrous oxide emission rates from different soils under anoxic conditions. Different uppercase letters represent statistically significant (tukey α=0.05) differences. Mean±standard error

2.3.5 The highest enzyme activities were observed at 1% O_2

Hydrolytic enzyme activities did not differ as O_2 concentrations declined from 20 to 5%, but were highest (in 4 soils) at 1% O_2 levels (Fig 2.7). The soils differed in the degree of change, and sensitivity of enzyme activity. AG2 was the only soil that showed significant increase on each level of O_2 in BG, CBH and NAC activities. The AG and BG activities remained unchanged in GR and DF soils at all O_2 levels.

The activities of oxidative enzymes also responded to changes in O_2 levels, however, with a different intensities. Phenol oxidase activity decreased for all soils except AG1 between 20.5% and 5% O_2, but increased at 1% O_2 compared to 20.5 and 5% for all soils. Peroxidase activity increased linearly with decreasing levels of O_2 for AG1 and DF soils, but did not respond to O_2 levels in AG2 and SF soils (Fig 2.7).

Among different soils types, DF soil had significantly lower activities of all hydrolytic enzymes, except NAC, than other soils types. On the contrary, GR soil showed significantly higher peroxidase activity than all other soils. Among different hydrolytic enzymes, β-glucosidase was the most active enzyme in all soil types, except DF and SF soils where activities of β-glucosidase and N-acetyl-glucosaminidase to some extent were comparable among soil types.

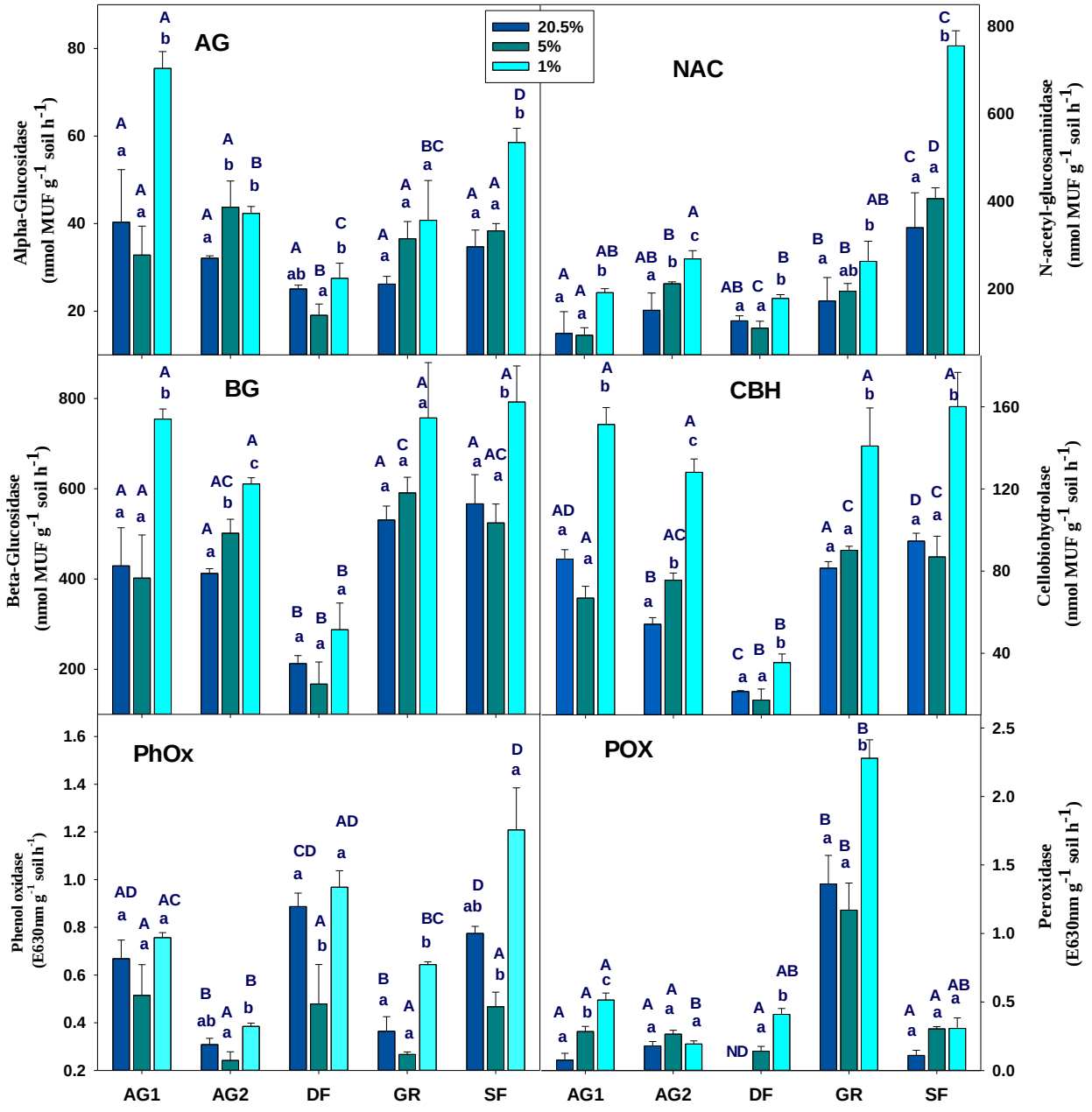

Figure 2.7: Specific enzyme activities of the five different soils incubated under continuous air inflow system with shifting O_2 concentrations. Abbreviations: AG = α-glucosidase, BG = β-glucosidase, NAC= N-acetyl-glucosaminidase, CBH= cellobiohydrolase, PhOx=phenol oxidase and POX=peroxidase. Mean± standard error (n=3), letters in small cases represent statiscal significance in enzyme activity among O_2 levels, whereas different large cases represent statically significant enzymatic activities among soil types.

2.4 Discussion

Soil microorganisms are divided into five different groups based on their demand for molecular O_2; 1). Obligate aerobes require atmospheric O_2 concentrations (~20%) for optimal growth. 2). Microaerophiles, that grow optimally only at sub-atmospheric O_2 concentrations. 3). Facultative anaerobes can respire aerobically but use alternative terminal electron acceptors for anaerobic respiration or grow via fermentation. 4). Aerotolerant anaerobes can survive in the presence of trace O_2, but grow optimally only anaerobically. 5). Finally, obligate anaerobes cannot tolerate O_2 and grow only under anoxic conditions (Morris and Schmidt, 2013). Differences in the abundance and activity of high and low O_2 affinity organisms with soil/land use types could be the best explanation for changes in GHG emissions with O_2 supply in our experiments. Significant differences in fluxes of trace gases (CO_2, CH_4) and soil enzymatic activities observed in this study specifically between the highest and the lowest O_2 levels (20.5% vs 1%), suggesting a decrease in activity of obligatory aerobes and a shift in relative abundance of microaerophiles. Field studies provided the presence physiologically different microbes, especially in soils where O_2 gradient is commonly found (Reim, 2012; Morris and Schmidt, 2013).

Heterotrophic decomposition of soil organic matter is the main process responsible for CO_2 emission in incubated soils. Stoichiometrically 0.7-1 mole of O_2 is required to produce each mole of CO_2 (Dilly, 2003). Regardless of soil type, O_2 concentrations in the inlet air stream were always in stoichiometric excess in our treatments, except during times when there was total anoxia. Our experimental design, which continuously flushed the soil column with air (residence time of air in the column was 10-14 minutes), also minimized the chance for anoxic microsites to develop in the soil column.

In total, the drop in CO_2 emission rates with O_2 concentrations (Fig 2.2) likely includes two signals: the depletion of a more labile fraction of organic carbon with time (e.g. (Conant *et al.*, 2008)), and also the reduction in oxidation rates with lower O_2 content expected by Michaelis-Menton kinetics. We were unable to separate influences of these two variables due to the absence of control treatments kept at constant O_2 levels the whole time. However, previous incubation studies carried out with similar soils in the same incubation system and continuously under atmospheric O_2 concentrations (Malghani *et al.*, 2013; Thiessen *et al.*, 2013), show less dramatic declines in the rate of CO_2 emission over the time period compared the declines observed in this study. Thus, it is plausible that most of the changes in CO_2 emission we observed were due to shifts in O_2 concentrations and substrate depletion played a more minor role. Proxies indicative of substrate availability like microbial biomass and DOC (Iqbal *et al.*, 2009; Iqbal *et al.*, 2010) which were similar at 20.5% and 1% O_2 in all soils except SF, which supports this idea. Isotopic measurements also indicated no shifts with O_2 level (except during complete anoxia), suggesting no large shifts among substrates with different C isotopes (Fig 2.4).

Metabolic quotient (qCO_2, the community respiration per biomass unit) values also indicated that changes in CO_2 production rates were largely due to shift in O_2 levels in inlet air. All soils except DF showed significant decrease in qCO_2 values at low O_2 concentrations (5% & 1% O_2), and the degree of change in qCO_2 was land use dependent (Fig 2.3). Studies reported positive correlation between qCO_2 values and labile carbon concentrations (Moscatelli *et al.*, 2005; Llorente and Turrión, 2010). However, it is most unlikely that the decrease in labile C was driving this change

in qCO_2, as evident from qCO_2 values from 1st and last week of incubation, during this period O_2 concentration was same (20.5% O_2) in-spite of differences in DOC and MB. The low qCO_2 at sub-oxic conditions is interesting and supports the idea that at this stage microaerophiles could be the main active group.

The most prominent effect of low O_2 concentration on activity of microorganism was evident from measurements of extracellular enzymes, as enzymes are direct products of soil microorganisms. Microbial taxa may vary considerably in their ability to produce higher or more efficient enzymes (Kramer, 2014). Similar to CO_2 fluxes, the activities of hydrolytic and oxidative enzymes were also significantly different between 20.5 and 1% O_2 but showing higher activities at 1% O_2 concentration (Fig 2.7). This could represent either a direct influence of O_2 concentrations on active microbial community structure or the result of growth stress exerted on microbial communities. In accordance with the concept that the microbial taxa present in the deeper soils are more efficient than microbial taxa present at surface (Baldrian, 2014; Qian *et al.*, 2014; Stone *et al.*, 2014), we can hypothesize that microaerophiles have higher efficiency in enzyme production than strict aerobes. A contrasting hypothesis would be that growth stress exerted by low O_2 concentration can trigger higher production of enzymes by strict aerobes as allocation of assimilated carbon to enzyme production increases under influence of external factors (Schimel and Weintraub, 2003). The qCO_2 values did not indicate presence of stress at low O_2 concentrations, as at sub-oxic conditions qCO_2 values were significantly low (Fig 2.3) contrary to literature which suggests high qCO_2 values under stress (Anderson, 2003).Taken together, we hypothesize that higher enzyme activity at low O_2 levels was largely due to shift in active microbial taxa rather than an impact of low O_2 stress on strict aerobes.

Unlike CO_2, CH_4 fluxes were rather insensitive to shift in O_2 concentrations and only significant impact was recorded either at 1% or under complete anoxia (Fig 2.5). Interestingly, the changes in CH_4 oxidation rates (presumably CH_4 oxidation was the only process that was responsible for changes in CH_4 concentration between inlet and outlet airstreams as no production of CH_4 was detected even at complete anoxia) at sub-oxic (1%) condition was land use dependent as contrasting respond was measured in all three soils (DF, SF and AG) that had capability to uptake CH_4 upon shifting O_2 concentrations from 2.5% to 1% (Fig 2.5). In addition intrinsic CH4 uptake capacity of these soils was also significantly ($p<0.001$) different. This contrasting behavior of land use types could be due to differences in methanotrophic community structure as evident from phylogenetic based studies (Knief and Dunfield, 2005; Mohanty *et al*., 2006; Nazaries *et al*., 2013). Arguably, soils may differ in their response to low O_2 concentrations depending on the most dominant methanotrophic taxa. Reim *et al* 2012 found taxa specific niche in paddy soil due to gradient in O_2 concentrations, highlighting exploitation of low O_2 and high CH_4 condition by *Type I* methanotrophs. An increase in CH_4 oxidation rates were also reported in other studies but soils used in these studies had intrinsic CH_4 production (Henckel *et al.*, 2000; Brzeziska *et al.*, 2012). The behavior of SF and AG soil to sub-oxic conditions indicate presence of *Type 1* methanotrophs as CH_4 oxidation rates were increased or remain unchanged in these soils. The dominancy of *Type 1* methanotrophs in agricultural soil is evident in other studies (Abell *et al*., 2009) however there is no report regarding SF land use. On other side, our results regarding DF soil are in consistent with previous reports that also reported significant decrease in CH_4 oxidation rates in a variety of unsaturated soils when exposed to low O_2 concentrations (Bender and Conrad, 1994; Czepiel *et al.*, 1996; Teh *et al.*, 2005).

In contrast to CO_2 and CH_4 fluxes, N_2O emission rates were similar at all levels of O_2 and emissions significantly increased (4-10 fold; Figure 2.6) only under complete anoxia (Fig 2.6). The presence of a linear correlation between N_2O emission rates and NO_3 content among soil types highlights denitrification as the sole process for N_2O production in these soils. Nitrifiers also can produce N_2O but require the presence of O_2, while denitrifiers are only active in the absence of O_2 (Khalil *et al.*, 2004). Indeed, the presence of O_2 even in trace amount can suppress denitrifier's activity (Murray and Knowles, 2004). Denitrifiers are efficient in production of N_2O and contribute dominantly to total emission of N_2O by a soil (Ussiri, 2013). We observed a lag of about 5 days between the change in O_2 supply and changes in N_2O fluxes. By this, we might fail to record immediate response of soils to sequential shifts in O_2 concentrations, as most of the microbial processes responsible for N_2O emissions respond rapidly to any shift in O_2 (Butterbach-Bahl *et al.*, 2013) or to other abiotic factors. Very low emission rates at 1% contradict with studies that showed significant increase in N_2O emission only at very low O_2 concentrations (Bollmann and Conrad, 1998).

2.5 Conclusions

In soils CO_2, CH_4 and N_2O are produced/consumed biogenically by soil microorganisms and their fluxes are controlled by soil physical characteristics and this complexity makes difficult to understand the direct influence of environmental factors on microbial processes. We incubated five contrasting soils in a continuous flow system and determined impact of shift in O_2 concentrations on the emissions of CO_2, CH_4 and N_2O. Our results showed significant impact of low O_2 (1%) on all gases emissions, particularly, on CO_2 and CH_4. All soils used in our study (two agricultural soils, 1 grassland and 2 forests soils) showed lowest respiration rate at 1% O_2 level compared to respiration rates determined at 20.5% O_2. Out of five soils, three soils acted as sink for CH_4 at atmospheric levels, unlike response of soils respiration to 1% O_2 level, contrasting results were observed in CH_4 uptake rates among soil types. A significant increase or decrease in CH_4 oxidation rates was observed in SF and DF soils, respectively, while AG1 soil showed no such effect. Moreover, CH_4 oxidation was completely halted when O_2 supply was switched off and CH_4 production was only observed during this anoxic period. On the other hand, strong shifts in N_2O emissions were only recorded in the absence of O_2. Soils with higher carbon content emitted more N_2O under anoxic conditions. Our study highlights the presence of soil processes that only dominate at low O_2 levels, most probably due to functionally redundant but physiologically different microorganisms. We recommend use of advanced O_2-sensing technology in environmental studies to define and quantify low-O_2 environments and provide accurate and sensitive measures of O_2 gradients, as these sites may play important role in emissions of GHGs from soils.

CHAPTER 3

Abundance of high affinity methanotrophs may not be substrate driven: laboratory based incubation study using soil from contrasting land use types

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Manuscpit in preparation

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Abstract

Variations in the rates of atmospheric CH_4 uptake in upland soils can arise from both abiotic and biotic factors. Among the less-studied biotic factors is the degree to which methanotroph activity and community composition are influenced by the long-term average supply of CH_4 to the soil. Here, we investigated whether the abundance of high affinity methanotrophs in a range of soils representing different land use types is substrate (CH_4) dependent. Field replicates of three soils sampled from deciduous forest (DF), spruce forest (SF) and agricultural (AG) sites were incubated in columns flushed continuously for 27 days with air at one of four CH_4 concentrations: <1 ppm (starvation), 1.8 (ambient), 30 (low elevated) and 60 (high elevated) ppm. Soil columns were flushed rapidly enough with inlet air to minimize the potential impact of soil-structural differences (i.e. diffusion) as a control for CH_4 supply to microbes. For all levels of CH_4 supply, CH_4 oxidation rates were the highest in DF soil followed by SF and AG soils. However, the magnitude of response in CH_4 oxidation rates upon CH_4 fertilization/starvation and the cell specific activity at each CH_4 level were similar among land use types and unchanged by the CH_4 concentration treatments. Terminal restriction fragment length polymorphism (T-RFLP) analysis showed distinct methanotrophic communities in soils with agricultural versus forest land use. In this regard, the T-RFs associated with *USCα* and *Type II* methanotrophs (*Methylocystis sp,Methylosinus sp.)* were the most abundant in forest soils while the AG soil was dominant by *Type 1a* associated T-RFs. Soil across land use type also differed in fractionation of stable isotopes, ^{13}C and D (^{2}H), during CH_4 oxidation. For instance the fractionation factor (ε) for D

was -129.5±7‰ versus -165.2±6‰ for DF and SF soils respectively. Altering CH_4 concentration in the inlet air did not change methanotroph abundance, as evidenced by three different assays, two qPCR and T-RFLP, that recorded no changes in the number of *pmoA* gene and/or relative abundance of T-RFs. Altogether, it is proposed that intrinsic differences in CH_4 oxidation rates between soils, particularly between temperate agricultural and forest soils, are driven by methanotroph community structure. However, the population size of methanotrophs in upland soils does not respond to CH_4 availability and is most probably regulated by some other factors, perhaps the availability of nitrogen, cross feeding or other carbon sources.

Key words: Atmospheric CH_4 oxiation, Stable isotopic fractionation, QPCR, T-RFLP, land use types, methanotrophs abundance

3.1 Introduction

Methane (CH_4) is a potent greenhouse gas with a global warming potential ~ 23 times greater than CO_2. Methane concentrations in the atmosphere have more than doubled since 1800 AD, which contributes about 15% to the current enhanced greenhouse effect (IPCC, 2013). Currently CH_4 concentrations in the atmosphere are increasing again after nearly a decade where they stayed constant. During the last decade, the growth rates of atmospheric CH_4 were 0.4% per year (Kirschke *et al*., 2013). Biological oxidation of methane by specialized microorganisms that are capable of consuming CH_4, in upland soils represents the second largest sink for atmospheric CH_4 after tropospheric chemical oxidation (Brevik, 2012). Global estimates of the soil sink vary more than five-fold, between 9 and 47 Tg/yr (Kirschke *et al*., 2013). The large uncertainties in global estimates of the methane oxidizing capacity of upland soils are mainly due to poor understanding of the factors that regulate CH_4 uptake rates.

Upland soils vary in their rates of atmospheric CH_4 oxidation, and the mechanisms underlying these differences can be broken down into factors that affect soil diffusivity and factors that affect microbial (methanotrophic) activity (Smith *et al*., 2003; Curry, 2007). Soil diffusivity controls the rate at which CH_4 and O_2 are supplied from the atmosphere to the methanotroph community. The rate of gas diffusion through soil is mainly controlled by soil moisture, although the relationship between diffusivity and soil moisture differs among soil types (Hiltbrunner *et al*., 2012; Luo *et al*., 2013). The role of methanotrophic activity is rather complex and reflects a suite of underlying features of the biological community. Methanotroph activity can be broken down into the per-capita (per microbe) rate of activity, which may be thought of as physiological state (e.g., in terms of response to temperature) or it may be a function of enzyme kinetics and therefore respond to substrate supply and/or inhibitor concentration (e.g., ammonium) (King and Schnell, 1994; Bodelier and Laanbroek, 2004; Menyailo *et al*., 2012). Variation in the per-capita activity may further be influenced by which individual strains comprise the methanotroph community because enzyme kinetics and physiological responses appear to vary among taxa or even genera (Knief and Dunfield, 2005; Mohanty *et al*., 2006; Tate, 2015). In addition to the per-capita mechanisms, the total activity in soils may also vary due to the overall abundance of methanotrophs (Maxfield *et al*., 2008b; Degelmann *et al*., 2009; Kolb, 2009).

In recent studies two main explanatory factors have been extensively discussed for their regulatory effect on methanotroph activity in contrasting land use types: methanotrophic community structure and methanotroph abundance. It is experimentally evident that particulate methane monooxygenase (pMMO) enzyme facilitates the conversion of CH_4 to methanol, which is the first step of CH_4 oxidation (Hanson and Hanson, 1996). Arguably, pMMO associated with different methanotrophs may vary in its affinity for CH_4 concentrations as evident from range of Km values exhibited by contrasting soils (Knief and Dunfield, 2005; Knief et al., 2006; Tate et al., 2012). The ability of the enzyme to perform at different substrate concentrations is determined by the Michaelis-Menten constant (Km). Lower Km values indicate higher affinity. In this regard, identified groups of methanotrophs such as the upland soil cluster (*USCα*, *USCγ*), *Methylocystis spp* and some other groups have particularly gained attention as their ability to oxidize atmospheric concentrations of CH_4 has been confirmed by stable isotope probing (Kolb, 2009; Nazaries *et al*., 2013). In addition, the inconsistent response of different soils to NH_4^+ amendment also found to be related to the dominant methanotroph community as certain taxa showed higher tolerance of NH_4^+ compared to others (Jang *et al*., 2011).

Overall methanotroph abundance can be positively correlated with CH_4 uptake rates (Knief and Dunfield, 2005; Degelmann *et al*., 2009; Ho *et al*., 2013; Bárcena *et al*., 2014). For example, the effects of land management practices, such fertilizer use in cultivated lands, afforestation or the type of vegetation, on the intrinsic CH_4 oxidation capacity of soil have been linked to altered biomass of the methanotrophic community (Singh *et al*., 2007; Maxfield *et al*., 2008b; Menyailo *et al*., 2010). However, the mechanisms underlying these relationships remain unknown.

CH4 oxidation produces large fractionations in both C and H isotopes. For example, pMMO discriminates strongly against heavy isotopes (^{13}C & D) (Feisthauer *et al*., 2011). However, the degree to which methanotroph community or activity can result in differences in kinetic isotopic effect (α) is not well known? Published values of α associated with CH_4 oxidation vary with soil type (α^{13C}= 1.005-1.022; α^{D}= 1.058-1.161) as well as among different organisms (α^{13C}= 1.006-1.028; α^{D}= 1.138-1.252) (Snover and Quay, 2000; Templeton *et al*., 2006; Feisthauer *et al*., 2011; Kato *et al*., 2013). Chamber-based field studies link this variability to both microbial and soil physical parameters, including diffusion (Maxfield *et al*., 2008a). To our knowledge, there is no study targeting the isotopic fractionation associated with only microbial uptake of atmospheric CH_4. The most commonly used approach estimates the value of α simultaneously with the influence of diffusion on isotopes through use of a mathematical model (e.g. Reeburgh *et al*. (1997) and Snover and Quay (2000)).

Given the very low concentrations of methane in the atmosphere and soil air, we hypothesize that methane supply limits methanotroph community size and therefore a soil's methanotroph activity. Previously, only a few attempts have been made to determine whether changes in ambient CH_4 concentration cause a change in methanotroph biomass (Roslev *et al*., 1997; Bull *et al*., 2000). These studies were hampered by methodological limitations in determining methanotrophic community size in terms of the relative abundance of certain PLFA, mainly18:0 and 16:0, used as indicators of methanotrophic bacterial population (Kolb *et al*., 2003). The resolution of PLFA based techniques is usually very low as similar PLFA are produced by organisms other than methanotrophs (Sundh *et al*., 2000; Kolb *et al*., 2003; Dedysh *et al*., 2007). In addition, CH_4 concentrations used in these previous studies were high enough that they could

potentially activate different taxa (e.g. low affinity) methanotrophs, but none of these studies include any phylogenetic analysis to test for shifts in methanotroph community structure.

To test this methane limitation hypothesis, we conducted an incubation study to quantify the impact of CH_4 concentrations on the abundance and community structure of methanotrophs in three upland soils sampled that varied in a range of characteristics, including land use. Soil samples were exposed to 4 levels of CH_4: <1, 1.8, 30 and 60 ppm, for a period of 24 days in a continuously flushed incubation system that eliminated the effect of diffusion as control of methane or O_2 supply to microbes. During this incubation period, the concentrations as well as the isotopic values of $\delta^{13}C$ and δD of CH_4 in inlet and outlet air were compared and used with the rate of air flow to calculate fluxes and fractionation factors. After prolonged exposure to high and/or low CH_4 levels, all treatments were shifted to a single CH_4 concentration (1.8 ppm) to see if the community had developed differences in their capacity to consume methane. On the 27th day, samples were flash frozen for measurement using DNA based methods to observe shifts in methanotrophic community size and composition.

3.2 Material and Methods

3.2.1 Sites description and soil sampling

For this study we chose soils from deciduous and spruce forests and an agricultural field sampled in the state of Thüringen, Germany. Previous experiments with soils from these sites indicated that they had different intrinsic CH_4 oxidation rates, and we have considerable information on their chemical and physical properties. The agricultural (AG) soil was collected from the site of the Jena biodiversity experiment, and includes a C3-C4 vegetation shift experiment established in 2003 (Steinbeiss *et al.*, 2009). At the time of sampling, the plot was sown with maize which was at seedling stage. The two forest sampling sites are part of the Biodiversity Exploratory (Fischer *et al.*, 2010) and soil sampling plots were the monocultures of deciduous beech (*Fagus Sylvatica*) (DF) and spruce (*Picea abies*) (SF)) located near the Hainich National park.

At each site, 16 field replicates of mineral soil (10 cm) were sampled from an area of 20 x 20 m using spade. A subsample was taken separately to determine field soil moisture and bulk density. Soil sampling was done 2-5 days before starting incubations and all samples were kept at room temperature. Each sample was separately passed through 4 mm sieve and large plant particles were removed by hand. Main characteristics of soils are described in Table 3.1.

Table 3.1: Basic characteristics of the soils used in present study

Soil	Type	Land use	SOC (%)	CN ratio	pH*	µgg^{-1} soil DOC	MB*	NH_4^+	NO_3^-	δ^{13}C-SOC **(‰VPDB)**	Ref.
AG	Eutric Fluvisol	Cropland	2.2±0.4^a	9.3±2^a	7.2±0.9^a	28.9±9^a	225±163^a	†2.0±0.1^a	9.1±4^a	-25.3±0.8	(Steinbeiss *et al.*, 2009)
DF	Luvisol	Beech forest	2.9±0.5^a	13.4±1^b	4.3±0.3^b	44.3±14^b	359±163^b	†6.6±3.3^b	†5.1±3^b	-27.1±0.3	(Fischer *et al.*, 2010)
SF	Cambisol	Spruce forest	†4.4±0.9^b	14.9±1^b	5.2±0.6^c	44.6±16^b	379±163^b	1.7±1.2^c	12.6±7^a	-26.3±0.5	

*pH was determined in 0.1M $CaCl_2$ solution (1:5 soil water), MB represents microbial biomass
Each value present means± Standard deviation (n=15), †missing values (n<15)

Different letters in uppercase represent statistical significance among land use types (Tukey α=0.05)

3.2.2 Soil incubation

Details of the incubation system and soil chambers have been described elsewhere (Malghani *et al.*, 2013; Thiessen *et al.*, 2013). Briefly, approximately 1 kg of field moist, sieved soil was placed in especially made PVC columns (10 cm diameter and 20 cm height) after thorough mixing and soil moisture was adjusted to 50% WFPS by adding distilled water using a sprinkler for even distribution. All soil columns were placed in a climate chamber at constant temperature (25°C). The bottom of each column was connected to an air inlet system that was used for continuously flushing of soil columns (30±3 ml/min) with air of known composition (see below). The headspace (top) of each column was connected to a gas analysis system via an automated multiport stream selection valve (Valco). The airstream leaving the headspace of each column was analyzed in an infrared carbon dioxide analyzer ("LI-6262" LI-COR Biosciences Lincoln, USA) and cavity ring down spectrometer (PICARO 2301). For stable isotopic analysis of gases, headspace air was sampled with 1 L glass flasks (See below). Moisture loss (~1 g per day) due to continuous flushing of dry air was compensated by adding water on every 4^{th} day using sprinkler.

For manipulation of CH_4 concentrations in the inlet air stream, an automated air mixing system was built in MPI-BGC. This system contained four small cylinders for gas mixing (20 L volume) which were connected to air sources (commercially purchased gas cylinders) on one side and with soil columns on other side via a set of mass flow controllers. Cylinders that were filled sequentially had air with same mixing ratios of O_2, CO_2 and N_2 (O_2~20%, CO_2~350 ppm, Rest N_2) but CH_4. Field replicates of each soil were divided into 4 sets that were linked with gas cylinders, each set comprised of 4 replicates. Four CH_4 concentrations; <1ppm (T1, sub-atmospheric level), 1.8 ppm (T2, atmospheric level), 30±1 ppm (T3, 15 times higher than atmospheric level) and 60±2 ppm (T4, 30 times higher than atmospheric level), were supplied for 24 days of incubation and at 25^{th} day CH_4 concentrations in T1, T3, T4 were switched to atmospheric level. For three days CH_4 oxidation rates were monitored at atmospheric level to determine any enhancement and on 27^{th} day experiment was ended. Due to defect in air mixing system, CH_4 concentrations in low CH_4 treatment were not constant at one mixing ratio but range between 0.2-0.8 ppm.

Fluxes of gases from soils were calculated by multiplying the concentration differences between the outlet and inlet air streams using by the flow rates from the mass flow controllers.

3.2.3 Soil analysis

On the last day of experiment, the soil in each column was thoroughly mixed and divided into three aliquots. The first aliquot was shifted immediately into sterilized tubes (5 g) and frozen using dry ice and stored at -20°C until DNA extraction for microbial analysis. The second aliquot was used for soil extractions; these were performed the same day. The third aliquot was air dried at 40°C and was ball milled (4 min, 70 rpm) for elemental analysis.

Extractable dissolved organic carbon (DOC) and soil microbial biomass were determined by extraction using 0.05 M K_2SO_4, with and without chloroform fumigation (Brookers *et al.*, 2007;

Karsten *et al.*, 2007) and analyzed with TOC analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). Extractable inorganic nitrogen contents (NH_4^+, NO_3^-) were extracted using 0.01M KCl and freezed until measured with flow injection system (Quikchem QC85S5, Hach Company, Loveland CO, USA)

Total soil carbon and nitrogen content were analyzed in ball milled samples by an elemental analyzer at 1150°C (vario Max CN, Elementar Analysensysteme GmbH, Hanau, Germany). Organic carbon concentrations were determined by calculating the difference between elemental analyses of the total carbon concentrations and soil inorganic carbon concentrations (Steinbeiss *et al.*, 2008).

3.2.4 Flask sampling and analysis of CH_4 isotopes

Air exiting soil chambers as well as in inlet air stream was sampled using 1 L glass flasks that were pre-flushed with synthetic air. Each flask was attached to the air stream exiting the soil columns or the empty column (to sample the inlet air stream) and flushed for more than 10 hours (Malghani *et al.*, 2013; Thiessen *et al.*, 2013). The valves were then closed to isolate the sample, and gas concentrations (CH_4) were analyzed by gas chromatography (Agilent technologies 6890, Santa Clara, USA) equipped with a flame ionization detector (FID). Isotopic signatures ($\delta^{13}C$) of CO_2 and CH_4 were determined by stable isotope ratio mass spectrometry (Finnigan MAT 252IRMS and GC-C-IRMS) (Jordan and Brand, 2001; Sperlich, 2013). Samples from high CH_4 treatments were diluted prior to analysis for high analytical precision.

3.2.5 Microbiological analysis

3.2.5.1 DNA Extraction

The total DNA was extracted from 0.3 g of frozen soil using the Nucleo Spin® soil Kit (Macherey-Nagel Düren, Germany) following instructions of manufacturer. The DNA yield was 14±2 from forest soils (DF, SF) and 9±1 µg g^{-1} from agricultural (AG) soil. The quality and quantity of extracted DNA was confirmed by determining the OD, 260/280 ratio (NanoDrop 1000 spectrophotometer, PeQLab Biotechnology GmbH, Erlangen, Germany) and ranged between 1.7 and 1.9.

3.2.5.2 QPCR

Real-time quantitative PCR (qPCR) with three replicates for each sample was performed to determine the copy numbers of the *pmoA* gene using the primer sets A189f-A682r for MOB (Holmes *et al.*, 1995) and A189f-Forest675r for MOB members of USCα (Kolb *et al.*, 2003). The composition of reaction mixture and thermal profile are presented in Table 3.1. The assays were performed on CFX connect real-time system (Bio-Rad) and the associated software. The Copy numbers of target sequences in unknown soil DNA extracts were determined from standard curves. Standards for qPCR were made by serial dilution of stocks of a known number of plasmids containing the *pmoA* gene and *USCα* cloned *pmoA* gene. The quality of primers (the occurrence of bands of unspecific size and primer dimers etc.) were confirmed by running melting curves for each primer sets. Furthermore random samples were also run in gel to observe any unspecific product.

Table 3.**2:** Detail about reaction mixture and thermal profile used for Real Time PCR assays

Gene	Reaction Mixture	Thermal profile
pmoA[b] (proteobacteria)	15 µl {7.5 µl IQ SYBR Green mix (Bio-Rad), 0.05 µl each primer (100 µM), 0.2 µl BSA (50 mg/ml), 2.4 µl $MgCl_2$ (25 mM), H_2O[a], 3 µl DNA template (1:10 diluted)	Denaturing (94°C, 30s), Annealing (62.5°C, 30s), elongation (72°C, 30s), Cycles (40) fluorescence data acquisition (80°C, 5s),
pmoA[c] (USCα)	15 µl {7.5 µl Kapa SYBR Fast (PeQlab), 0.2 µl each primer (100 µM), H_2O[a], 3 µl DNA template (1:10 diluted)	Denaturing (94°C, 25s), Annealing (67°C, 20s), elongation (72°C, 45s), Cycles (40s), fluorescence data acquisition (75°C, 6s)

[a] Nuclease free water, [b] (Holmes *et al.*, 1995), [c] (Kolb *et al.*, 2003)

3.2.5.3 Terminal restriction fragment length polymorphism (T-RFLP)

For the investigation of methanotroph community structure, terminal restriction fragment length polymorphism (T-RFLP) fingerprint patterns *pmoA* genes were generated. Primer details and thermal profile of PCR reactions are given in Table 2. For *pmoA* gene amplification, 50 µl reaction mixture consisted of 25 µl of Premix F (Epicentre Biotechnologies), 5 µl of bovine serum albumin (10 mg ml^{-1}) (Roche), 0.5 µl dimethylsulfoxide (DMSO), 0.5 µl of each primer (20 µmol l^{-1}), 1 µl Taq DNA Pol. (Invitrogen), 12.5 µl DNase free water (Sigma) and 2 µl of 10-fold diluted extracted DNA as template.

Table3.3: Details about primers and thermal profile used for PCR cycle

Primer Pair	Sequence (5'–3')	Thermal profile [a]	Molecular Analysis
A189f-FAM*	GGNGACTGGGACTTCTGG	Denaturing (94°C, 45s), Annealing (52°C, 45s), elongation (72°C, 90s), Cycles (25) Final elongation (72°C, 7min)	T-RFLP pmoA
A650r	CCGGMGCAACGTCYTTACC		

* 5-carboxyfluorescein.

Approximately 100 ng of PCR amplicons were purified by using GenElute PCR Cleanup kit (Sigma) and were digested with 10 U of the restriction endonuclease MspI (Fast Digest enzyme, Taq FD). The digestion was carried out in a total volume of 15 µl for 15 mins at 37 °C. The restriction digests were purified using a Sigma spin clean up kit (Sigma Aldrich, USA). Aliquots (3 µl) of the purified digested amplicons were mixed with 11 µl of deionized HiDi Formamid (Applied Biosystems) and 0.2 µl of an internal DNA fragment length standard (MapMarker®1000, Bioventures). The mixtures were denatured at 94 °C for 3 min and then chilled on ice. The fluorescently labeled terminal restriction fragments (T-RFs) were size-separated on an automatic sequencer (3130 Genetic Analyzer, Applied Biosystems). The length of each fluorescently labeled T-RF was determined by comparison with the internal standard using GeneMapper Version 4.0 (Applied Biosystems). The accuracy of size calling between replicates was ±1 bp. The relative abundance of T-RFs in a given T-RFLP pattern was determined as the peak height of the respective T-RF divided by the total peak height of all T-RFs (Lüke *et al.*, 2011; Shrestha *et al.*, 2012). The terminal restriction fragments (T-RF) were binned only when relative abundances were presented. Tentative assignments of organism

identity were made by comparison to previous published literature where similar primer set and digestion enzymes were used (Table 3.6).

3.2.6 Data analysis and Statistics

The fractionation factor (α) for carbon (^{13}C) and Hydrogen (D) isotopes for CH_4 oxidation process was determined using Rayleigh equation as described in (Mariotti *et al.*, 1981).

$$\varepsilon\ (‰) = \left[\frac{10^3 Ln\left\{\frac{(10^{-3}\delta_{outlet} + 1)}{(10^{-3}\delta_{inlet} + 1)}\right\}}{Ln(F)}\right] \quad (1)$$

Where; ε is slope of linear curve fit after adjusting intercept to 0 and represents value of isotope enrichment factor for CH_4 oxidation process. F is the fraction inlet CH_4 concentration that remained after passing soil column $CH_{4\ (inelt)}/CH_{4\ (outlet)}$.

The kinetic isotope effect (α, ^{13}C & D) associated with high affinity methanotrophy was estimated using values of the enrichment factor ε;

$$\varepsilon\ (‰) = (1 - \alpha) * 1000 \quad (2)$$

Significance of differences among/between treatments was determined using one way analysis of variance (ANOVA) followed by a post-hoc test (Tukey or LSD, $\alpha = 0.05$). All statistical analyses were carried out either in SPSS (PASW statistics-18) and graphs were prepared in SigmaPlot (Version 12.5) and MS excel (Microsoft office, 2010).

3.3 Results

3.3.1 CH_4 oxidation rates were correlated with CH_4 concentrations but intrinsic CH_4 oxidation capacity differed among land use types

At atmospheric CH_4 concentrations, there were significant differences in CH_4 oxidation rates among land use types. DF soil had the highest intrinsic atmospheric CH_4 oxidation capacity followed by SF and AG soil (Table 3.4). The observed differences in CH_4 uptake rates among soil types remain similar at all levels of CH_4 concentration in inlet air (Table 3.4). SF soil had CH_4 oxidation rates similar to AG soil at sub-atmospheric concentration while rates were similar to DF soil at 30 ppm concentration.

Table 3.4: CH_4 uptake rates (ng-CH_4 g^{-1} soil.day^{-1}) exhibited by three contrasting soils incubated under four different levels of CH_4

Treatment	AG	DF	SF
T1 (<1 ppm)	2.4 ± 0.4^{Aa}	6.8 ± 0.8^{Ba}	3.3 ± 0.4^{Aa}
T2 (1.8 ppm)	4.3 ± 0.1^{Ab}	20.7 ± 2.1^{Bb}	7.4 ± 1.4^{Cb}
T3 (30 ppm)	45.4 ± 4.4^{Ac}	157.7 ± 22.0^{Bc}	73.1 ± 34.5^{ABb}
T4 (60 ppm)	90.5 ± 17.5^{Ad}	370.7 ± 46.5^{Bd}	188.1 ± 11.8^{Cc}

Each value represents Mean±S.E.M (n=4); Different capital and small alphabets in uppercase values show statistical significance (Tukey α=0.05) among soil types and treatments respectively.

There was linear correlation between CH_4 oxidation rates and CH_4 concentration in the inlet airstream, indicating that first order kinetics for the range of CH_4 we used in the experiment (Fig 3.1). The values of regression parameters (slope and intercept) varied among soil types, with highest slope in the DF soil followed by SF and AG, indicating different rate constants for the different soils. The scale of shift or change in CH_4 oxidation rates as result of CH_4 fertilization or starvation was similar in all soil types compared to their intrinsic rates of oxidation at atmospheric CH_4 levels (Table 3.4).

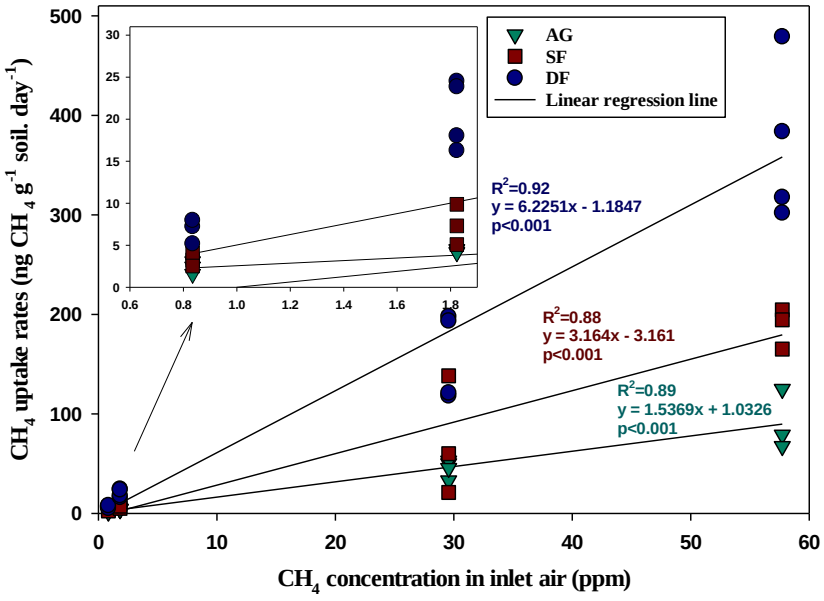

Figure 3.1: Change in CH_4 oxidation rates in three different soils when incubated under lower or higher level of atmospheric CH_4 concentrations.

Daily CH_4 uptake rates recorded throughout experiment are presented in figure 3.2 on a log scale excluding uptake rates of sub-atmospheric CH_4 (<1 ppm) treatment. The data showed constant rates of oxidation at each level of CH_4 in all land use types during 24 days. However an immediate and sharp decline was recorded when CH_4 concentrations in high CH_4 treatments were adjusted to atmospheric levels (1.8 ppm). Thus the 27 days of incubation at different CH_4 concentrations did not alter the intrinsic sink capacity of soils (Fig 3.2). The single exception

was the DF soil, where samples incubated at 30 ppm CH_4 had significantly ($p<0.05$) lower CH_4 oxidation rates upon readjusting CH_4 levels than samples continuously incubated at 1.8 ppm (Fig 3.2).

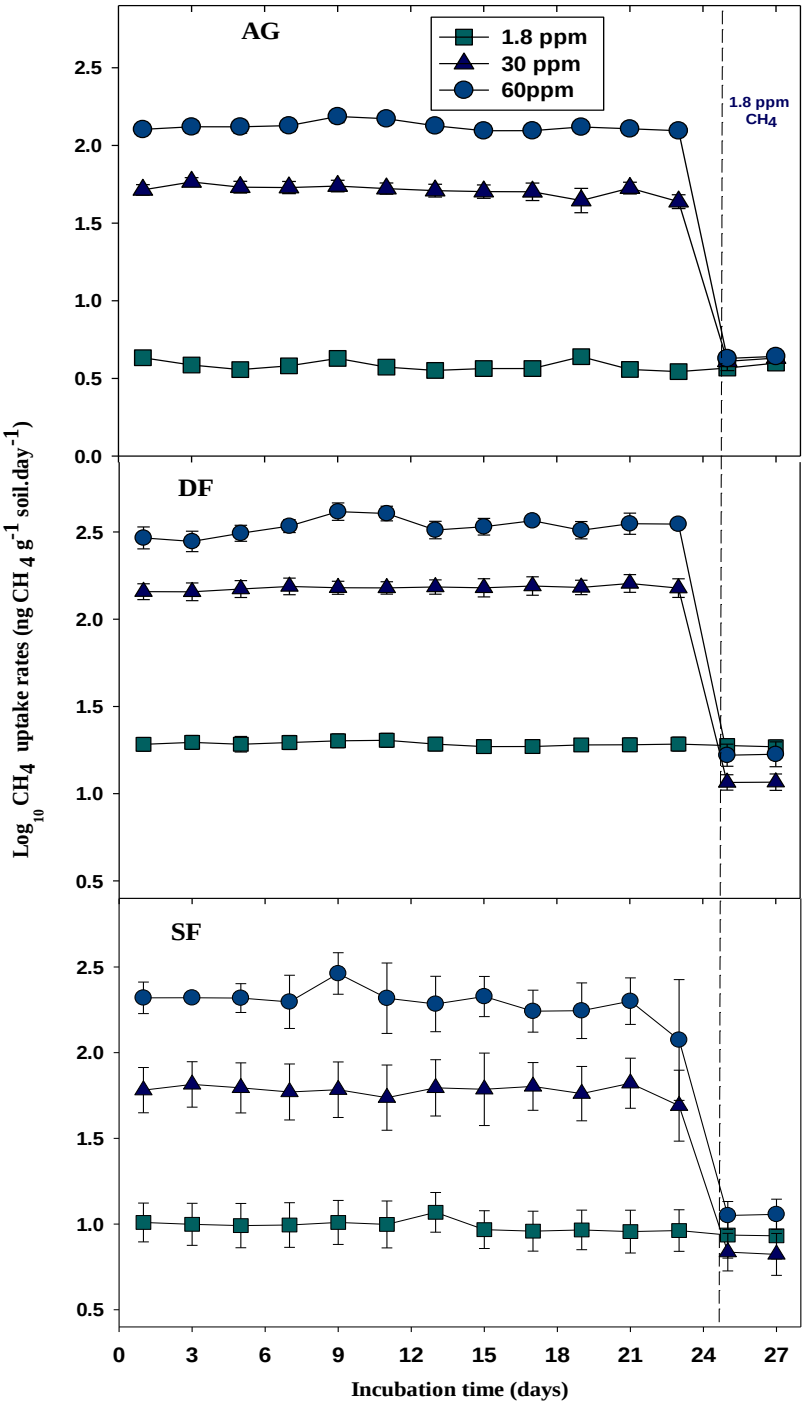

Figure 3.2: CH_4 uptake rates in AG, DF and SF soils incubated at different levels of CH_4 for 24 days. From 25 to 27 days all CH_4 levels were switched to atmospheric level (1.8 ppm). Each data point represent mean±S.E (n=4).

3.3.2 Percent of inlet CH_4 removed (removal efficiency) differs between low and high CH_4 treatments

Differences in CH_4 concentrations between inlet and outlet airstreams were used to calculate the CH_4 removal efficiency in each land use type under different treatments. CH_4 removal efficiency (basically a measure of the rate of methane oxidation given the residence time of air in the soil column) was below 50% for all soil types and treatments. However differences were observed between high and low CH_4 treatments by soil type (Fig 3.3).There was a general decline in removal efficiency with CH_4 concentrations, consistent with what is expected for Michaelis-Menten kinetics control of methane uptake. However, due to limited concentration range Km and Vmax were not determined. A two way ANOVA indicated land use type explained the most variance (SS=1364) in CH_4 removal efficiency followed by CH_4 treatments. However, no interaction was measured between land use type and CH_4 treatments ($p<0.05$).

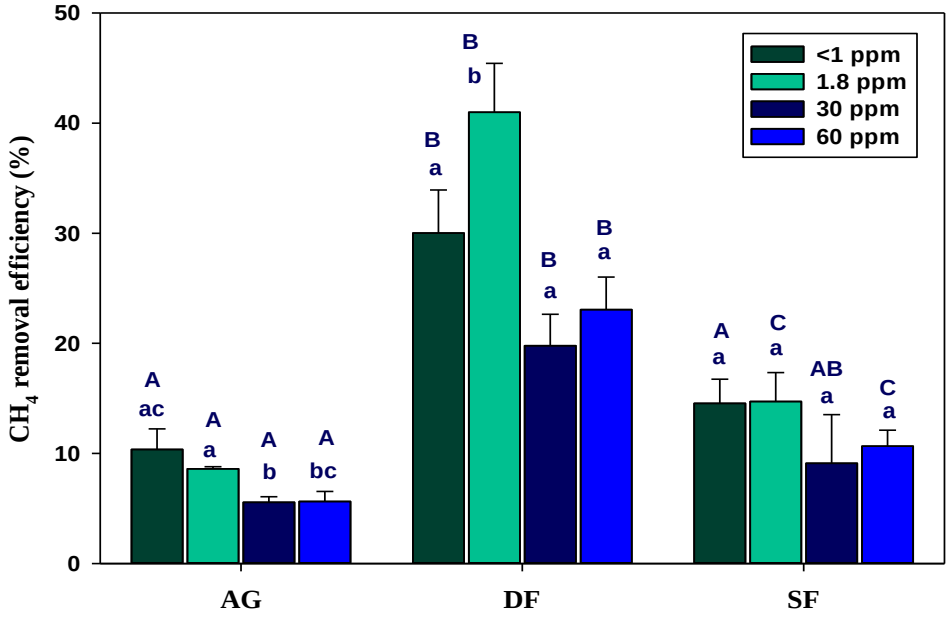

Figure 3.3: Variation in % CH_4 removal efficiency of different soils as a function of incubation CH_4 concentration; AG. Agricultural soil, DF. Deciduous forest soils, SF. spruce forest soil. Mean± Standard error (n=4). Different lower case and capital alphabets indicate significant differences among treatments and land use types respectively (Tukey $\alpha = 0.05$)

3.3.3 Variable carbon and hydrogen isotope fractionation expressed by contrasting land use types

Isotopic values of CH_4 in inlet and out air stream differed significantly in all soils, CH_4 leaving the soil column was enriched in ^{13}C and D (^{2}H) highlighting preference of lighter isotopes for CH_4 oxidizing enzyme (Fig 3.4). Fractionation values for ^{13}C were -10 to -14.6‰ and for D were -130 to -165‰ depending on land use type. SF showed statistically (Tukey, $\alpha=0.05$) distinct fractionation factors for both D and ^{13}C as compared to the AG and DF soils. Fractionation factors were not correlated with the CH_4 removal efficiency.

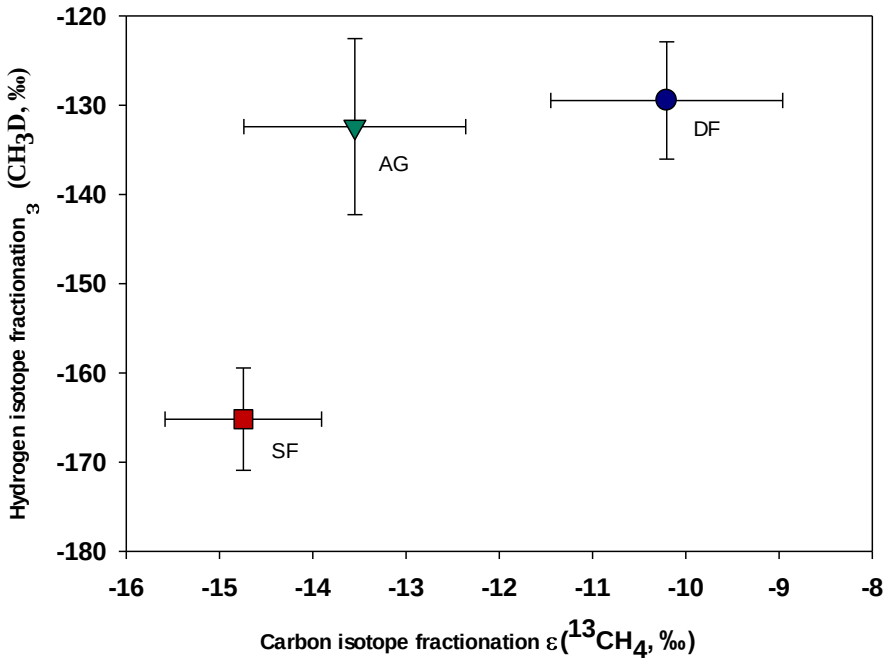

Figure 3.4: Differences among land use types in stable isotopic fractionation ($\delta^{13}C$ and δD) during CH_4 oxidation process, error bars are one standard error.

The values of isotopic fractionation factors (α^{13C} & α^{D}) associated with soil CH_4 oxidation or microbial uptake, either observed in present study or reported in literature, are listed in Table 5. The fractionation values observed in our study vary when compared to previous studies which did not separate the impacts of isotope discriminating factors, diffusion and microbial activity. We only recorded similarities in the values of α (^{13}C & D) associated with microbial CH_4 oxidation proposed by our study (1.010-1.015 for α^{13C} & 1.130-1.165 for α^{13C}). Interestingly, the fractionation values of AG and SF soils were similar to the values expressed by *Type II* methanotrophs as reported by a pure culture based study (Table 3.5). Overall differences in values of α (^{13}C and/or D) between our study and pure culture (methanotrophs) based studies highlight the variability in isotopic fractionation associated with low and high affinity methanotrophy.

Table 3.5: Stable isotope fractionation factor (α) associated with atmospheric CH_4 oxidation by upland soils or with different groups of methanotrophs.

Land use or methanotroph type (vegetation) (climate)	**Method**	**Isotope fractionation factor (α) for CH_4 oxidation**		**Reference**
		α^{13C}_{Soil} / $\alpha^{13C}_{mic.}$	α^{D}_{Soil} / $\alpha^{D}_{mic.}$	
Agriculture (corn) (Temperate)	Continuous flow through soil column	^{NA}/1.014±0.001	^{NA}/1.132±0.010	Present study
Forest (Beech) (Temperate)		^{NA}/1.010±0.001	^{NA}/1.130±0.007	
Forest (Spruce) (Temperate)		^{NA}/1.015±0.001	^{NA}/1.165±0.006	
Grassland (Meadow) (Temperate)	Static closed chamber	^{NA}/1.007		(Kato *et al.*, 2013)
Grassland (shrubs) (Temperate)		^{NA}/1.005		
Grassland (Temperate)	Soil probe (bottom up)	1.0220±0.002 / 1.0211±0.002		(Maxfield *et al.*, 2008)
Forest (Beech) (Temperate)		1.0218±0.002 / 1.0219±0.001	NA	
Forest (Pine) (Temperate)		1.0220±0.002 /$_{NA}$		
Forest Tropical rainforest	Lab. incubation	^{NA}/1.0102±0.001		(Teh *et al.*, 2006)
Grassland (Sub-tropical)	Static closed chamber	1.0168±0.002 / 1.0143±0.003	1.084±0.006 / 1.149±0.012	(Snover and Quay, 2000)
Forest (Coniferous) (Sub-tropical)		1.0181±0.0004 / 1.0168±0.0008	1.066±0.008 / 1.114±0.018	
Forest (Aspen) (Boreal)	Soil probe	1.019 / 1.022		(Reeburgh *et al.*, 1997)
Forest (Spruce) (Boreal)		1.019 / 1.026	NA	
Forest (Deciduous) (Temperate)	Soil probe	1.022±.004 /$_{NA}$		(Tyler *et al.*, 1994)
Grassland (Tundra)	Static closed chamber	1.026/$_{NA}$		(King *et al.*, 1989)
TypeI methanotrophs	Pure cultures at 5% CH_4	$_{NA}$ / 1.028±0.002	$_{NA}$ /1.228±0.024	(Feisthauer *et al.*, 2011)
Type II methanotrophs		$_{NA}$ /1.019±0.001	$_{NA}$ /1.152±0.014	
TypeI methanotrophs	Pure cultures at 12% CH_4	$_{NA}$ / 1.006	NA	(Templeton *et al.*, 2006)
Type II methanotrophs		$_{NA}$ / 1.015		

3.1.1 Methanotrophic community size (pmoA gene copies) did not differ between low and high CH_4 treatments

The two qPCR assay performed in this study targeted all microorganisms that possessed *pmoA* and *amoA* genes and/or specific group of methanotrophs related with *USCα* as described in MM section. Results of both assays showed somewhat similar results; we found no significant ($p<0.05$) differences in *pmoA* gene copy numbers among samples exposed to different CH_4 concentrations (Fig 3.5). However, a second assay identified a large difference between the two forest soils, at <1 ppm and 30 ppm CH_4 treatments: DF soil had significant ($p<0.05$) high number of *USCα* associated *pmoA* gene copy numbers. In addition, we found weak but a positive correlation between *USCα* and CH_4 treatments in DF and SF soils.

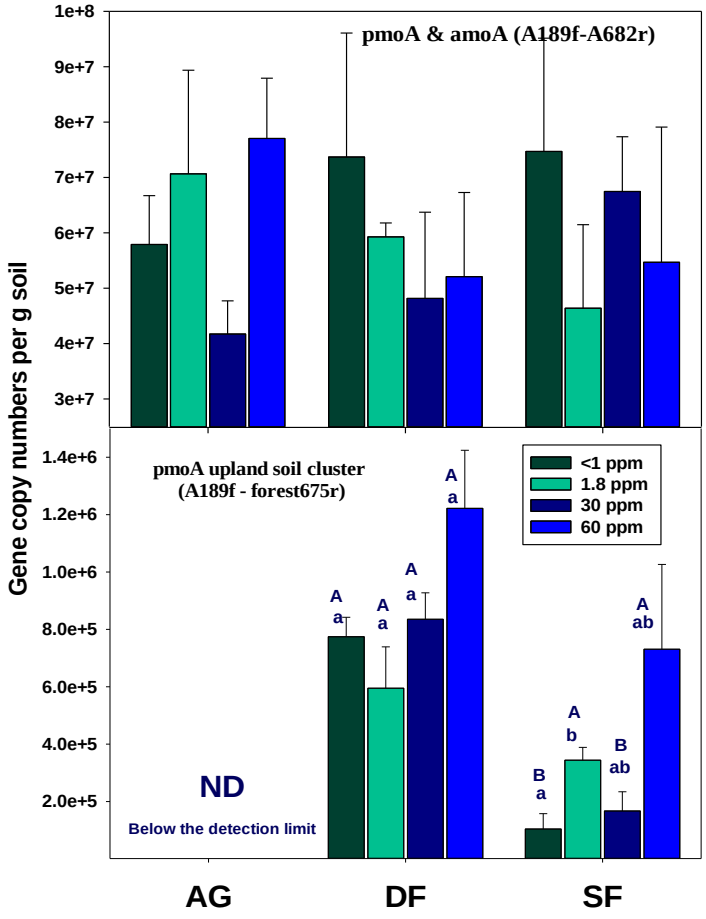

Figure 3.5: Gene copy number (A) *pmoA* with primer set 189f-682r (B) *pmoA* upland soil cluster with primer set (186f-forest675r). Mean±standard error. Different capital and small letter represent statistical significant soil type and treatments respectively.

3.1.2 Composition of microbial community

The most prominent difference observed in pattern of T-RFs associated with contrasting methanotrophic organisms were between agricultural and forest soil samples. No significant difference in abundance of any particular T-RFs was recorded in soil samples incubated at different CH_4 levels (Fig 3.6). In total 18 different T-RFs were measured, excluding a few exceptions almost all TRFs were present in all soil samples but the abundance of particular T-RFs differ considerably among land use types. In the AG soil the most dominant T-RFs size was 337-bp, associated with *Type Ia* methanotrophs (Table 3.6), constituting about 32-60% of total T-RFs. While, both forest soils were dominated by the small size T-RFs largely associated with *USCα*. Among all T-RFs present in forest soils, 27 bp associated with *USCα* (Table 3.6) was the most dominant constituting 15-40% and 14-36% in DF and SF soils respectively. The type and abundance of all T-RFs were almost identical in both forest soils except 75-bp which was only present in DF soil. Other dominant T-RFs in forests soils were 33-34 bp, 36-37 bp, 65 bp and 504 bp constituting 3-11%, 6-32%, 6-26% and 3-16% respectively. Methanotrophs associated to dominant T-RFs observed in forest soils were *USCα*, *Methylocapse acidiphila*, *Methylobacter sp.* and *Methylocystis sp.* (Figure 3.6). T-RFs sizing 36-37, 65, 240 and 504-bps were also present in AG soil but their relative abundance were far below than 337-bp.

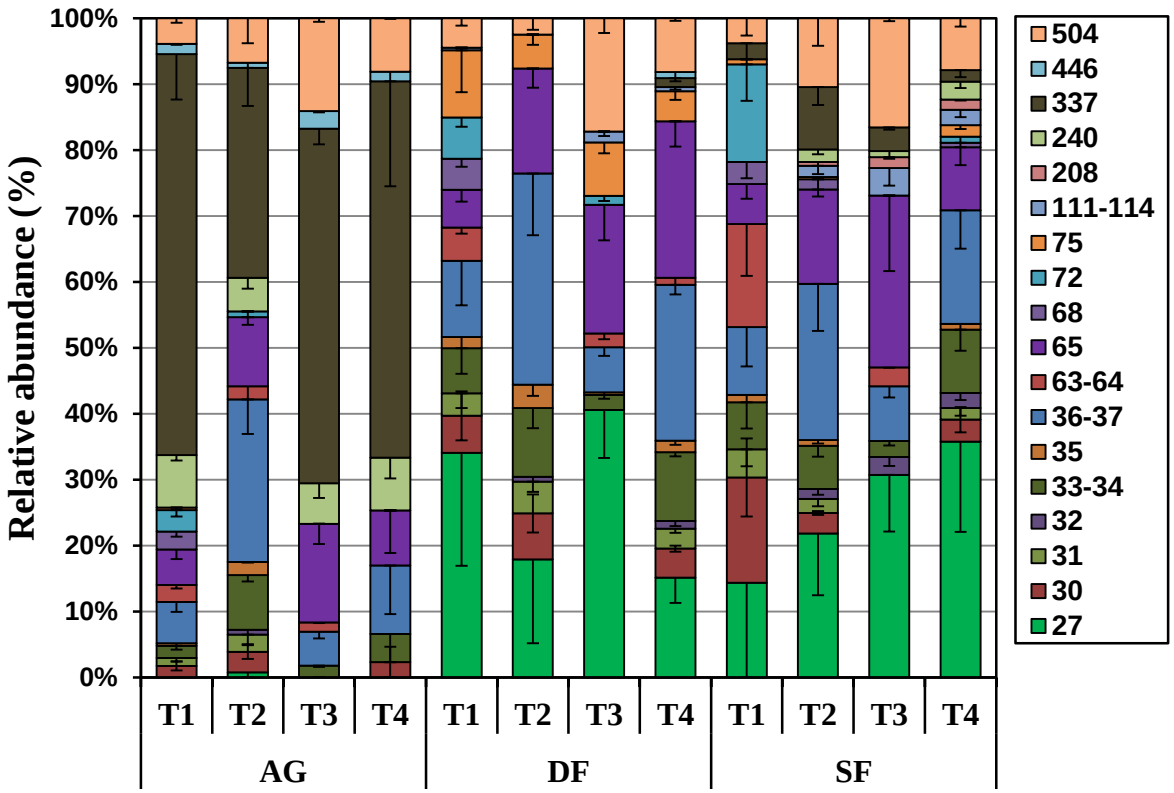

Figure 3.6: Bar diagrams of *pmoA*-based T-RFLP fingerprint patterns obtained from all subsamples representing the 3 different upland soils. The T-RFLP patterns were generated using the primer set A189f-A650r in PCR. The percentage abundances (mean ± s.dev., n = 4) of 18 distinguishable T-RFs are indicated by different colors.

Table 3.6: Phylogenetic affiliation of the most abundant T-RFs (digestion of *pmoA* with *MspI* using same primer set A189-FAM, A650r) found in soils.

T-RF-size (bp)	Associated organism	Reference*
27	*Methylocapsa sp./USCα clones*	(Nazaries, 2011)
33-4	*RA14 (SL1,2,3,4)/ Methaylocapsa sp. (USCα)*	(Horz *et al.*, 2005; Lüke, 2009; Shrestha *et al*., 2012)
75-6	*Methylocapse acidiphila,*	(Nazaries, 2011)
208	*Type II Methylocystis sp,Methylosinus sp.*	(Horz *et al.*, 2005)
240-2	*Methylocystis sp,Methylosinus sp.*	(Nazaries, 2011)
337-8	*Type Ia*	(Henneberger *et al*., 2012)^
446	*MHP clade*	(Shrestha *et al*., 2012)
504-6	*Methylobacter sp.*	(Shrestha, 2007)

*Literature that used same digestion enzyme and primer set was used to assign specific group names to T-RFs of similar size or with 1-2 bp differences.
^same digestion enzyme but different reverse primer (A682r or mb661)

3.4 Discussion

3.4.1 Variability among soils of contrasting land use types in intrinsic CH_4 oxidation rates could be associated with differences in methanotrophs community composition

We measured significant differences in intrinsic CH_4 oxidation rates of soils from contrasting land use types (Fig 1, Table 4) and the observed trend was similar to previous field studies (Borken and Beese, 2006; Kizilova *et al*., 2013), with highest CH_4 uptake rates exhibited by DF soils, followed by SF and AG soils (Fig 3.1, Table 3.4). Factors that have been proposed to be responsible for differences in CH_4 uptake capacity among land use types include soil physicochemical characters i.e. pH, NH_4^+, air diffusivity (Borken *et al*., 2003; Borken and Beese, 2006), methanotrophic community population size or biomass (Menyailo *et al*., 2010; Bárcena *et al*., 2014) and community structure (Nazaries *et al*., 2013). Since our experimental design eliminated differences in diffusivity between the soils as a variable, the factors other than physical characters could be responsible for our results.

Our results highlight the role of methanotroph community structure among soils of contrasting land use type i.e. forest & agriculture for their different CH_4 oxidation capacity and other factors for the differences between soils of similar land use type but vegetation i.e. SF & DF. The T-RFLP results concur with other studies that proposed a direct role of methanotroph community structure on CH_4 uptake capacity of soils especially the presence and absence of *USCα* in soil under different land use types (Kolb, 2009; Nazaries *et al*., 2013; Tate, 2015).T-RFs associated with *USCα* were the most dominant in forest soils (Fig 3.5 & 3.6). Previously *USCα* were also known as uncultured forest cluster as their presence was initially detected only in forest ecosystems (Kolb *et al*., 2003). The presence and active participation of *USCα* in atmospheric CH_4 oxidation in temperate forest soils is a well-established phenomenon (Kolb, 2009; Shrestha *et al*., 2012). However, it is also clear from CH_4 uptake capacity of AG soil that methanotrophic

groups other than *USCα* i.e. *Type I*, *Type II*, also actively participate in atmospheric CH_4 oxidation as *USCα* were not detected in molecular assays i.e. qPCR and T-RFLP of AG soil (Fig 5 & 6).

The absence of *USCα* in agricultural soils is also a well-known phenomenon. Studies based on land use change reported that *USCα* are extremely sensitive and their population size (abundance and biomass) decreases rapidly in response to agricultural practices especially plowing (Maxfield et al., 2008b; Shrestha *et al*., 2012; Nazaries *et al*., 2013). Some studies reported re-emergence of *USCα* in soils where agricultural practices were abandoned (Ho *et al*., 2011; Levine *et al*., 2011) but in spite of microbial community recovery, soil capacity to uptake atmospheric CH_4 at pre-disturbance levels takes decade to century (Priemé *et al*., 1997; Tate, 2015). Our sampling site for AG soil is under agricultural use from its all known history, more than 15 years, and is plowed at each cropping season. It is plausible that continuous cultivation has eliminated high affinity methanotrophic group *USCα* was absent in AG soil as other factors that may influence abundance and activity of high affinity methanotrophs were either similar to other soil types i.e. NH_4^+ content (Table 3.1) or were not in practice i.e. irrigation, pesticide & fertilizer application. The governing role of soil plowing in determining the community structure of methanotrophs in AG soil is further supported by dominance of T-RFs associated *Type 1a* in this soil (Fig 6). The presence of *Type 1a* methanotrophs is usually associated with perturbed environments (Abel 2009; Dorr *et al*., 2010). Although, the role of soil pH is rather inconclusive as *USCα* have been detected on range soil pH (Knief *et al*., 2003) but we cannot completely rule out its role based on our data.

Variability in CH_4 oxidation rates between forest and agricultural soils could be explained with methanotrophic community structure assuming that *Type II* and *USCα* dominant soils probably have higher intrinsic CH_4 uptake capacity but this cannot imply on the observed differences between SF and DF soils as both samples contain almost identical methanotrophic community structure (Fig 6). In this regard, studies that were specifically conducted on deciduous and spruce forest soils proposed that low abundance of *USCα* and presence of inhibitory compounds i.e. monoterpene could be responsible for low sink capacity of spruce forest soils for atmospheric methane (Maurer *et al*., 2008; Degelmann *et al*., 2009). We did not measure production of monoterpenes but their inhibitory role in SF soil cannot be ruled out. Our sample preparation method, samples were passed from 4mm mesh and only large plant particles were hand-picked, do not guarantee complete removal of needle leaves associated litter which promote production of monoterpenes when decompose (Asensio *et al*., 2012). In case of relative abundance of *USCα* between soils of two vegetation types, we recorded considerable low number *pmoA* gene copy numbers related with *USCα* in SF soil (Fig 5).

3.4.2 Isotopic fractionation factors, α^{13C}_{mic} & α^{D}_{mic}, associated with high affinity methane oxidation and variability among land use types

This work presents values of isotopic fractionation factor α^{13C} & α^{D} associated with high affinity methanotrophs because other factors that could affect αmic values, were either absent or eliminated. In normal static chamber, the most common method used in upland soils, based studies (Reeburgh *et al*., 1997; Maxfield *et al*., 2008a; Kato *et al*., 2013) the parameters other than microbial oxidation i.e. diffusion, native CH_4 production, could also result into enrichment or shift in isotopic values ($\delta^{13}C$ & δD) of headspace CH_4 (von Fischer, 2002; Gonzalez-Gil *et al*.,

2008; Kayler *et al*., 2010). These methodological differences could be the main reason that the values of α^{13C} proposed by chamber based studies were significantly larger than what we recorded in present study (Table 3.5).

Our measured α_{mic} (^{13}C & D) values are similar in magnitude to KIEs reported by Snover and Quay (2000) but differ when compared studies that normalized αsoil values by using hypothetical values of α_{diff} i.e. Kato *et al* (2013), Maxfield *et al* (2008) in order to find α_{mic}. These findings point out that use of one single value of α_{diff} for soils of variable land use types and textural classes is not the best choice. To our knowledge, Snover and Quay (2000) is still the only study which measured α_{diff} associated with atmospheric CH_4 oxidation. Bottom up study reported α_{diff} =1.013 for tundra wetland soils (Preuss *et al*., 2013).

The isotopic fractionation values observed in this study were similar to those reported in studies using pure cultures of low affinity methanotrophs (Coleman *et al*., 1981; Templeton *et al*., 2006). This highlights that the mode of action of low and high affinity enzymes is probably similar. On other hand we also found differences in values of our study with those proposed by pure culture based reports (Table 3.5). This advocate further investigations not only for isotopic fractionations associated with high affinity methanotrophs but also for low affinity organisms.

We observed no empirical trends in α (α^{13C} & α^{D}) among land use types as we recorded in intrinsic CH_4 oxidation rates (Table 3.4, 3.5). Variations in α^{13C} & α^{D} were noted between DF and SF soils, while, comparisons of AG with the mentioned soils exhibited no significant differences (Table 3.5). Isotopic fractionation for ^{13}C in AG soil was similar to SF soil and differs significantly when compared with DF soil, however, α^{D} were found to be similar to the latter. In our opinion the proposed factors, variability in the structure of the active methanotrophic community between forest and agricultural land use type and different per capita activity of similar organisms in two forest types (DF & SF), for the variability in intrinsic CH_4 sink capacity among our soils could also be the reason for the differences in values of isotopic fractionation. However, lack of particular trend among land use types in values α^{13C} and α^{D}, other factors may also have played their role i.e. location of methanotroph niche, size of the active methanotroph community etc.

3.4.3 CH_4 may not be the only source of carbon and energy for high affinity methanotrophs

Methanotrophs are functionally unique organisms in that they are the only soil microbes capable of utilizing CH_4 to fulfill carbon and energy requirements. Several previous studies have found a positive correlation between CH_4 oxidation rates and total methanotrophic biomass or abundance of methanotrophs related with *USCα* (Menyailo *et al*., 2008; Menyailo *et al*., 2010; Nazaries *et a*l., 2013; Bárcena *et al*., 2014). Thus it is important to find what factors limits methanotroph growth and population size in soils under different land use type.

Because CH_4 is the primary energy substrate for methanotrophs, our experimental manipulation of available CH_4 concentrations could have considerably affected energy supply required for their growth and cell maintenance processes. However, we did not observe any significant differences (Tukey α=0.05) in *pmoA* gene copy numbers in qPCR or in relative proportion of T-RFs associated with *USC*α and other methanotrophs type among soil samples that were exposed to different CH_4 concentrations (Fig 5, Fig 6). In addition, soils from different land use types showed significant differences in CH_4 oxidation rates at all CH_4 levels (Table 3.4, Fig 3.1) but had identical *pmoA* gene copy numbers (Fig 5, Fig 6). Together, these lines of evidence suggest that atmospheric CH_4 oxidation may not be the only source of energy for metabolic activities of these organisms and point out one of several possible underlying mechanisms. First, methanotrophs may utilize an alternate energy source (e.g. locally produced CH_4, alternative C compounds). Second, methanotroph population size may depend on growth or population controlling factors i.e. nutrient limitation, grazing by soil bacteriovores. Third, the growth rates of methanotrophs in natural environment are too slow to be detected (oligotrophic life style). In addition, it also point towards existence of active methanotrophs below the detection limits of DNA based molecular techniques.

Methane monooxygenase (MMO) is a broadly nonspecific enzyme and can catalyze a wide range of oxidative reactions (Rosenzweig, 2011). Based on stable isotope (^{13}C) labelling and other traditional methods the facultative use of carbon compound, i.e. acetate, by high affinity methanotrophs have been experimentally evident and some studies report induced activity in the presence of simple compounds like acetate, methanol etc. (Benstead *et al*., 1998; West and Schmidt, 1999; Belova *et al*., 2011; Pratscher *et al*., 2011; Shrestha *et al*., 2012). We did not measure concentrations of other compounds in this study but the role of other energy sources cannot be completely ruled out. It is well evident that even well aerated soils possess anoxic microzones that serve as ecological niche for methanogens (von Fischer, 2002; Angel, 2010). Several studies proposed that this extra source of CH_4 which is difficult to detect by normal CH_4 flux measurements act as energy booster for starving high affinity methanotrophs (Shukla *et al*., 2013). And successful adaptation of high affinity methanotrophs to feast-feminine conditions is the key for their survival. However, presence of active methanogenesis is the most unlikely in our experiment for the reason of continuous flushing by air composed of 20.5% O_2. In our pre-experiment incubations, using samples from same sites, we did not observe CH_4 production (CH_4 concentration and $\delta^{13}CH_4$ were identical between inlet and outlet air samples) even exposing samples to anoxia for 4 days. Active methanogenesis may not be the source for methanotrophs in present study but it potentially could be an extra source of energy and carbon in natural soil conditions.

Biological factors such as competition and predation are the key players in regulating microbial population and thereby could be the reason for no significant change in methanotrophs abundance in spite of high CH_4 oxidation rates. In this regard several studies point out presence of growth limiting factors for methanotrophs including those mention above nonetheless there is

no direct evidence so far. For instance, Ho and Frenzel (2012) observed recovery of methanotrophs, *Type I* & *Type II*, after heat shock but population size did not exceed than original size indicating insufficient space or nutrient limitation of recovering community of methanotrophs. Similarly, stable isotope based i.e. PLFA-SIP, studies observed depleted signals of $\delta^{13}C$ in some of the known microbial predators i.e. nematodes, amoeba, flagellates but did not clear whether these signals were by direct feeding of methanotrophs or it was due to cross feeding (Murase *et al*., 2011). Cross feeding include assimilatory decomposition of intermediate products of methane oxidation i.e. methanol by other heterotrophic communities (Qiu *et al*., 2009).

Physiological traits, slow growth strategy (oligotrophic nature) and presence of large number of population in dormancy could affect outcomes of methanotrophs population dynamics. The oligotrophic nature of methanotrophs demand whether incubation period used in present study or in previous studies (Benstead and King, 1997; Bull *et al*., 2000; Pratscher, 2010) was long enough to observe differences in methanotroph population by enhanced growth rates due to increased availability of CH_4. In this regard, there is scarcity of literature that could propose population doubling time in high affinity methanotrophs due to absence of pure culture. We decided 24 days incubation period simply based on calculations assuming assimilation of 40% of oxidized CH_4-C (assimilation of 30-54% of oxidized CH_4-C is proposed in literature (Roslev *et al*., 1997)). The observed differences in CH_4 oxidation rates in samples under low and high CH_4 treatments (Table 3.4), 24 days were sufficient for methanotrophic growth. On other hand, no considerable differences in *pmoA* gene copy numbers among treatments could also be constrain due to measurement of DNA which includes active and dormant community and do not truly representative of metabolically activity community (Wang *et al*., 2014). The observations like; detection of methanotrophic community in soils without intrinsic oxidation capacity (Shrestha *et al*., 2012, present study, unpublished results), no seasonal shift in methanotrophic community abundance (Shrestha *et al*., 2012) but in methane oxidation rates and resilience of methanotrophic population after heat stress clearly indicate that at least a proportion of methanotrophs population may only exist in dormant form and act as seed bank against environmental extremes (Ho *et al*., 2013). In such scenario a shift in size of small number of active methanotrophs is practically impossible to determine with DNA based techniques. This will be a future challenge for molecular biologists to use next generation sequencing techniques i.e metatranscriptomics for observing dynamics of relatively very small microbial community (<0.01% or even low).

3.4.4 Induced cell specific activity after exposure to high CH_4 concentrations is probably a phenomenon for low affinity methanotrophy

The efficiency (CH_4 consumption per microbial cell) of aerobic methanotrophs increases when exposed to high CH_4 concentrations and this phenomenon is well described in paddy soil based studies (Bender and Conrad, 1992; Steenbergh *et al*., 2010). Induced enzyme activity and

increased methanotroph cell numbers are principle reasons explaining this phenomenon (Steenbergh *et al*., 2010). We did not observe changes in any of the above mentioned parameters among samples that were exposed to different CH_4 concentrations. On one hand, *pmoA* gene copy numbers were similar among treatments (Fig 3.5) and other hand CH_4 oxidation rates (as mirror of enzymatic activity) were almost identical when CH_4 concentrations were shifted to atmospheric levels (Fig 3.2). These outcomes suggest that induced enzymatic activity due to high CH_4 availability is most probably a phenomenon for low affinity methanotrophy. Interestingly, a fewer upland based studies also reported relative persistency in induced methanotrophic activity (Schnell and King, 1995; Bengtson *et al*., 2009; Kammann *et al*., 2009) but in these studies a concentration of more than 1000 ppm CH_4 was used which on other hand could lead to activation of low affinity methanotrophs (Benstead and King, 1997; Shrestha *et al*., 2012). We did not observe any significant shift in methanotrophic community structure among samples exposed to different CH_4 concentrations (Fig 3.6).

3.5 Conclusion

This study highlights several aspects of high affinity methanotrophy though it was primarily designed to determine impact of CH_4 availability on methanotrophic community abundance. Our results provide evidence that the intrinsic capacity of soil to oxidize atmospheric CH_4 is biologically driven. Large differences in methanotroph community structure among land use types, specifically, between forest and agricultural soils, suggest its dominant role in setting the sink capacity for a particular land use type. In this regard, the most prominent results were the absence of *USCα* and the presence of *Type 1a* methanotrophs in the agricultural soil. Interestingly, differences were recorded also in isotope fractionation values among land use types. Most probably these results were driven by differences in physiochemical characteristics of soils (clay content, tortuosity of soil pores) and methanotroph community structure. The main finding of this study is that no significant differences in methanotroph community abundance occurred in samples incubated at contrasting CH_4 concentrations (<1 ppm -60 ppm) for 24 days. This suggests that either methanotrophs depend on other carbon source for their growth or the amount of CH_4-C used for C-assimilation was lower than expected. For future research in high affinity methanotrophy, we suggest that the new investigations must include other carbon sources in order to determine main driving factor for methanotrophic population.

CHAPTER 4

Carbon sequestration potential of hydrothermal carbonization char (Hydrochar) in two contrasting soils; results of a one-year field study

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This study has been published in ***Biology and fertility of soils.*** 51(1):123-134

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Abstract

Soil amendment with hydrochar produced by hydrothermal carbonization of biomass is suggested as a simple, cheap and effective method for increasing soil C. We traced C derived from corn silage hydrochar ($\delta^{13}C$ of -13‰), added to "coarse" and "fine" textured soils ($\delta^{13}C$ of -27‰) over two cropping seasons. Respiration rates increased in both soils ($p<0.001$) following hydrochar addition; most extra respiration was derived from hydrochar-C. Dissolved losses accounted for ~ 5% of added hydrochar-C ($p<0.001$). After one year, cumulative loss of hydrochar-C was 33±8% for both soils, while hydrochar amended soils preserved 15±4% more native SOC. Hydrochar negatively affected plant height ($p<0.01$) and biomass ($p<0.05$) in the first but not the second cropping season. Our results confirm previous laboratory studies showing that initially hydrochar decomposes rapidly and limits plant growth. However, the negative priming effect, and persistence of added hydrochar-C after one year highlight its soil C sequestration potential.

Key words: Hydrochar, plant growth, soil respiration, soil leachate, carbon isotopes, physical soil parameters.

4.1 Introduction

Conversion of biomass into biochar and its application to soil is an emerging mitigation technique that has received much recent interest. Several methods are known for biochar production and they differ in factors such as the type and pre-treatment of biomass or feedstock used, and the temperature, pressure and other conditions during conversion. Thus, chars produced by different methods also vary significantly in chemical and physical characteristics and in their potential for C sequestration (Malghani *et al*. 2013). Biochar produced by dry pyrolysis (pyrochar) is the most documented material used for carbon sequestration. Pyrochar can persist in soil for decades or longer (Lehmann 2007), and also affects other soil properties (Sohi *et al*. 2009) including bulk density (Abel *et al*. 2013), soil aeration, water retention capacity (Abel *et al*. 2013), pH (Rillig *et al*. 2010), nutrient availability (Farrell *et al*. 2014; Prayogo *et al*. 2014) and the microbial community composition (Khodadad *et al*. 2011; Song *et al*. 2014). It has been estimated that each ton of dry biomass pyrolysed and applied to soil could offset up to 800-900 kg of CO_2 emissions (Roberts *et al*. 2010).

Pyrolysis techniques are derived from methods of conventional charcoal production (Ogawa and Okimori 2010) and generally require dry biomass. The use of the moist biomass in these processes wastes a large portion of the added energy to evaporate water (Antal and Grønli 2003). Hydrothermal carbonization (HTC) has recently been suggested as a simple, cheap and effective way of increasing the C content of biomass (Titirici *et al*. 2007). This method of carbonization is of particular interest for wet biomass that is produced in large quantities and is not suitable for other carbonization methods, such as sewage sludge, industrial bio wastes and green household wastes (Ramke *et al*. 2009; Berge *et al*. 2011; Liu *et al*. 2013; Oliveira *et al*. 2013). HTC uses low temperatures that produce very low gas yields, with the majority of the biomass either converted into brown coal or dissolved in liquid (Ramke *et al*., 2009). The overall end product yield varies depending on the treatment time, feedstock type and other parameters like process temperature, pressure and the presence of catalysts (Hu *et al*. 2010;Cao *et al*. 2011).

Hydrothermal carbonization (HTC) is a well-documented process in terms of process conditions and characteristics of end products (Hoekman et al. 2011) but very few studies exist for evaluating its proposed use as a tool for C sequestration in soils. The final product is a "slurry-like" material that consists of solid products (suspended brown coal) of different sizes, shapes and surface functional groups (Libra *et al*. 2011). It has been suggested that this material may have low potential for C sequestration because a large fraction of the C remains in dissolved form and is subject to rapid decomposition (Libra *et al*. 2011; Malghani *et al*. 2013). Compared to pyrochar produced at high temperatures, hydrochar has higher H/C and O/C ratios making it more susceptible to microbial degradation (Schimmelpfennig and Glaser 2012) and it may undergo microbial transformation before it can act as long term storage for the C content.

Because sequestration timescales may depend on interactions between dissolved or microbially transformed C with soil mineral surfaces, soil properties, particularly texture, may play an important role in the fate of hydrochar C amendments. In this context, a previous laboratory incubation study found no impact of soil type on hydrochar mineralization when incubated at 1% mass HTC/mass soil ratios (Malghani *et al*. 2013). To date, studies on the potential of hydrochar for C sequestration and the role of soil type are limited to either lab-based soil incubations or

greenhouse-based mesocosms. The objective of this study was to estimate the C sequestration potential of hydrochar and its impact on plant growth in arable field settings. Hydrochar-C derived from C4 plants (here corn silage) was added to soil that developed under C3 plants, and its fate was subsequently monitored using C stable isotopes measured in all major C pools such as CO_2, DOC in soil leachate and bulk soil carbon (SOC).

4.2 Materials and Methods

4.2.1 Hydrochar Characteristics

Hydrochar (HTC) produced from corn silage was obtained commercially from Carbon Solutions Ltd, Kleinmachnow Germany. Material was produced in two stages; one at 230°C and 2nd at 180 °C (Naisse et al. 2014). Hydrochar was applied as it was delivered as slurry containing ~10% dry matter, with a considerable fraction of the total C in dissolved form (2.85±0.06 g-C/L). The slurry was acidic with a pH of 4.15, which was measured directly in the slurry after thorough shaking. The dry matter of hydrochar had 51% C, 1.9% N, 5.7% H and 19% O. The O/C ratio of 0.28 suggests the suitability of this HTC for potential C sequestration (Schimmelpfennig and Glaser 2012). Detail of hydrochar characteristics has been described elsewhere Malghani *et al*. (2013). The use of corn silage as feedstock was mainly to obtain hydrochar with C4 isotopic signals to allow us for tracking hydrochar-C.

4.2.2 Soil characteristics and experimental setup

The experiment used a long-term field experimental site maintained at the Max Planck Institute for Biogeochemistry, Jena, Germany. In 2002, two soils with contrasting texture, coarse and fine, were placed in separated 48 m^2 plots and were under free succession of local C3 plants. Initial $\delta^{13}C$ values of -27.8‰ and -26.7‰ (V-PDB), respectively (Table 1) indicated no prior influence of C4-C. The average annual temperature at the site was 9.87 °C and mean precipitation was 580 mm during the experimental period in year 2011-12.

Each large plot was sub-divided into 6 smaller plots (3.45 m^2) and two rings (D=30 cm, H=15 cm) were installed within each small plot, for a total of 24 rings. To collect soil leachate, suction plates (D = 9 cm) were installed in the center of each ring at a depth of 15 cm. In addition, sensors were also installed at 10 cm depth in each ring for continuous measurement of soil moisture (% v/v, ML3x probes, DeltaT, Campbell scientific) and temperature (NTC resistance thermometer, Campbell scientific). For soil respiration measurement, two small collars (D=7 cm, H=9 cm) were inserted permanently into the soil inside each large ring (the total of 48), the bottom of one of two respiration collars was sealed with mesh (25 µm) to prevent root ingrowth. Respiration rates from this root exclusion collar were assumed to include predominantly heterotrophic respiration while the paired collar with no mesh was assumed to measure both autotrophic and heterotrophic component (Moyano *et al*., 2008).

For the hydrochar treatment, soil within each large ring (12 rings in total) was excavated up to the depth of the suction plate (15 cm) and mixed thoroughly with hydrochar. The amount of hydrochar added to the soil was calculated corresponding to a C addition of approximately

20±2% and 30±2% of the initial SOC content of coarse and fine soils respectively. After thorough mixing soil samples were filled back into respective places. For the control treatment, soil was excavated from the remaining 12 large rings and mixed with water but no HTC was added. There were 6 replicates of each treatment in each soil type.

Table 4.1: General characteristics of two agricultural soils

Soil	SOC	TN	SIC	DOC	Microbial biomass	$\delta^{13}C$(SOC)	pH	Soil texture (%)		
	%	%	%	(µg-C/g soil)	(µg-C/g soil)	‰VPDB		Clay	Silt	Sand
Coarse	4.30	0.27	0.03	26.8	65.6	-27.8	6.09	6	44	50
Fine	2.06	0.22	0.74	30.4	106.3	-26.7	7.89	16	75	9

Mean (n=6); SOC, soil organic carbon; TN, total nitrogen; SIC, soil inorganic carbon

*All characters were analyzed in samples taken before starting the experiment.

4.2.3 Plant growth and plant biomass characteristics

Three replicates of each treatment were planted either with wheat (*Triticum aestivum*) or colza (*Brassica rapa*) according to typical German cropping practices. Identical seed numbers, determined according to common agricultural practice, were used on each plot, including in the large rings. The sowing of seeds was relatively late in the first season, with the result that the 2011 growing seasons was shorter than the 2012 season. In 2011, crops were sown 1 week after hydrochar application and the first harvest took place in the first week of October. In the next cropping season, crops were rotated as they would be in agricultural practice, i.e. treatments planted with wheat in first season were sown with colza and vice versa. The harvest of the winter crops occurred in the first week of July 2012. During each harvest we measured above ground plant height and total biomass after drying at 70°C for each treatment ring.

4.2.4 Soil analysis

Soil samples were analysed three times, at the start of the experiment (June 2011, right after the establishment of treatments), after harvesting the first crop (October 2011) and at the end of the experiment (July 2012). Each soil sample was homogenized and divided into two aliquots, the first was used to determine extractable DOC and microbial biomass, while the second was air dried at 40°C and used for further soil analysis. Total soil C content was analyzed on ball-milled subsamples (time 4 min) by an elemental analyzer at 1150°C (vario Max CN, Elementar Analysensysteme GmbH, Hanau, Germany). Organic C concentration was determined by calculating the difference between elemental analyses of the total C concentration and soil inorganic C concentration (Steinbeiss *et al*. 2008). Extractable dissolved organic carbon (DOC) and soil microbial biomass were determined by extraction using 0.05 M K_2SO_4 without and with chloroform fumigation (Brookers *et al*. 2007; Karsten *et al*. 2007) and analyzed with "high TOC" analyzer (Elementar Analysensysteme GmbH, Hanau, Germany).

The ^{13}C content of SOC was measured by coupling an elemental analyzer (EA 1100, CE Instruments, Milano, Italy) to an isotope ratio mass spectrometer (Delta^{Plus}XL, Thermo Finnigan MAT Bremen, Germany). Values were reported as $\delta^{13}C$ in per mill (‰) calibrated relative to the VPDB reference standard using NBS19 (Werner and Brand 2001), and represent repeated measurements with a standard deviation of less than 0.3‰. To avoid inorganic C impact on $\delta^{13}C$

values, samples were pre-treated with week acid, 6% H_2SO_3 (Bisutti *et al*. 2004; Steinbeiss *et al*. 2009).

4.2.5 Soil leachate collection and analysis

Soil leachate was collected using borosilicate glass suction plates (thickness 9 mm, diameter 90 mm, pore size 1 µm; UMS, Germany) at 15 cm depth in all treatments. A starting vacuum of 200 mbar was applied to suck soil solution into 2-L glass flasks. Sampling was carried out biweekly throughout the experimental period June 2011-July 2012 however, during dry period soil moisture was too low for collecting soil leachate.

After volume measurement, the collected leachate samples were divided into two aliquots. One aliquot was used to measure DOC and DIC and the other was freeze dried, after acid treatment to remove DIC, and subsequently combusted for $\delta^{13}C$ measurement (Delta^{Plus}XL, Thermo Finnigan MAT Bremen, Germany). When only a small volume of soil leachate was collected, only carbon concentrations were measured.

4.2.6 Gas sampling and analysis

Soil respiration was measured using a closed chamber technique (LiCOR 6400-09, Li-COR, Nebraska, USA) and all measurements took place during the day, between 9-12am, except in the winter season, when measurements took all day due to very low respiration rates. To collect gas samples for determining $\delta^{13}C$ of respired CO_2, a closed chamber equipped with a fan to mix air was placed on the small collars installed within each treatment. Three minutes after placing the chamber, 50 ml of headspace air was removed and used to flush and fill a 12 ml glass vial that was previously flushed with N_2 to remove any CO_2 from the vial. Background air samples were taken at 2 m height above the soil. All carbon dioxide concentration and $\delta^{13}C$ values were determined on a Gasbench II (Finnigan MAT, Bremen, Germany), equipped with a CTC PAL-80 auto sampler (CTC Analytics AG, Zwingen, Switzerland) that was connected to a continuous-flow isotope ratio mass spectrometer (IRMS, Finnigan MAT DeltaPlusXL, Bremen, Germany). This method was highly precise with a standard variation of 0.05‰ (Knohl et al. 2004).

Fluxes and the $\delta^{13}C$ values of respired CO_2 were mostly measured once per week initially and later fortnightly except for three months during the winter (Dec 2011, Jan, and Feb 2012) when fluxes were measured monthly. In winter, sampling time was also extended to get minimum difference of 20 ppm (CO_2) between soil air and back ground.

4.2.7 Calculations and Statistics

To calculate $\delta^{13}CO_2$ value of respired C from each treatment, the equation from Miller and Tans (2003) was applied (Miller and Tans 2003):

$$(\delta_{obs} * C_{obs}) - (\delta_{bg} * C_{bg}) = \delta_t(C_{obs} - C_{bg}) \quad (1)$$

Where, C and δ refers to CO_2 and $\delta^{13}C$ respectively and subscripts obs, bg and t refer to observed, background and treatment values.

To determine the contribution of hydrochar to respired C, a two component mixing model was used (Balesdent and Mariotti 1996; Gleixner et al. 2002). In this experiment SOC has a $\delta^{13}C$ signature reflecting C3 plants, while hydrochar has a signature reflecting the C4 origin of corn used in production.

$$\delta^{13}C_{(mixture)} = (f_{char} \times \delta^{13}C_{char}) + (f_{soc} \times \delta^{13}C_{soc}) \quad (2)$$

since: $f_{soc} + f_{char} = 1$

The fraction of hydrochar in respired carbon (*f*char) was calculated as:

$$f_{char} = (\delta^{13}CO_{2\ treatment} - \delta^{13}CO_{2\ control})/(\delta^{13}CO_{2\ char} - \delta^{13}CO_{2\ control}) \quad (3)$$

Where, $\delta^{13}CO_{2\ control}$ = unamended and $\delta^{13}CO_{2\ treatment}$ = amended hydrochar

To derive the $\delta^{13}CO_{2char}$ value, hydrochar was incubated at room temperature and the $\delta^{13}C$ value of evolved CO_2 was measured. This $\delta^{13}C$ value equaled that of the initial char to ±1‰ (Malghani *et al*. 2013). We implicitly assume that any fractionation associated with mineralization of SOC was the same with and without hydrochar amendment (Steinbeiss *et al*. 2009).

The presence of two C pools was assumed to determine the size and turnover rate of two C pools in hydrochar-C. Based on amount of hydrochar C remaining in soil, a double-exponential equation was used (Johnson et al. 2007),

$$C_t = C_a(e^{-k_a t}) + C_b(e^{-k_b t}) \quad (4)$$

Where, C_t is fraction of hydrochar remaining in soil at any given time (t), C_a and C_b represent fractions of fast and slow pool respectively. k_a and k_b represent decomposition or decay rates (day^{-1}) of fast and slow C fractions. To determine half-life of fast and slow pools, decomposition rates k_a and k_b were used in following equation;

$$t_{1/2} = \frac{Ln(2)}{k_i} \quad (5)$$

To determine the fraction of carbon derived from hydrochar in soil leachate and bulk soil C, we used two pool mixing model:

$$(fchar) = \frac{\delta^{13}C_{DOC\ treatment} - \delta^{13}C_{DOC\ control}}{\delta^{13}Cchar - \delta^{13}C_{DOC\ control}} \quad (6)$$

Fraction of hydrochar-C in soil leachate determined at different interval was simply summed up to get cumulative amount of hydrochar-C loss via leaching. For missing data points where the collected volume was too low to measure $\delta^{13}C$ values of DOC, values were calculated by interpolating linearly between sampling events.

The contribution of hydrochar to bulk soil C was measured twice, after the first harvest (October 2011) and at the end of experiment (July 2012). Comparing these two time points allowed us to calculate the total hydrochar-C mineralized during the experiment:

$$fchar_{min} = fchar_{start\ (June2011)} - fchar_{end\ (Oct\ 2011\ or\ July\ 2012)} \quad (7)$$

The impact of hydrochar addition on native soil C (priming effect; PE) was also determined by comparing the SOC contribution to overall respiration in the treatment compared to the control. The PE was calculated only in coarse soil (see above) using following equation:

$$PE = f_{soc} * CO_{2\ (amended)} - CO_{2\ (control)} \quad (8)$$

All data were expressed as means of the six replicates ± the standard error. Significance of differences among/between treatments was determined using one way analysis of variance (ANOVA). This was followed by a post-hoc test (Tukey, $\alpha = 0.05$). All statistical analyses were carried out using SPSS (PASW statistics-18) and graphs were prepared in SigmaPlot (Version 12.5) or MS-Excel 2010.

4.3 Results

4.3.1 Respiration and leaching losses of hydrochar-C

Rates of soil respiration in control treatments were slightly lower in fine soils, averaging 1.72 g-$Cm^{-2}day^{-1}$ compared to 2.26 g-$Cm^{-2}day^{-1}$ in coarse soils (Fig. 4.1). Soil respiration rates increased immediately after HTC amendment and remained significantly ($p<0.05$) higher for the next 12 weeks in the coarse soil ($p<0.05$; Figure 4.1), though less consistently in the fine soil (Fig. 4.1A). After these initial 12 weeks, HTC-amended and control soils emitted CO_2 at the same rate.

The CO_2 respired from hydrochar amended coarse soil remained significantly ($p<0.01$) enriched in ^{13}C from the start to end of the experiment (June 2011-July 2012) except during the month of May 2012 (Fig. 4.2). The $\delta^{13}CO_2$ values of respired C in the hydrochar treatment reached close to those of the hydrochar-C during first month of experiment and then gradually decreased, although remaining significantly higher than coarse control treatment.

In coarse soil, the main route of hydrochar-C loss was soil respiration as the proportion of hydrochar-C calculated in cumulative CO_2 emissions was equal to 37±2 % of the initial hydrochar- C concentrations (Fig 3A). While, relatively small proportion of hydrochar-C was recorded in soil leachate of comprising only 5% of the total added hydrochar C (Fig 4.3B). For the fine soil, we could not use the isotopes for respiration partitioning due to unknown contribution of carbonates, approximately 3.5% of the hydrochar-C was lost via soil leachate (Fig 4.3B).

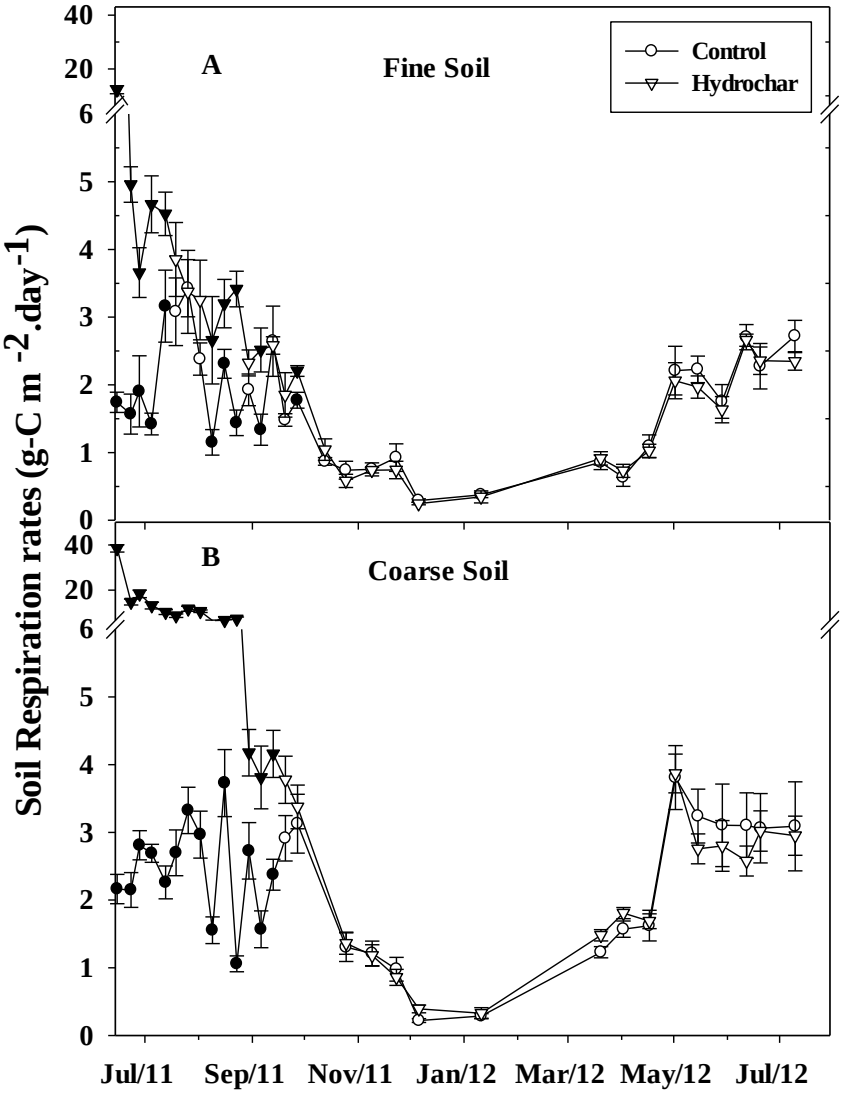

Figure 4.1: Heterotrophic soil respiration rates measured using close chamber technique during one year of field experiment (A) Fine soil (B) Coarse soil. Each measurement point represents mean respiration rate and vertical bars represent standard error (n=6). Filled symbols shows statistical significance (Tukey, $\alpha = 0.05$).

4.3.2 Priming effects of hydrochar on native soil carbon

The two phase of hydrochar decomposition showed contrasting effect on native soil carbon mineralization. A positive priming, i.e. higher CO_2 fluxes derived from native soil C in the amended soil compared to the control, occurred in the coarse soil, during a period following the initial pulse of respiration after hydrochar addition (which was nearly 100% derived from the hydrochar-C) and lasting through the subsequent 3-4 months. While, at 2nd phase of hydrochar mineralization, the control treatment of coarse soil emitted more soil C than the hydrochar treatment showing negative priming (Fig 4.2B).

The net effect of hydrochar amendment on native soil carbon was measured from bulk soil carbon data which showed a protective impact of hydrochar on native soil carbon. The SOC content of hydrochar treatments were considerably higher than control treatments of both soil types (Table 4.2) but this impact was statistically significant only in fine soil (Table 4.2).

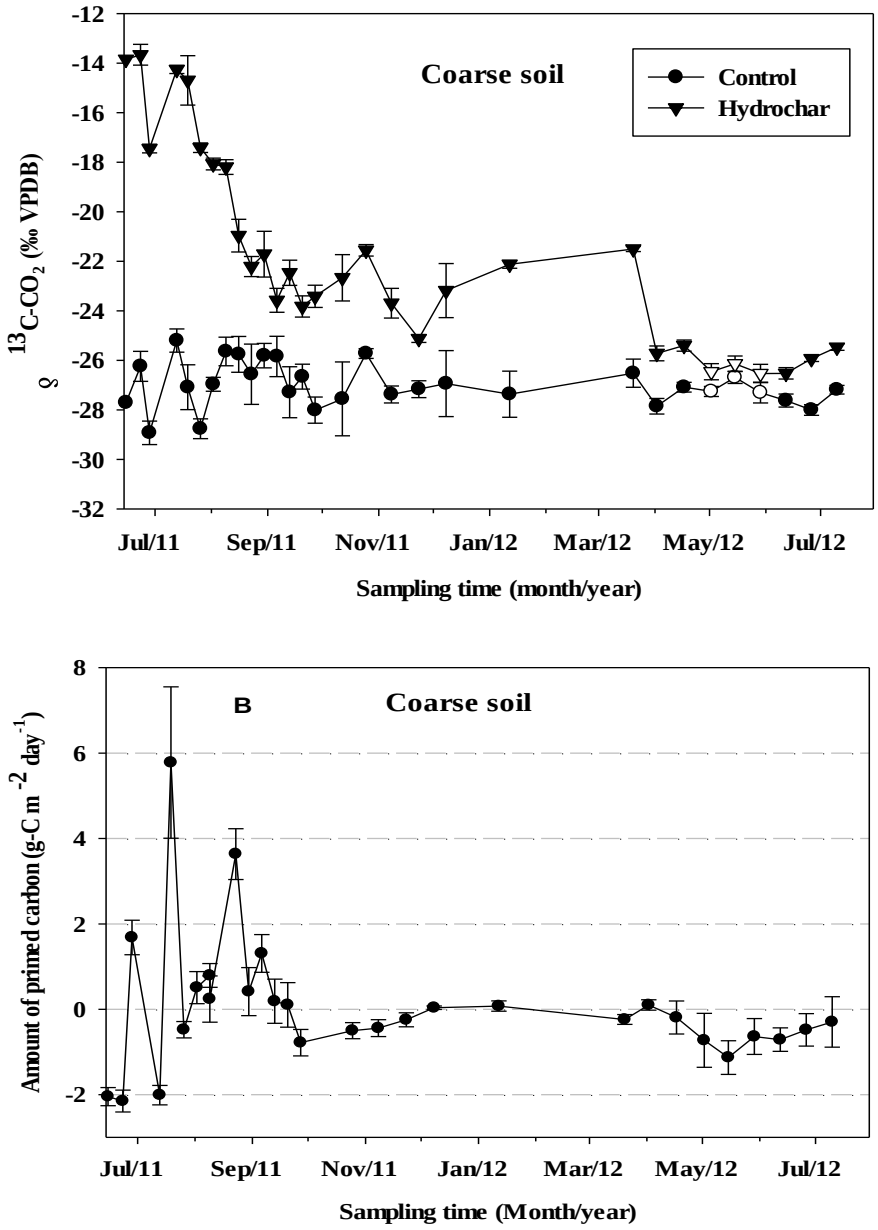

Figure 4.2: (A) Stable isotope ratio ($\delta^{13}CO_2$) of respired C from coarse soil calculated based on Miller and Tans 2003 model. Unfilled symbols represents statistical insignificance (Tukey, α = 0.05). (B) Amount of native SOC emitted or preserved due to positive or negative priming of hydrochar in coarse soil. Vertical bars represent standard error (n=6).

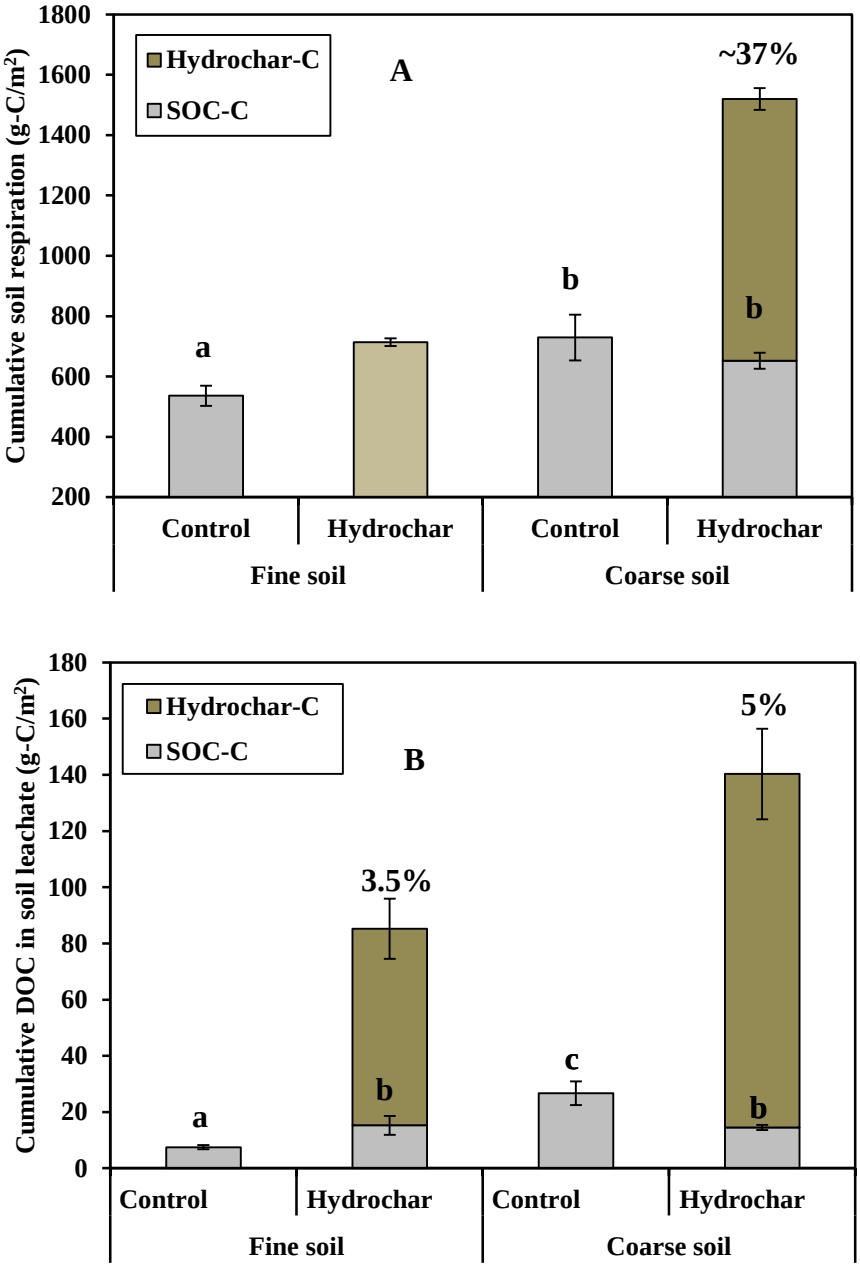

Figure 4.3: Cumulative amount of C loss via (A) soil respiration (B) DOC in soil leachate. In soil leachate, moving average of ($\delta^{13}C$) for two measurement dates was used for missing values. While, for cumulative soil respiration rates, values between measurement dates were calculated by simple mathematical integration. Fraction of hydrochar and native soil C were calculated using two pool C model. Different letter represent statistical significance in amount of soil derived C among treatments (Tukey α=0.05).

4.3.3 Stability of hydrochar in different textured soils

Calculation of remaining hydrochar based on soil respiration have two major drawbacks, 1) hydrochar-C loss via soil leachate is not considered; 2) interpolations based on linear regression between two measured values may over/under estimate the hydrochar-C losses. To overcome this issue we also calculated the remaining hydrochar-C using the isotopic mass balance of bulk soil (Fig. 4.4). Hydrochar-C sharply decreased during first 60 days of the experiment and then with a much smaller rate in the following 340 days (Fig. 4.4). The double exponential decay fractions (Eq. 4) suggested that almost 31% of hydrochar-C belong to easily degradable C. The half-lives of fast and passive C pools were approximately 0.08 and 19 years respectively (Fig. 4.4).

Losses of hydrochar-C based on the mass balance of soil C and ^{13}C agreed in general with the measurements of hydrochar-C lost (Fig 4.5). Initially hydrochar-C was lost rapidly, with 21±8% lost in coarse and 14±3% in fine soils between 15 June 2011 to 4 Oct. 2011 (Fig 4.5). Subsequent loss rates slowed in both soils (Fig 5). Variability among replicates was large in both types of soils, with no significant difference in overall loss between fine and coarse textured soils. A range of 48-77% of the originally added hydrochar C remained in individual collars after 1 year of the field experiment.

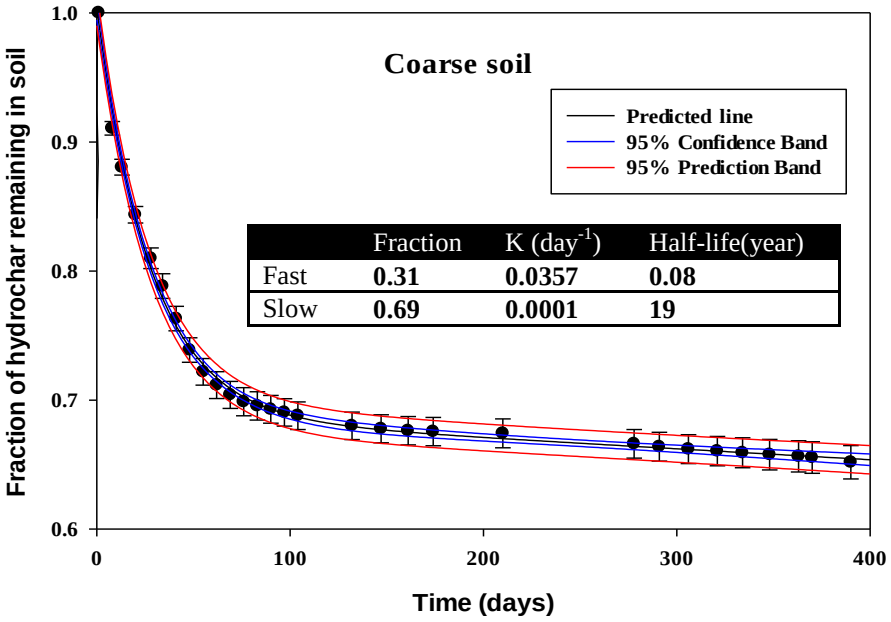

Figure 4.4: Fraction of hydrochar-C remaining in soil based on hydrochar fraction in cumulative CO_2 emissions and initial hydrochar-C input. Vertical bars are SE, n = 6. Decomposition parameters presented as table within the figure were calculated using double exponential equation: $Ct = C_a \exp^{(-kat)} + (C_b) \exp^{(-kbt)}$, where k_a is the decomposition rate for Ca, which represents the rapidly decomposing or fast pool, kb is the decomposition rate of C_b, which represents the slowly decomposing hydrochar-C, and C_a and C_b are the fractions of fast and slow pools of hydrochar remaining in the soil.

4.3.4 Impact of hydrochar on soil characteristics

Hydrochar addition resulted in considerable changes in the physical, chemical and biological properties for both amended soils. Soil moisture was the main soil physical property that differed significantly among treatments, mostly due to the fact that the hydrochar is known to be hydrophilic. As a result, the hydrochar-amended rings had significantly (p<0.01) higher moisture, persisting for more than 40 weeks in the fine soil (Fig. 4.6A), but with more variable effects in the coarse soil (Fig. 4.6B).

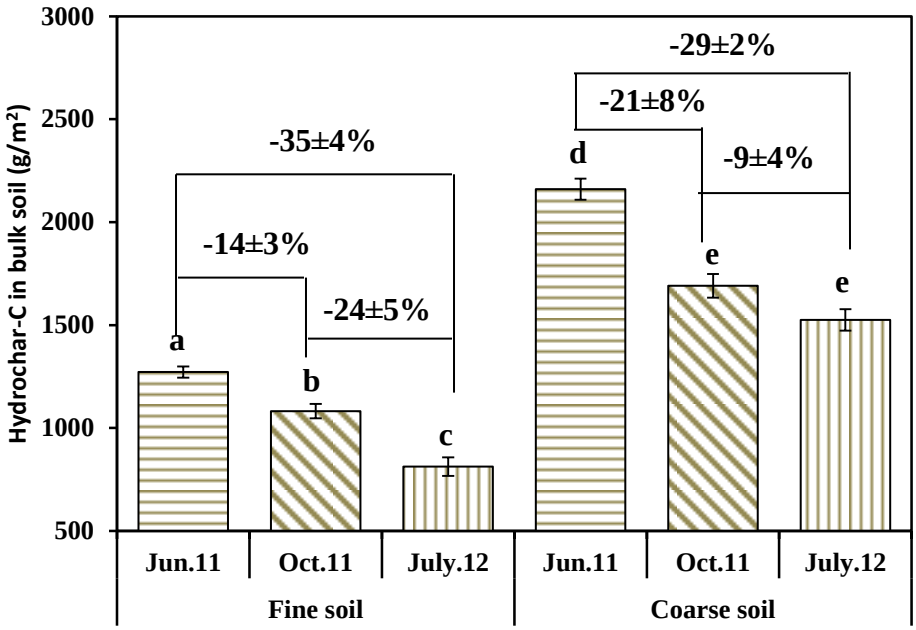

Figure 4.5: Amount of hydrochar C present in bulk soil at different time intervals, initial concentration at start of experiment, after 3-4 months and after 1 year of experiment. Amount of hydrochar C was calculated by exploiting bulk SOC amount and $\delta^{13}C$. Different letter represent statistical significance among treatments (Tukey α=0.05).

Microbial biomass, DOC and TN were significantly higher (p<0.05) in hydrochar treatments of both soils compared to their respective controls (Table 4.2) even after one year of hydrochar application. The impact of hydrochar on soil pH varied with soil type, with significantly higher pH (p<0.05) in the more acidic coarse soil compared to its control at the end of experiment. In contrast, the carbonate-containing fine soil amended with hydrochar had lower pH relative to its control but this difference was not significant (p>0.05). Microbial biomass and total N content were considerably higher (p<0.05) in the fine soil than in the coarse soil though DOC concentration in K_2SO_4 extracts was similar in both soils (Table 2). The differences between unamended and amended soils were larger than the differences between the two soil types or two different cropping schemes.

Table 4.2: Differences in general characteristics of two contrasting soils with or without hydrochar, analyzed after 1 year of field trial (July 2012).

Soil	Treatment	SOC (%)	TN (%)	TIC (%)	(µg-C/g soil) MB	DOC	pH	δ^{13}C(SOC) ‰VPDB
Coarse	Control	4.40	0.27	0.03	316.8±17	24.6±1.8	6.09	-27.7±0.04
	Hydrochar	†4.76	0.32***	0.03	440.4±32**	57.1±2.1***	6.37*	-26.1±0.07***
Fine	Control	2.01	0.22	0.82	601.7±41	20.1±2.2	7.88	-26.7±0.04
	Hydrochar	†2.32***	0.26***	0.95***	804.6±56*	38.9±5.01**	7.82	-24.8±0.12***

*Tukey (α 0.05, 0.01, 0.001), mean ±standard error (n=6) SOC, soil organic carbon; TN, total nitrogen; TIC, Total inorganic carbon; DOC, dissolved organic carbon; MB, microbial biomass, †represents proportion of native soil carbon

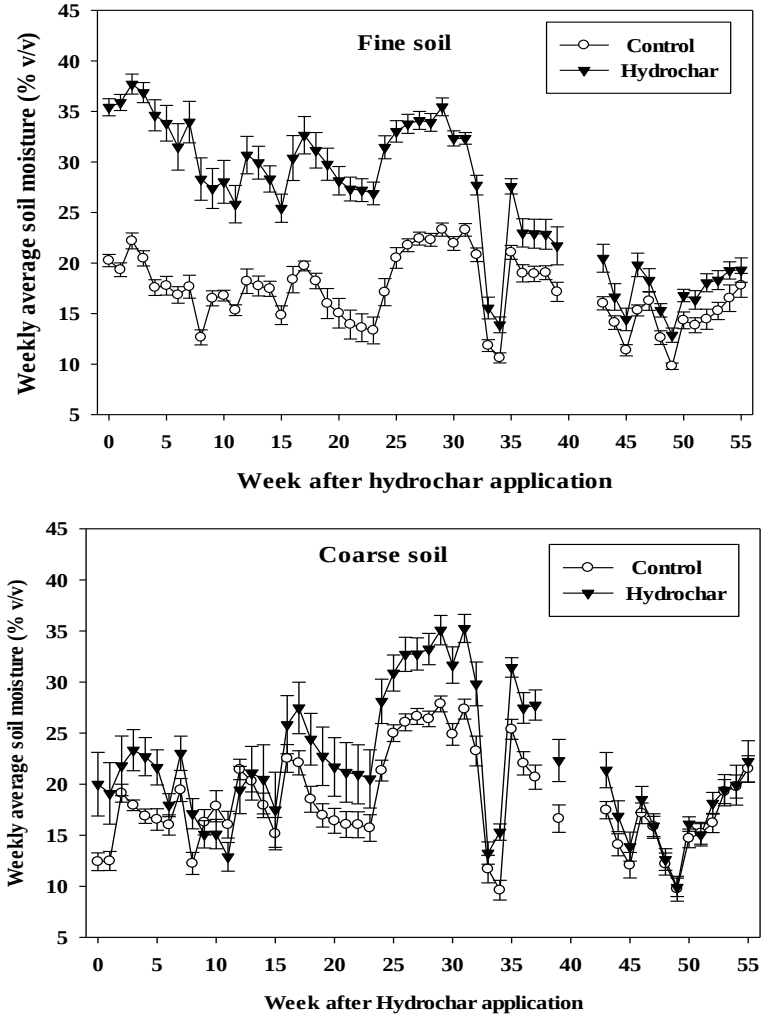

Figure 4.6: Weekly mean soil moisture contents observed at 10 cm depth during complete 1 year of field trial (A) Fine soil (B) Coarse soil. Data for missing dates was lost due to malfunctioning of logger. Each point represents Mean±S.E (n=6).

4.3.5 Impact of hydrochar treatment on plant performance

A negative impact of hydrochar amendment on plants growth was recorded during the first cropping season on both colza and wheat (Fig 4.7A). This impact was highly significant (p<0.01) for the colza crop, while the wheat crop had less biomass only in the coarse soil (p<0.05). In the second crop, sown in winter 2011-12, no differences in crop height or biomass were recorded in the amended plots (Fig 4.7B). In control plots, the initial crops were had less biomass in the first season due to the late sowing of the seed and relatively shorter growth period (105 days), compared to the second crop growth season (~250 days).

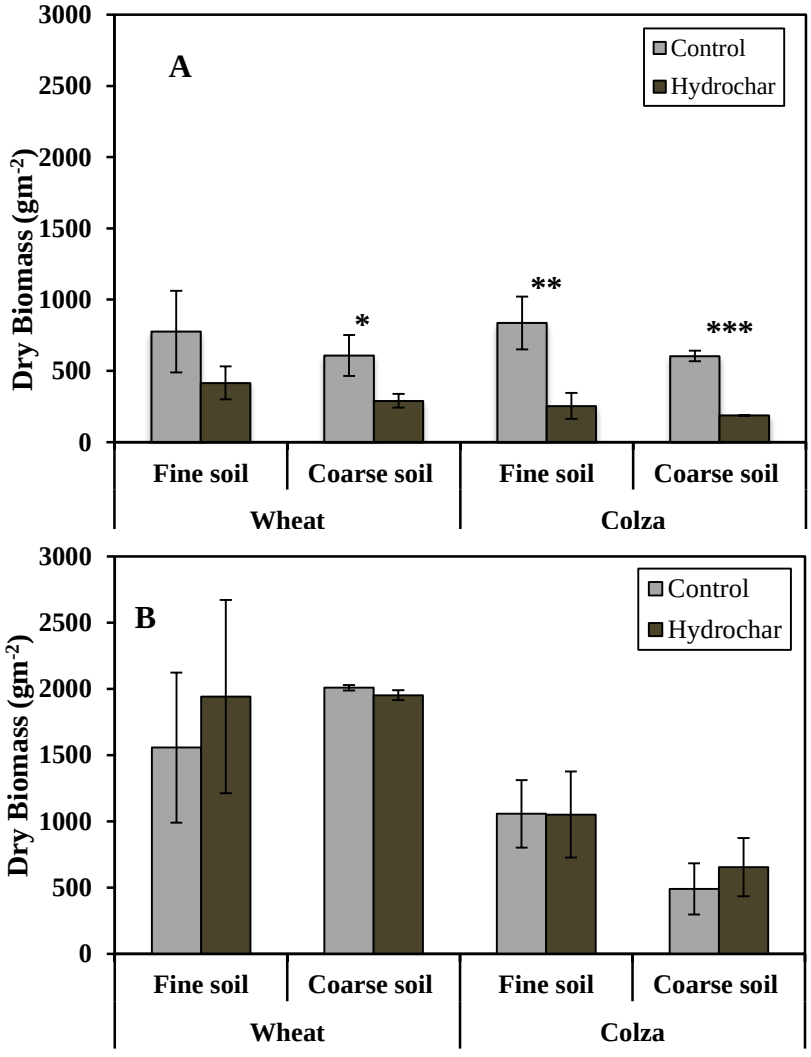

Figure 4.7: Dry biomass (gm^{-2}) of wheat and colza crops after drying at 70°C measured after (A) First cropping season (B) second cropping season. Symbol on top of the column represents statistical significance between control and hydrochar treatment of each soil type. Mean±S.E (n=3)

4.4 Discussion

4.4.1 Carbon sequestration potential of hydrochar

The C sequestration potential of hydrochar includes all direct and indirect effects of hydrochar on soil properties and C cycling processes. In this study, we focused on two main points: first, on the stability of hydrochar C itself and second, on the impact of hydrochar on the stability of native soil C. Hydrochar has a number of properties in that make it very reactive in soils, such as the presence of large amounts of oxygen-containing groups (Fuertes *et al*. 2010; Sevilla *et al*., 2011). Moreover, 15-30% of the biomass-derived C in hydrochar is in dissolved form (Ramke *et al*., 2009) and this liquid phase of hydrochar is rich in organic acids, especially formic, lactic acid and sugars like glucose/xylose and arabinose (Hoekman *et al*., 2011). These properties or characteristics indicate high degradability of a large portion of hydrochar-C (O'Toole *et al*., 2013; Malghani *et al*., 2013), and generally hydrochars are not recommended as amendments for soil C sequestration (Schimmelpfennig and Glaser 2012).

Thermo-gravimetric analysis of the hydrochar used in this study indicated that ~50% of the C was stable at low temperature, with the other half was comprised of aliphatic or thermally instable compounds (Malghani *et al* 2013). The presence of a large labile C pool in hydrochar could be the source of C lost rapidly as increased respiration and DOC losses (Fig 4.1 & 4.3). Within 3 months after application, approximately17±5% and 24±4% of the applied hydrochar C was lost in fine and coarse soils respectively (Fig.4.5). Initially (i.e. in the first 3 months) the rate of hydrochar mineralization was lower in fine soil, possibly due to higher water content (Fig 4.6) and improved aggregation ability due to promotion of AM fungi growth (Rillig *et al*., 2010; George *et al*., 2012). However, 1 year after its application, there was no difference in the amount of hydrochar-C loss between the two soil types (Fig.4.5), suggesting that composition of the added C source may exert a stronger influence on its fate than soil type as seen in similarly short-term incubation studies (Bai *et al*., 2013; Malghani *et al*., 2013; Naisse *et al*., 2014) but further studies with large variety of soil types may allow to conclude this effect. Approximately two thirds of the added hydrochar-C remained in both coarse and fine soils even 1 year after its application (Fig. 5), although the stable isotopic signatures of respired CO_2 and DOC in soil leachate showed significant contributions from hydrochar-C (Fig. 4.2 & A3.2) which were otherwise not detectable as differences in bulk C fluxes (Fig. 4.1).

All parameters regarding decomposition of hydrochar-C showed two phases, initially fast losses followed by slower losses. Decomposition kinetics of hydrochar-C was determined from nonlinear regression of the fraction of hydrochar-C remaining in coarse soil with time (Fig. 4.4). The inferred half-life (~19 years) of the passive component of hydrochar-C determined in our study was similar to that determined by incubation studies (Qayyum *et al*., 2012), highlighting similar behavior of hydrochar materials in contrasting experimental set ups. However, expected decadal time stability of passive pool of hydrochar is much less than pyrochar which could probably persist for centuries (Kuzyakov *et al*., 2014).

Another important factor associated with char or biomass amendment is the effect of its addition on the decomposition rate of native soil C (Keith *et al*., 2011). Priming is known as the short term acceleration or decline of native C decomposition associated with the addition of a readily decomposable substrate (Fontaine *et al*., 2003; Kuzyakov 2010). As discussed above, hydrochars

generally contain a large fraction of labile or easily decomposable compounds, and we observed both positive and negative priming effects in the amended coarse soil (Fig 4.2B). Positive priming was observed in the first three months, when a major fraction of the hydrochar-C was respired, and is often associated with the availability of rapidly decomposable substrate (Fig. 4.1 & 4.4). At second phase of experiment when hydrochar decomposition rates were slow, opposite effect on native SOC was evident (Fig 4.2). A protective impact of hydrochar C was prominent in bulk soil C results, amount of soil related C was considerably high in hydrochar treated plots, fine and coarse soils had 13.8±1.4% (n=4, two outliers were not included in calculations) and 8.8±3.4% (n=5) more native soil C than their respective controls (Table 4.2). These results indicate that a positive priming effect of hydrochar as proposed by short term incubation studies (Steinbeiss *et al*., 2009; Kammann *et al*., 2012) may not persistent for long term and soon after decomposition of labile pool, hydrochar could play a protective role. This positive role of hydrochar could be related with enhanced aggregation in hydrochar treated soils, due to promotion of fungal growth by hydrochar material (Rillig *et al*., 2010; George *et al*., 2012).

4.4.2 Potential changes in soil properties caused by hydrochar

Hydrochars are unique in their physical and chemical characteristics depending on feedstock type and process conditions and one can expect that their addition would have considerable impact on soil properties. Hydrochars can increase soil water by enhancing soil porosity (Abel *et al*., 2013), aggregate formation (Rillig *et al*., 2010) and by changing soil tortuosity, large particles of char can block pores. Moreover, hydrochar particles are known for having more porosity due to their spherical shape and deformability and could hold water (Abel *et al*., 2013). We recorded significantly higher moisture contents in hydrochar amended treatments but these differences were time and soil dependent (Fig. 4.6). In this study no differences in bulk densities were recorded between amended and control soils of both coarse and fine soils (data not presented) and thus the observed increase in water content could be associated with hydrochar particle characteristics and changes in soil tortuosity/hydraulic conductivity in the fine soil. After 10 months, diminshed effects of amendment on soil water content may have been related to the loss of the labile pool of hydrochar-C or changes in the surface chemistry of hydrochar particles, though this requires further exploration.

Another unique property of hydrochars is their liming effect on soils in spite of their acidic nature (Rillig et al. 2010). The hydrochar production processes are sensitive to pH and generally, an acidic pH (<7) is a pre-requisite for the hydrothermal carbonization method (Meyer *et al*., 2011). Lower pH is achieved with addition of low strength acids (e.g. citric acid) (Hu *et al*., 2010; Cao *et al*., 2011) and usually hydrochar has a pH similar to the liquid added to biomass prior to carbonization (Liang *et al*., 2011). The hydrochar used in this study had pH of 4.75 but a significant increase in soil pH was recorded 1 year after its application. The pH of soil leachate of hydrochar and control treatments, collected after 1 week of hydrochar experiment were 8.03±.2 and 7.4±.2 respectively. This suggests that enhanced microbial reduction reactions may be the reason for the liming effect of hydrochars (Rillig *et al*., 2010), however it is still unknown what reactions are responsibel for such impact.

4.4.3 Initial negative impact of hydrochar on plant growth

Our observation of reduced plant height and dry biomass yield in the first crop after hydrochar amendment has also been reported in other studies (Busch *et al*., 2012, 2013; Gajic and Koch 2012). These studies proposed two explanations. The first involved the production of phytotoxic and/or volatile compounds from easily degradable portions of hydrochar that have a negative impact on seed germination rate (Busch *et al*., 2012). Jandl *et al* (2013) proposed that the phytotoxic compound could also be ethylene produced by degradation of long C-chain aliphatic compounds. In addition, hydrochar's surface adhered volatile organic acids such as lactic acid, formic acid and laevulic acid that may present in large amount in crude or fresh hydrochar (Hoekman *et al*., 2011) could also be responsible for low plants growth as later are known for their growth retardant functions. The second explanation is related to nutrients available to seeds that do successfully germinate (Bargmann *et al*., 2014). Hydrochar application may inhibit N-availability due to N-immobilization and ultimately could cause negative impact on plants (Gajic and Koch 2012; Bargmann *et al*., 2014). We did not measure changes in inorganic nitrogen (NO_3^-, NH_4^+) in this study but we observed significantly ($p<0.05$) higher TN content in hydrochar treated soils (Table 4.2). We observed no differences in N content of plant biomass harvested in the first cropping interval, so we assume that N related impacts were limited to the initial stages of plant growth. Moreover, the negative impact of hydrochar on plant growth was not observed during the 2nd cropping season, though we also did not observe fertilization effects of hydrochar that have been reported by some studies (Busch *et al*., 2012; Bargmann *et al*., 2014). Consequently, agricultural use of hydrochar must be carried out carefully and application management like the use of nitrogen fertilizer (Gajic and Koch 2012) or composting of hydrochar (Busch *et al*., 2013) should be performed.

4.5 Conclusion

The addition of hydrochar C in amounts initially sufficient to raise overall soil C content by 20 - 30% showed considerable effects on soil properties as well as on plant growth. Total organic C, TN, DOC, water content and microbial biomass were among the measures that increased as a result of hydrochar amendment, even one year following its application. Although hydrochar-C was initially lost very rapidly through decomposition and leaching, roughly two thirds of the added C remained in the soil after two cropping seasons. Interestingly, hydrochar protected native soil C from decomposition (negative priming). On the other hand, it initially had a negative impact on plant performance. We conclude that hydrothermal carbonization has a high potential for its proposed use of C sequestration for two reasons; first, this method is especially useful to produce carbonaceous products from unconventional sources like bio wastes or sewage sludge. Second, the passive fraction of hydrochar-C may persist in soil over three decades or more. For future work, there is need to standardize hydrothermal method to produce hydrochar specifically its use as soil amendment. There is scarcity of literature on fate of hydrochar material in field conditions; more work is needed using contrasting soil types.

CHAPTER 5

Chars produced by slow pyrolysis and hydrothermal carbonization vary in carbon sequestration potential and greenhouse gases emissions

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This study was published in *Soil Biology & Biochemistry* 62(0); 137-146

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Abstract

Bio-char, biomass that has been deliberately charred to slow its rate of decomposition, has been proposed as an amendment with the potential to sequester carbon and improve certain soil properties. Slow pyrolysis (temperature ≤500°C) and hydrothermal carbonization (low temperature, high pressure) are two efficient methods to produce bio-char with high yield and are applicable to a broad range of feedstocks. Chars made using slow pyrolysis (PC) and hydro thermal carbonization (HTC) of the same feedstock material (corn, C4) differed in physical appearance, chemical properties and decomposition behavior. We added these HTC and PC chars as amendments to three soils with C3-derived organic matter that differed in clay content, pH, and land use (managed spruce forest, unmanaged deciduous forest and agriculture), and compared their impacts on carbon sequestration and net greenhouse gas (CO_2, $^{13}CO_2$, N_2O and CH_4) emissions. HTC addition (1% w/w) significantly increased CO_2 emissions in all three soils ($p<0.001$), with much of the extra C derived from HTC decomposition. In contrast, PC addition (1% w/w) had almost no impact on deciduous forest soil and actually decreased CO_2 emission from the agricultural soil. HTC treatment resulted in increased CH_4 emission from all soils but reduced N_2O fluxes in the agricultural and spruce forest soils. PC amendment had no significant effect on CH_4 emission, and resulted in intermediate levels of N_2O emission (between control and HTC treatments). Although both HTC and PC chars were produced from the same feedstock, PC had markedly higher potential for carbon sequestration than HTC.

Key words: Dry and wet pyrolysis; $\delta^{13}C$; GHGs; laboratory incubation; carbon sequestration; bio-char; soil organic matter.

5.1 Introduction

The annual growth rate of atmospheric CO_2 was 1.9 ppm in the past decade (2000-2009), reflecting a continuing, large, imbalance between carbon (C) release to the atmosphere and removal by natural sinks (Peters *et al*., 2012). One proposed mitigation strategy is to enhance land C sinks by removing C from the atmosphere and storing it in a form that is stable over a long period of time. Soils provide a large global reservoir of C stabilized for decades to centuries (Schmidt *et al*., 2011) and therefore practices that increase soil C storage has received much attention. Recent attention has been to add charred biomass (bio-char), which has been demonstrated to persist in tropical 'anthroposols' for up to thousands of years, in order to store C in soils (Lehmann, 2007). Bio-char, resulting from pyrolysis of biomass that enriches overall carbon content and slows degradation, has been proposed as an amendment that can potentially sequester carbon and improve certain soil properties such as soil fertility (Sohi *et al*., 2010).

The concept of bio-char amendment was derived from the study of Amazonian dark earth soils, also known as anthroposols. These soils were managed by indigenous people living in the Amazon basin between 600 and 8700 years ago (Grossman *et al*., 2010), and are characterized by higher C content and greater microbial diversity compared to unamended adjacent soils with similar mineralogy (Grossman *et al*., 2010; Navarrete *et al*., 2010; O'Neill *et al*., 2009). The soils are also characterized by the presence of charred particles, suggesting that char lasts hundreds to thousands of years at these sites. Thus amendment with bio-char is widely hypothesized to increase carbon storage capacity, although this effect is largely unquantified and depends on many factors (Liang *et al*., 2010). One such factor is the method used for bio-char production. Large differences in the composition of bio-char produced using different methods can result in timescales for persistence in soils, ranging from millennia (Forbes, 2006; Liang *et al*., 2008) to decades (Steinbeiss *et al*., 2009).

Two thermal degradation processes, in the presence and absence of water, are most commonly used to carbonize biomass. Both methods efficiently produce large amounts of char, have high rates of carbon recovery, and can be applied to a broad range of feedstock. These properties make them optimal from a soil amendment point of view (Fuertes *et al*., 2010; Titirici *et al*., 2007).

The most efficient process for char production under dry conditions is slow pyrolysis. This method derives from methods for charcoal production used by mankind for millennia (Ogawa and Okimori, 2010). Slow pyrolysis uses moderate heating rates over a long period of time, and ultimately leads to 30–45% C yield as bio-char (Bruun *et al*., 2012). However, this process is not suitable for carbonization of most agricultural wastes due to the requirement for drying of the feedstock prior to and/or during the reaction.

In contrast, hydrothermal carbonization (HTC) makes use of a range of unconventional biomass feedstocks, such as sewage sludge, animal wastes and compost (Titirici *et al*., 2007), without the need for drying prior to char production. Although HTC was discovered in the early 20th century during studies of natural coal formation, to date there are only a few studies about its potential use for C sequestration (Funke and Ziegler, 2010; Rillig *et al*., 2010; Schimmelpfennig and Glaser, 2012). In hydrothermal char production processes, the wet biomass mixture is heated to

temperatures of up to 220-240°C in a high-pressure reactor. Steam pressures reach up to 20 bar, and very little gas (1–5%) is generated, so that most organics remain either in dissolved form or transform into brown coal (Libra *et al*., 2010). Various carbonaceous materials with different sizes, shapes, and surface functional groups are synthesized during HTC but a large proportion of the initial carbon (40-54%) remains in soluble form (Hu *et al*., 2010). Among the advantages of the HTC process is the use of non-traditional feedstock that could provide a continuous feedstock stream for this process and less carbon losses during the char generations.

The net greenhouse gas effects of char amendment depend not only on the potential to sequester atmospheric CO_2, but also the changes in the overall consumption or emission of methane and nitrous oxide. Biophysical processes responsible for CH_4 and N_2O emissions from soils are considerably altered with incorporation of biomass, fertilizer or bio-char into soils. A large proportion of published literature agrees that pyrolysis char suppress N_2O emissions from soil majorly due to its effect on soil moisture, soil aeration and NO_3^- runoff/leaching (Kammann *et al*., 2012; Taghizadeh-Toosi *et al*., 2011; van Zwieten *et al*., 2010). The only reported results about HTC char showed initial decrease in N_2O emissions but this effect was not observed on later stages of field experiment (Kammann *et al*., 2012) In contrast, the reported impacts of pyrolysis char on CH_4 fluxes are inconsistent, with positive (Feng *et al*., 2012; Yu *et al*., 2012), negative (Spokas and Reicosky, 2009; Zhang *et al*., 2010), or neutral influence on emissions (Kammann *et al*., 2012; Yoo and Kang, 2012). These effects were highly moisture dependent, and a full explanation of the impact of HTC on CH_4 fluxes is lacking.

Chars made from slow pyrolysis and HTC differ in physical appearance and chemical properties (Fuertes *et al*., 2010). The objective of this study was to evaluate the overall greenhouse effect of amendment with the two types of char by continuous monitoring of trace gases emissions from three different soils. The two chars used were produced from the same corn-based feedstock. We used differences in the natural abundance of $\delta^{13}C$ to track bio-char carbon (reflecting C4 origin of corn) from the organic matter in the amended soil, which reflected a pure C3 origin. In addition to CO_2, we monitored the effect of soil amendment on the production of CH_4 and N_2O. To our knowledge, this is the first study to compare the net greenhouse gas effect of soil amendment with slow pyrolysis and HTC chars.

5.2 Materials and Methods

5.2.1 Soil sampling and characterization

Two forests soils (Cambisols) and one agricultural soil were collected from three different locations within the Thüringen state in Germany: a deciduous forest (DF) within the Hainich National Park; a spruce forest (SF) near Ölknitz village; and an agricultural soil (AG) from research plots located near the Max-Planck Research Institute for Biogeochemistry in the city of Jena. Together, these soils span a broad range of vegetation types and soil properties, including soil texture, which ranged from clay loam (DF) to sandy loam (AG). At each site 5-6 subsamples of the upper 15 cm of mineral soil were collected. The SF and AG soils were processed at field moisture. The DF soil was very wet when sampled, and was dried at room temperature to 20% of gravitational moisture content prior to processing. Soils were first passed through a 4 mm

mesh size sieve to remove all plant material and large roots. Samples were then homogenized to produce a single, composite sample and stored at 4°C prior to incubation. Sub-samples of the homogenized soils were dried at 40°C for physical and chemical analyses. .

Soil water holding capacity was measured by the volumetric method using char free soils (Livingston and Topp, 2007). Soil carbon and nitrogen concentrations were measured from ball-milled sub-samples by elemental analysis ("Vario Max", Elementar Analysensysteme GmbH, Hanau) before and after incubation. Organic carbon concentration was determined by calculating the difference between elemental analyses of the total carbon concentration and soil inorganic carbon concentration (Steinbeiss et al., 2008). Soil mineral N (NO_3^- and NH_4^+), dissolved organic carbon (DOC) and soil microbial biomass were determined by extraction before and after incubation (Karsten et al., 2007). For DOC analysis, 10 g of moist soil sample was suspended in 50 mL 0.05M K_2SO_4 and shaken on a low speed reciprocal shaker for 1 hr. Supernatant was filtered and analyzed ("high TOC" Elementar Analysensysteme GmbH, Hanau). Soil microbial biomass was determined by chloroform fumigation-extraction (Brookers et al., 2007). Unless otherwise stated, all measurements and are reported as the mean and standard variation of triplicate analyses.

The $\delta^{13}C$ of soil organic carbon was measured by a coupling an elemental analyzer to an isotope ratio mass spectrometer ("Bianca" Delta^{Plus}XL). Values are reported as $\delta^{13}C$ in per mill (‰) calibrated relative to the VPDB reference standard using NBS19 (Werner and Brand, 2001), and represent repeated measurements with a standard deviation of less than 0.3‰.

5.2.2 Bio-char production and characterization

Slow pyrolysis char (henceforth pyro-char, or PC) was produced from corn silage that was air dried (70°C) and ground to less than 4 mm. The ground silage was sealed in aluminum foil to avoid aeration, with a small hole on one side of the foil to let gaseous products out. Samples were heated from room temperature to 500°C at a rate of 10°C per min and held at 500°C for 2 hrs. After cooling, the resulting PC was passed through a 2 mm sieve and stored at 4°C until the incubation experiment.

Hydro-char (HTC) from the same corn silage was obtained commercially from the carbon solutions Company Ltd, Kleinmachnow Germany. The delivered material was slurry (10% solids). Before we used it as an amendment, this slurry was freeze-dried at -50°C and the resulting solid material was passed through a 2 mm sieve.

Elemental concentrations of C, N, and H in both types of char were measured from ball-milled subsamples using an elemental analyzer (VarioMax Elementar Analysensysteme GmbH, Hanau). Oxygen was estimated as follows: O = 100 - (C + H + N + ash) (all expressed in weight %). Thermo gravimetric (TG) curves (Mettler Toledo, TGA/SDTA851) were used to characterize the relative lability of the char materials. Volatiles were assumed to equal the mass lost between 105°C and 850°C in an N_2 atmosphere, and the mass lost at 850°C after introduction of O_2 was considered to be the stable fraction. Ash content was defined as the mass remaining after combustion in air. No inorganic C was detected in the chars. The $\delta^{13}C$ of both char and plant material was determined using same procedure as for soil samples.

5.2.3 Soil incubation

Prior to incubation, the approximatley15 kg of prepared soil sample from each soil was divided into three aliquots. The first aliquot was used as a control; the second received an amendment with PC (10 g/kg soil) and the third was amended with HTC (10 g/kg soil) and samples were homogenized to equally distribute the amendments. Each treatment (Control, +PC, +HTC) was then divided into four replicates of ~ 1 kg each and placed into incubation chambers (total 3 soils x 3 treatments x 4 replicates, or 36 chambers). The chambers used for incubation were constructed from PVC columns (10 cm diameter and 20 cm height).

Chambers were placed in a custom-built continuous flow incubation system, the details of which have been reported elsewhere (Thiessen *et al*., 2013). Briefly, this automated system continuously (~30 mL/min) flushes the chambers holding soil samples with CO_2-free synthetic air (20.5% O_2 and N_2). Fluxes of gases are determined from the concentration of gases in headspace air exiting the chambers and the rate of airflow. The outlet air sampling the head space of the column was connected to an automated multiport stream selection valve (Valco) that directed the airstream from different columns sequentially to an infrared carbon dioxide analyzer ("LI- 6262" LI-COR Biosciences Lincoln, USA). Other greenhouse gases and isotopes of CO_2 were sampled less frequently using flasks (see 2.4 below).

The chambers were installed in a temperature-controlled climate chamber, where we maintained temperatures at 20°C throughout the incubation period. The soil water content of each column was initially adjusted to equal 70% water holding capacity by adding water to the field moist (SF and AG) or pre-dried (DF) soils, using the previously determined water holding capacity for each soil without char amendment to determine field capacity. Addition of 1% pyrochar char resulted increase in WHC of DF, AG and SF soils by 2.89%, 1.65% and 2.43% respectively. Similarly HTC-amended DF, AG and SF soils have 1.51%, 1.47% and 1.77% higher WHC respectively than respective controls. But this increase in WHC of bio-char amended soils was not accounted during initial soil moisture adjustment. The continuous stream of synthetic air that flowed through the incubation columns dried the soils at a rate of (~1 g H_2O/d), This water was replaced by sprinkling the surface of the column with distilled water every 4th day to replace the measured mass loss from the column.

5.2.4 Gas sampling and analysis

Other gases, including $\delta^{13}C$-CO_2, CH_4 and N_2O, were measured from flasks collected from the air exiting the chambers. The gas samples were taken on days 1, 5, 10, 20, 25, 35, 45, 55, 70, 85 and 100 after starting the incubation. Gases were collected using either 2.3 L or 1 L glass flasks (flushed with synthetic air prior to sampling) equipped with two stopcocks and connected via a capillary to the soil columns. The exhaust gas of each flask was passed through water filled diffusion traps to prevent back diffusion of atmospheric air, and the air was subsequently dried using magnesium perchlorate before entering the flask. After allowing ~2 hours to flush the flasks with headspace air (~30 ml/min flow rate), the stopcocks were closed. Possible dilution from incomplete purging of synthetic air in the sampling flasks, especially the 2.3 L flasks, was corrected by comparing the CO_2 concentration in the flask with the simultaneously measured CO_2 measured by LiCOR. It was assumed that N_2O and CH_4 were diluted in the same ratio as CO_2 and no dilution correction was applied for $^{13}CO_2$ as there was no additional source of CO_2.

Flasks where this dilution correction exceeded 25% were discarded and were not used in calculations.

The analysis of N_2O and CH_4 were made using gas chromatography (Agilent technologies 6890, Santa Clara, USA) equipped with an electron capture detector (ECD), and a flame ionization detector (FID). The $\delta^{13}C$ of CO_2 was determined by stable isotope ratio mass spectrometry (Finnigan MAT 252IRMS). Both analyses were carried out in laboratories at MPI- BGC (Jordan and Brand, 2001; Rothe et al., 2003). Cumulative emissions of CH_4 and N_2O were calculated by interpolating linearly between sampling events (see below).

To observe impact of char on N_2O emissions, we performed an additional experiment on the AG soil. Approximately 20 mL of fertilizer solution ($(NH_4)_2SO_4$ with a concentration of 200 mg-N/L) was added to AG soil after 51 days of incubation.

5.2.5 Calculations and Statistics

The soils used in our incubations each had a long history of C3 vegetation inputs. In contrast, both PC and HTC were produced from corn silage, which is a C4 plant. We used two methods to quantify the contributions of HTC, PC and native soil organic matter to carbon mineralized during incubation period using the difference in ^{13}C from native SOC and amendment char. In both cases we used a two component mixing model to calculate the relative fraction of evolved CO_2 derived from bio-char (f_{char}) and from native soil organic matter (f_{soc}) in soil-bio-char mixtures (Balesdent and Mariotti, 1996; Gleixner *et al*., 2002):

$$\delta^{13}C \text{ (soil bio-char mixture)} = f\text{char}* \delta^{13}C\text{char} + f\text{som}*\delta^{13}C\text{som} \quad (5.1)$$

Since: $f\text{soc} + f\text{char} = 1$

$$f\text{char} = (\delta^{13}CO_{2\ \text{treatment}} - \delta^{13}CO_{2\ \text{control}}) / (\delta^{13}CO_{2\ \text{char}} - \delta^{13}CO_{2\ \text{control}}) \quad (5.2)$$

To derive $\delta^{13}C_{char}$, we assumed that the $\delta^{13}C$ of CO_2 derived from decomposition of the char equaled that of the bulk $\delta^{13}C$ of the respective PC or HTC char. We tested this assumption by incubating pure char materials and found that the $\delta^{13}C$ of CO_2 evolved equaled the $\delta^{13}C$ of the initial char to ±1‰ (data not shown). However, we note that when we incubated pyrochar in ambient air, it showed net absorption of CO_2; to obtain a $\delta^{13}CO_2$ value, we therefore incubated the PC in air that was initially CO_2-free.

Our first method used direct measurements of CO_2 evolved and its $^{13}CO_2$ signature over the period of the experiment to derive $\delta^{13}C_{treatment}$ and $\delta^{13}C_{control}$. The automated incubation system measured CO_2 fluxes 2 times per day for each chamber. To calculate cumulative CO_2 fluxes, we interpolated fluxes linearly between these very frequent sampling times. Soil respiration results from days 41-45 were lost due to malfunctioning of the data logger, and we filled this data gap with linear interpolation.

The $\delta^{13}CO_2$ was sampled less frequently than CO_2 fluxes. The cumulative $\delta^{13}C$ signature of the evolved CO_2 was obtained by multiplying the amount of CO_2 evolved between ^{13}C sampling events by the measured $\delta^{13}CO_2$ signature, summing over the entire period of the incubation, and

dividing by the cumulative total CO_2 produced. By using the ^{13}C signature of the cumulative CO_2 evolved in the control as the "SOC" end member, we implicitly assume that any fractionation associated with mineralization of SOC carbon is the same with and without char amendment (Steinbeiss et al., 2009).

The second method we used compared the amounts and ^{13}C signatures of the carbon remaining in the incubated samples at the end of the experiment ($C_{end,}\delta^{13}C_{end}$) with those at the beginning of the experiment ($C_{start,}\delta^{13}C_{start}$) to estimate the amount and ^{13}C signature of the C mineralized ($C_{min,}\delta^{13}C_{min}$):

$C_{min} = C_{start} - C_{end}$;

$\delta^{13}C_{min} = (C_{start}\delta^{13}C_{start} - C_{end}\delta^{13}C_{end})/ C_{min}$

We then use $\delta^{13}C_{min}$ calculated for treatment and control incubations as $\delta^{13}C_{treatment}$ and $\delta^{13}C_{control}$, respectively, in Equation (1) to estimate the fraction of organic matter and char mineralized in the incubations. We again implicitly assume that any fractionation associated with the mineralization of SOC will be the same in both control and amended soils, and that there is no isotopic fractionation on decomposition of the chars.

The two methods sometimes gave slightly different results, especially in terms of the significance when comparing control and amended incubations or soils. We have used the results using the method that yielded the greatest significance in the results and discussion, and also indicate where the two methods disagree. For example, a very small loss of char mass due to decomposition during the incubation will be difficult to detect by changes in mass and ^{13}C from beginning to end of the incubation (method 2), while it may be more sensitively measured in the relatively smaller amount of CO_2 evolved (method 1).

The amount of extra soil organic carbon released or suppressed as a result of the addition of char (primed carbon) was calculated as the difference between amounts of soil carbon respired from the pyrochar amended treatment (µg C/g dry soil) and soil carbon respired (µg C/g dry soil) in the control. For this calculation we report results using f_{char} calculating using both methods.

All data were expressed as means of the four replicate incubations ± the standard error. Significance of differences among/between treatments was determined using one way analysis of variance (ANOVA). This was followed by a post-hoc test (Tukey, $\alpha = 0.05$). All statistical analyses were carried out using SPSS (PASW statistics-18) and graphs were prepared in SigmaPlot (Version 10.0) or MS-Excel 2010.

5.3 Results

5.3.1 Soil characteristics

Although all soils sampled for this study were classified as *Cambisols*, they differed in physical, chemical and biological properties (Table 5.1). The soil sampled in spruce forest (SF) had the

highest sand content and lowest pH, with intermediate C content (4.59% by weight) compared to the other two soils. The deciduous forest (DF) soil had higher clay content and lower C content than the other soils (Gleixner, 2009; Tefs and Gleixner, 2012). The agricultural field soil (AG) had the highest overall pH and C content (Malik *et al*., 2012).

Microbial biomass, reported only for control soils, was highest in the DF soil (522.8±27.43 µg/g soil) followed by SF and AG soils (355.8±75.12 and 340.3±32.92 µg/g soil respectively). Inorganic carbon contents were very small in all cases: zero in the spruce forest, and 0.09 and 0.03% in the deciduous and agricultural soils, respectively. Dissolved organic carbon content was lowest in AG soil. The ^{13}C signature of all three soils confirmed that the major source of C in all sites was C3 plants and $\delta^{13}C$ values ranged between -26.51‰ to -28.02‰ (VPDB).

Table 5.1: Soils characteristics; DF (deciduous forest, Hainich National Park), SF (spruce forest, Olknitz), AG (agricultural soil, in Jena)

Soils	Textural class	(%) TOC	(%) TIC	TN	(ug/g) DOC	(ug/g) Microbial biomass	pH	$\delta^{13}C$ (‰VPDB)
DF	Silty clay	3.61	0.03	0.29	14.2±0.1	523±27.4	6.26	-26.51
SF	Sandy loam	4.59	ND	0.19	39.4±4.0	356±75.1	4.70	-28.02
AG	Silty loam	5.11	0.09	0.27	21.3±2.5	340±32.9	6.68	-27.91

Values represent means ± S.D (n=3) and are expressed on a dry weight basis.
TOC, total organic carbon, TIC, total inorganic carbon; TN, total nitrogen; DOC, dissolved organic carbon

5.3.2 Char characterization

Both char amendments used in this study were derived from the same feedstock (corn silage), with a $\delta^{13}C$ signature typical for C4 plants (Table 5.2). The PC (slow pyrolysis) and hydrothermal carbonization (HTC) chars produced differed in physical and chemical properties from each other as well as from the feedstock. Both types of bio-char had higher C and N concentrations and lower H and O concentrations than the feedstock. In particular, the PC had very low H:C and O:C atomic ratios compared to the HTC. The $\delta^{13}C$ signature of the char materials was depleted compared to the feedstock by ~0.5‰ (PC) to 1.3‰ (HTC). A large proportion of the HTC char was soluble, based on its high DOC content the fact that before freeze drying the HTC char was in the form of slurry with 10% (w/w) solid material. The HTC showed strong initial weight loss during thermogravimetry analysis (TGA) compared to PC (Fig. 5.1). Weight loss on heating followed the order feedstock>HTC>PC (Fig. 5.1). Ash content increased with the degree of charring; PC, HTC and corn had 11.39%, 21.35% and 3.07% of ash respectively.

Table 5.2: Characteristics of bio-chars and its feedstock; PC (slow pyrolysis char), HTC (Hydrothermal char) and Corn silage (feedstock for both types of chars)

	Chemical composition (wt %)					Atomic ratio		mg/g		(VPDB)
Char	C	H	O	N	Ash	O/C	H/C	DOC	pH	$\delta^{13}C$(‰)
PC	77.88	2.29	6.45	1.99	11.39	0.06	0.35	0.033	9.73	-13.11
HTC	51.63	5.70	19.42	1.90	21.35	0.28	1.33	12.99	4.15	-13.87
Corn S.	45.14	6.78	43.88	1.13	3.07	0.73	1.80	-	-	-12.69

Values represent means (n=3) and are expressed on a dry weight basis. C,carbon; H, hydrogen; O,oxygen; N, nitrogen. Corn S, corn silage

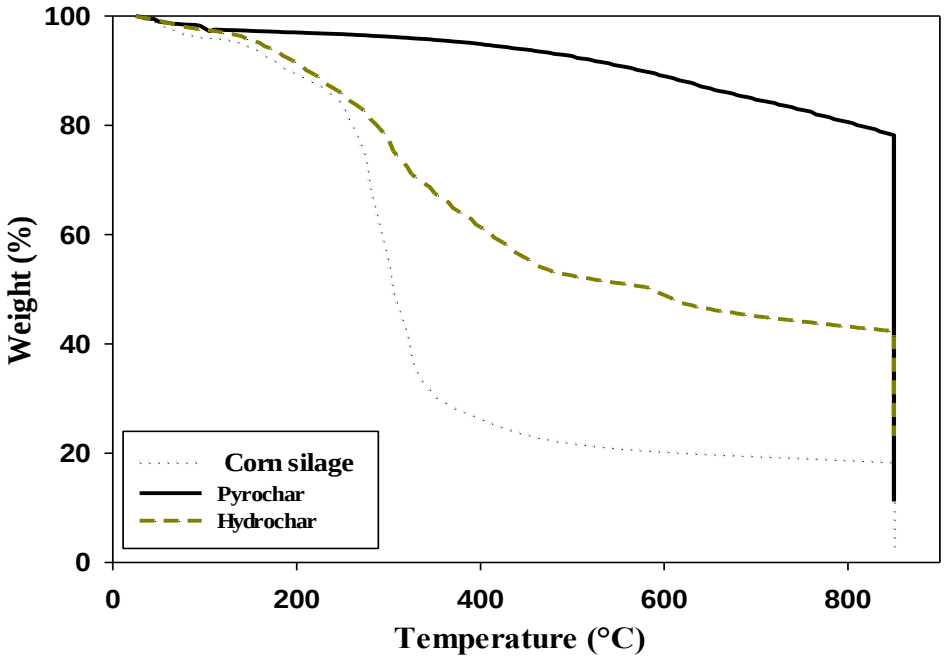

Figure 5.1: TG curves for feedstock and bio-char samples (Pyrochar (PC), slow pyrolysis char; Hydrochar (HTC), hydrothermal char)

5.3.3 Soil respiration

During the first 50 days of incubation, the hydro-char (HTC) amendment resulted in higher production of CO_2 (p<0.001) compared to control or PC treatments. This effect was most pronounced during the first week of incubation where the rates of CO_2 production were 66.8± 2.8, 83.4± 15.6 and 62.0± 14.9 µg-C/g soil in SF, DF and AG soils, respectively (Fig.5.2 A, B and C). During the last 7 weeks of incubation, the increased respiration from HTC treated soils was statistically significant only in forest soils (DF and SF; p≤0.01). Amendment with slow pyrolysis (PC) char produced results that differed by soil type. The PC amended SF soil respired more than the control and this increase was significant during the first 50 days of incubation

(p<0.01), However, no increase was observed in DF soils, and overall caused a decline in the evolution of CO_2 from AG soils.

Overall, the CO_2 output over 105 days of incubation was significantly higher in all HTC amended soils (2.66, 2.11 and 1.89 times higher than the respective controls in AG, DF, and SF soil (Fig. 5.4A). PC amendment increased CO_2 production in the SF soil specifically during the initial 7 weeks ($p \leq 0.01$), but this PC effect was not observed in another forest soil. Surprisingly, PC addition resulted in lower cumulative CO_2 production in the AG soil compared to unamended soil.

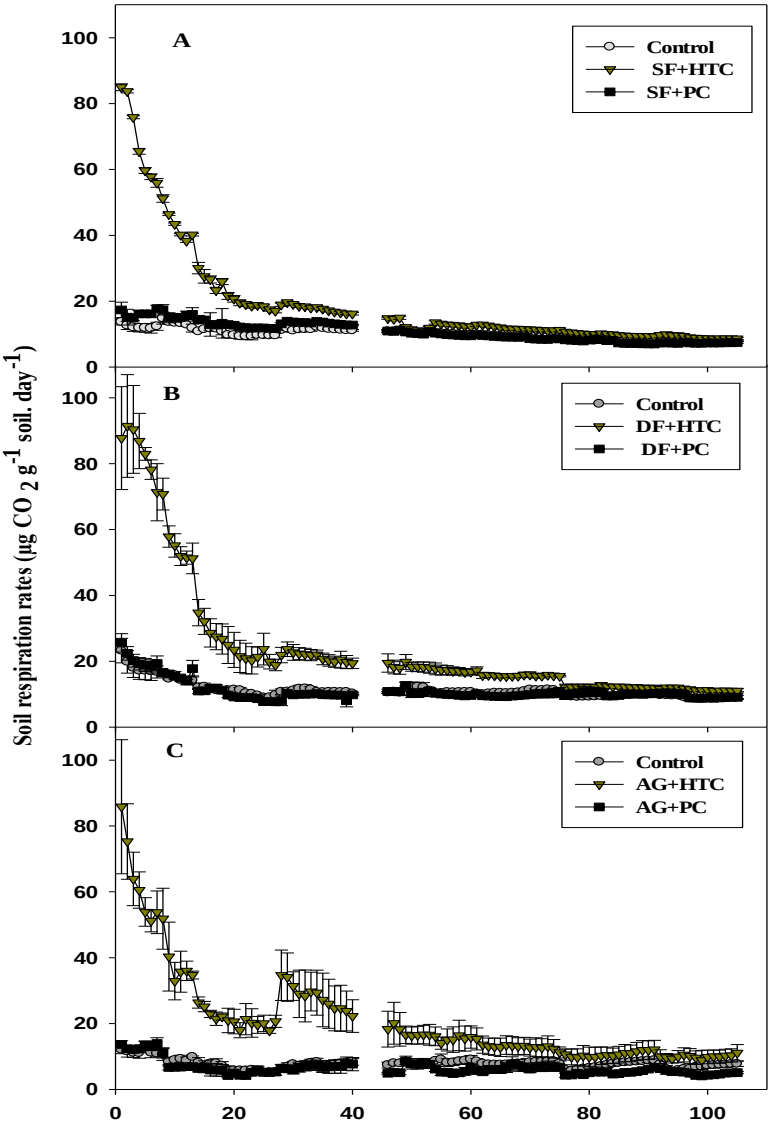

Figure 5.2: Respiration rates in two forests soils and one agricultural soil; (A) respiration rates in SF soil treatments (B) respiration rates in DF soil treatments (C) respiration rates in AG soil treatments. Error bars represent standard error of mean (n=4)

5.3.4 Source of respired CO_2 and char mineralization

The $\delta^{13}C$ of bulk SOM (DF, SF and AG) and bio-char (PC and HTC) reflected their respective C3 and C4 plant origins (Table 5.1 & Table 5.2), with a 14‰±1 difference in $\delta^{13}C$ between the soil and the char. The CO_2 respired from HTC treatment of all soils (Fig. 5.3 A, B, C) was enriched in $\delta^{13}C$ compared to that respired from the respective unamended soils, indicating that the increased respiration from HTC-amended soils resulted at least in part from char decomposition. In contrast, no significant difference in $\delta^{13}C$-CO_2 was observed for PC-amended soils compared to their respective controls, except in the initial CO_2 effluxes from the spruce forest soil. Variability in CO_2 production rate as well as ^{13}C-CO_2 was largest in the agricultural soil, especially the difference between first and final date was 7‰ among replicates of control.

For the two forest soils, PC amendments did not increase the respiration of native soil carbon ($p>0.05$) calculated using either cumulative respiration (method 1) or mass balance of solids (method 2). The greatest effect of PC addition was observed in the AG soil, where native carbon mineralization decreased after PC addition and this effect was persistent for most of the measuring dates (method 1; $p<0.05$). However, the HTC-amended AG soil showed accelerated native-soil SOM decomposition (priming); this effect was significant in cumulative CO_2 emissions (method 1; $p\leq0.01$) though it was not detectable from comparing the amounts of HTC and SOC remaining in the bulk soil at the end of the incubation (method 2; Fig.5.4B). During the initial days of incubation the effect of PC amendment on native soil carbon fluxes was inconsistent and varied among the three soil types. Initially there was positive impact and native (C3) soil organic carbon was respired at higher rates than the control treatments in both forest soils. In particular the SF soil showed positive priming for the first 25 days of incubation (Fig 5.3B). However, given the errors involved, this positive priming effect was not detectable in the cumulative respiration (method 1) but it was in the mass balance of the residual soil material (Fig 5.4B; method 2).

Based on the bulk soil carbon analysis at the end of the experiment (method 2), roughly 50% of the added HTC was decomposed into CO_2 during the incubation, and this mineralization rate was consistent irrespective of soil types (Fig.4B). The loss of PC-C was only significant in the SF soil where a 7.07% (S.D= 1.35 %) decrease in added carbon was observed (Fig.5.4B).

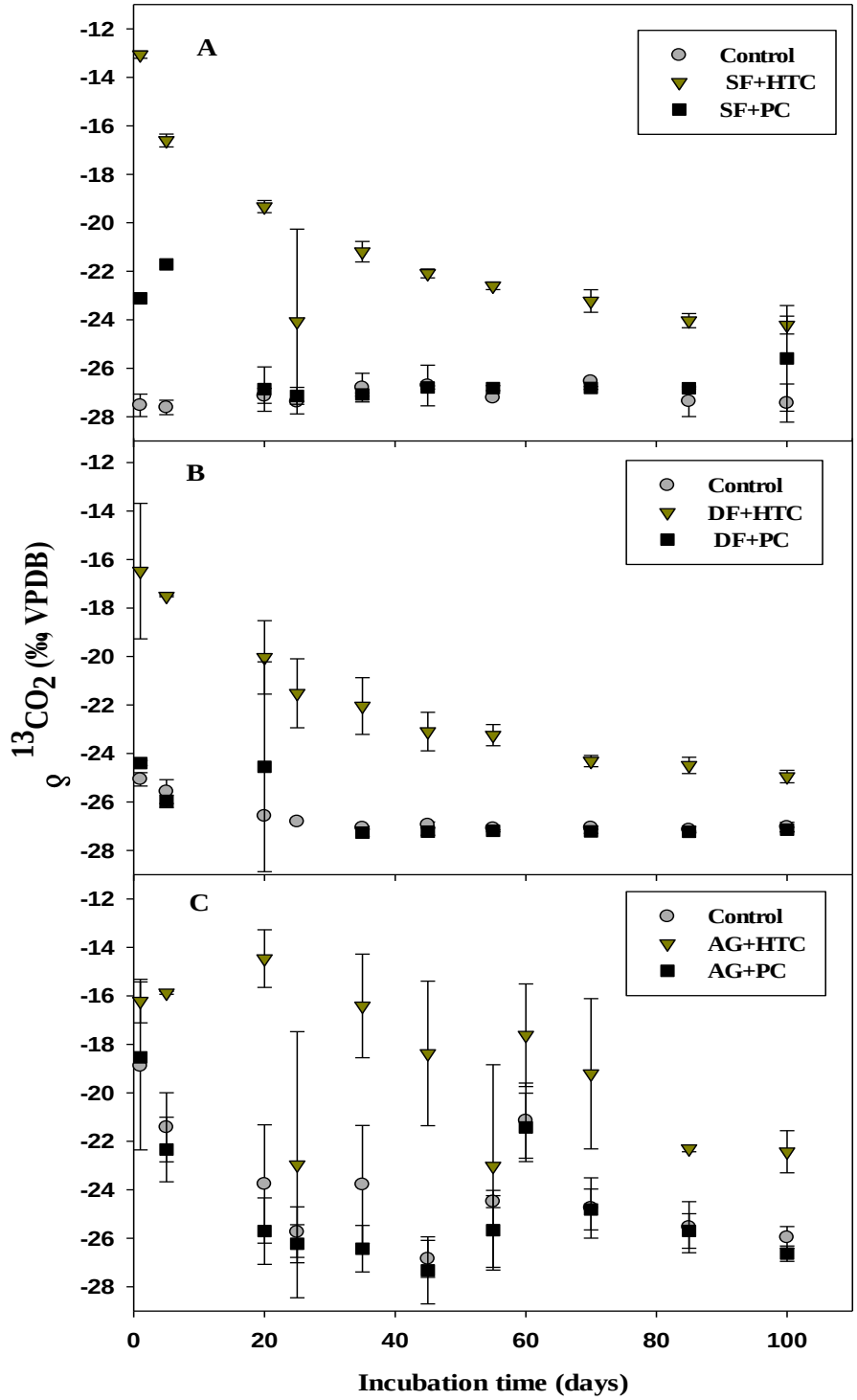

Figure 5.3: Stable carbon isotope signatures in aerobic laboratory incubations of two forest soils and one agricultural soil. Shown are the $\delta^{13}C$-signatures of CO_2 emitted from soil column. Vertical bars represent standard deviation of the mean (n=3). A; SF soil treatments, (B) DF soil treatments and (C) AG soil treatments

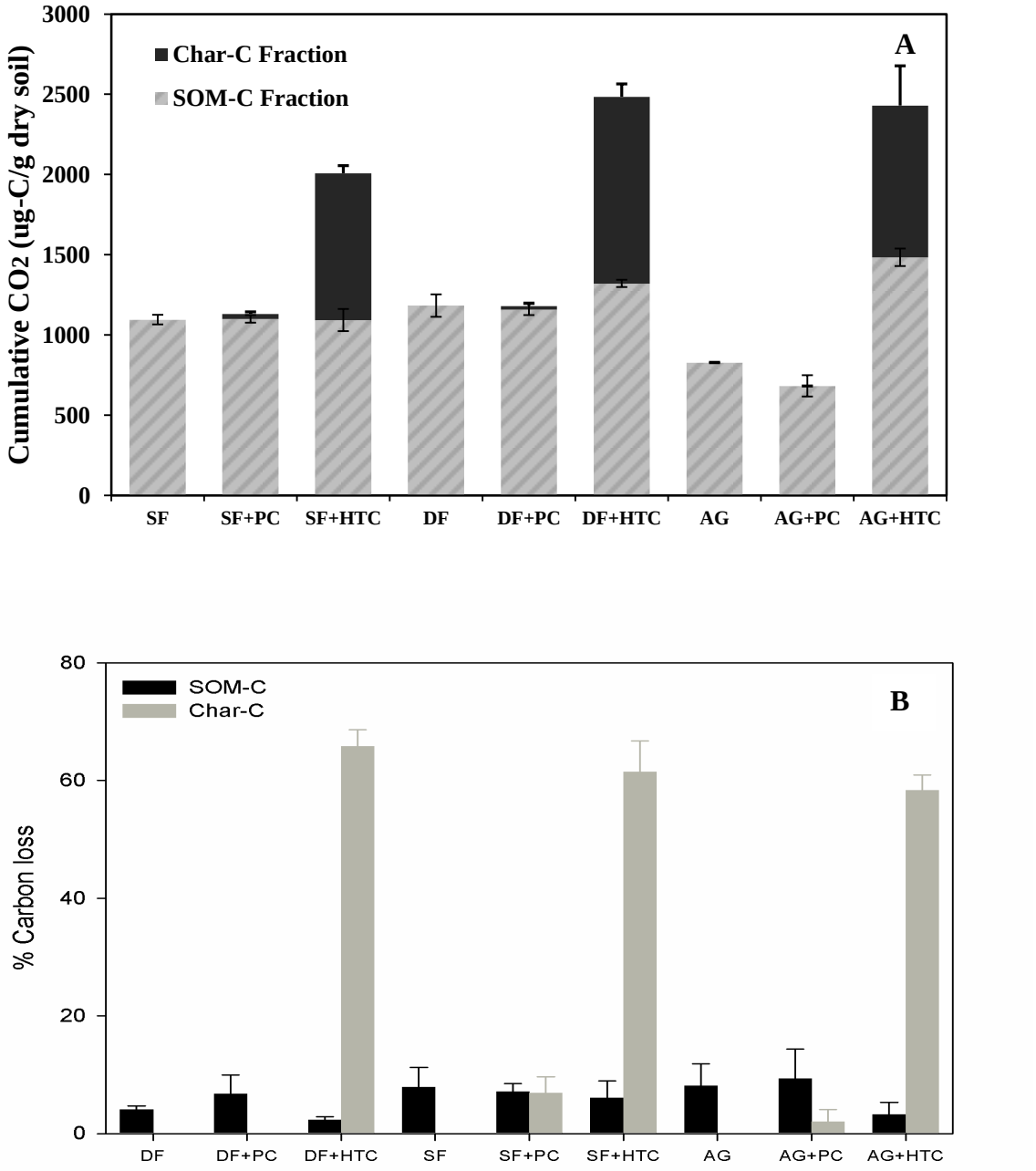

Figure 5.4: (A) Cumulative respiration (CO_2-C ug/g dry soil) from different treatments and relative carbon losses of bio-char and soil organic matter after 105 days of laboratory incubation. Vertical bars represent standard deviation among replicates (n=3) (B) Losses of bio-char carbon and soil organic carbon after 105 days of incubation given relative to the respective initial amounts in the treatments (bulk soil) . Vertical bar represents deviation among replicates (n=4)

5.3.5 Methane and N_2O fluxes

Nitrous oxide emissions were suppressed in HTC and PC amended agricultural and spruce forest soils. This effect was enhanced in the AG soils following ammonium sulfate fertilizer addition, with reduced N_2O emissions from HTC and PC treatments compared to fertilized control soils (Fig. 5.5). This reduction was more pronounced for the HTC compared to the PC amended soils (p <0.01). In contrast, the opposite was found for the DF soil, where HTC amendment increased N_2O emissions (p<0.05). There were large variations in N_2O and CH_4 emissions among all replicates of AG soil treatments.

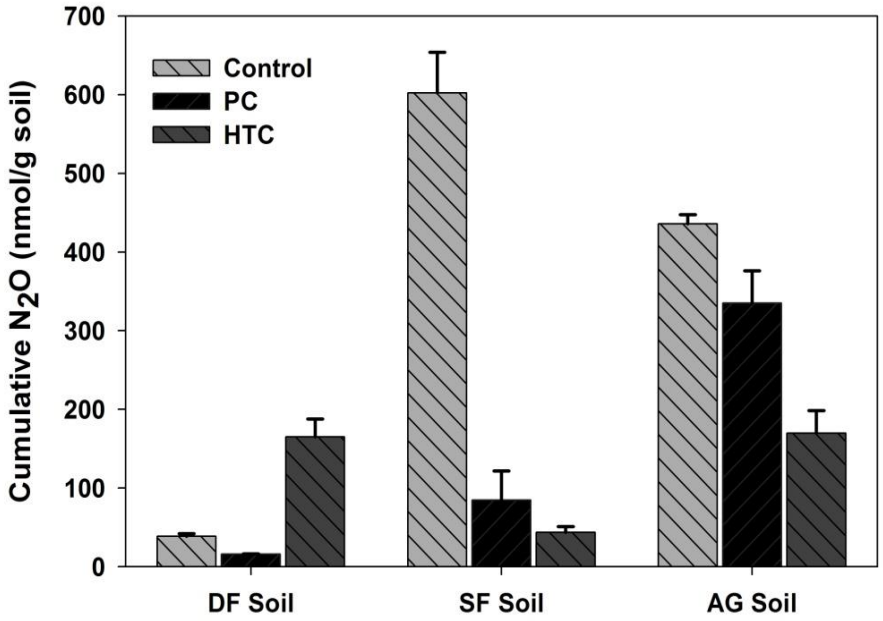

Figure 5.5: Cumulative emissions of N_2O different treatments of two forest soils and one agricultural soil after 105 days of aerobic incubation. Error bars represents standard error (n=3)

No CH_4 was emitted in DF, DF+PC, SF and SF+PC treatments (Fig. 5.6); the use of zero-methane air as our inlet gas did not allow us to test whether these soils would have consumed methane. Although all treatments were incubated under aerobic conditions and at the 60% of their respective field capacities, CH_4 emissions were recorded in HTC treatments of all soils, with highest effluxes from the AG soil (p<0.01). The only impact of pyrochar on CH_4 emissions was observed in the AG soil but there was large variability among replicates (Fig. 5.6).

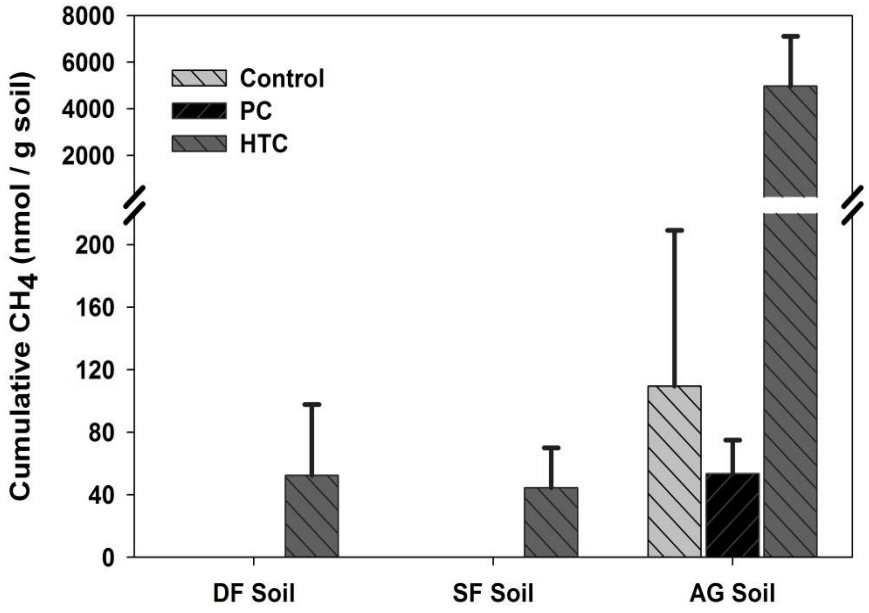

Figure 5.6: Cumulative emissions of CH_4 different treatments of two forest soils and one agricultural soil after 105 days of aerobic incubation. Error bars represents standard error (n=3).

5.4 Discussion

5.4.1 Links between chemistry and decomposition rates for PC and HTC

The major goal in char production is to increase the carbon content in products compared to the original biomass (Sohi *et al*., 2010). To be the most beneficial for carbon sequestration, however, the char produced must not only be stable (not rapidly decomposed) when added to soil, but also must use the less energy during production the carbonization (production) process. Slow pyrolysis char and hydrothermal char are both methods known to have high rates of carbon recovery from biomass but also use less energy. HTC does not require wet biomass pre-drying, and so saves additional energy in production (Meyer *et al*., 2011). In our study, the carbon recovery (percentage of C originally contained in the raw material that is retained in the carbonized sample) of dry pyrolysis process was 44.88% similar to other PC chars reported (Brown, 2009; Fuertes *et al*., 2010; Meyer *et al*., 2011).

Chars produced from various pyrolysis techniques differ in their physical structure and chemical composition even when the same feedstock was used in their preparation (Brewer *et al*., 2009; Bruun *et al*., 2012). In our study, the physical structure of the corn silage feedstock was maintained until the final product during slow pyrolysis. For the HTC, however, the end product was a slurry and no structures from the original corn vegetation were visible. These characteristics likely resulted from the very high pressures reached during the hydrothermal processes.

The PC and HTC chars differed not only in physical form but also chemical composition. The carbon and ash content of HTC was much higher than PC, and had higher hydrogen and oxygen concentrations and were similar to those were recorded by other studies (Cao *et al*., 2010; Fuertes *et al*., 2010; Schimmelpfennig and Glaser, 2012).

The biggest differences between the two chars were in pH and in the amount of soluble/volatile carbon. The PC and HTC chars had pH of 9.89 and 4.70, respectively. The hydrothermal carbonization reactions are sensitive to pH and generally, an acidic pH (< 7) is a pre-requisite for the HTC method (Meyer *et al*., 2011). The end products usually have pH similar to the liquid added to biomass prior to carbonization (Liang *et al*., 2011). Lower pH is achieved with addition of low strength acids (e.g citric acid) which also enhance dehydration and improve the overall reaction rate of hydrothermal carbonization (Cao *et al*., 2010; Hu *et al*., 2010). In contrast, slow pyrolysis produces char with pH values generally above neutrality, and these generally increase, along with ash content, with the temperature of pyrolysis (Ueno *et al*., 2008). With pH values above 9, there may be some carbonates that add inorganic C with the PC to our soil amendments.

The more rapid decomposition of HTC compared to PC is in accord with the differences in elemental composition and volatile content of the two chars. Elemental ratios i.e. O: C, C: H and C: N provide a reliable measure of the stability of chars in soil and correlate with initial decomposition rates of substrates (Novak *et al*., 2010; Singh *et al*., 2010a). A correlation between O: C ratio and char stability has also been reported, and it has been suggested that the half-life of the char with O: C molar ratio lower than 0.2 could be more than 1000 years (Spokas, 2010).

It has been recommended that char with O/C ratio <0.4, H/C ratio <0.6 and black carbon >15% is best suited for soil application as a method for sequestering C (Schimmelpfennig and Glaser, 2012). While we did not measure black carbon, the O/C of both our HTC and PC fall within the range of suitability; for our materials the O:C ratio declined from 0.73 to 0.28 to 0.06 for corn silage feedstock, HTC and PC chars, respectively (Table 5.2).

The HTC char also had much higher volatile compound content, as measured by thermogravimetry, compared to the PC in this study. It also contained large amounts of soluble carbon (DOC), which is presumably more available for microbial degradation. Pyrolysis –gas chromatography mass spectrometry (PY-GC-MS) analysis of HTC and PC (Julia Baumert and Gerd Gleixner, MPI-Biogeochemistry, personal communication 2012) showed that the char prepared from pyrolysis (PC) had higher aromatic C content (benzene, styrene, phenol etc.), where the char produced from HTC was mainly comprised of heteroatomic compounds like furans, pyrans, dihydropyranones, pyrroles, imidazoles suggesting biomass contribution. These results are consistent with previous reports that found slow pyrolysis produced char rich in aromatic compounds, whereas HTC contained precursors of cellulose, hemicellulose and lignin (Fuertes *et al*., 2010; Schimmelpfennig and Glaser, 2012; Titirici *et al*., 2008).

5.4.2 Behavior of amendments in different soils

The three soil types in our study differed considerably in their characteristics, including control (unamended) respiration rates. The two forest soils emitted significantly higher amounts of carbon than the agricultural soil ($p \leq 0.001$; method 1), although more CO_2 was emitted from the

DF soil than the SF soil ($p<0.05$; method 1). Microbial biomass was the only characteristic that showed the same pattern as the respiration rates (Table 5.1). Soil microbial biomass is closely related to soil fertility and is considered as sensitive indicator of soil quality (Iqbal *et al*., 2010). Lower respiration rates in AG soil might be impact of low available carbon pool (Table 5.1) as substrate limitation may cause stability in SOC (Marschner *et al*., 2008).

During 105 days of incubation, we observed more than 50% loss of the HTC char (Fig. 5.4B; method 2), and this loss was slightly higher in DF soil followed by SF and AG soil respectively. In contrast, the pyrolysis char (PC) was either inert or its mineralization rate was too low to be measured by either mass balance method we used (cumulative CO_2 loss or comparison of initial and final organic matter), except for a 7.07% (s.d 1.35%; method 2) loss from the SF soil (Fig 5.4B). Instability of HTC char in soil was found in number of recent studies (Kammann *et al*., 2012; Qayyum *et al*., 2012) and would be predicted from its chemical content as described above.

The enhanced respiration in PC amended SF soil could arise from abiotic as well as biotic factors (Jones, *et al*. 2011). The high pH of PC may result in inorganic C release during the incubation (Jones *et al*., 2011), and soils with lower pH showed higher PC mineralization in other studies (Luo *et al*., 2013). The observed short-term acceleration of PC mineralization in acidic SF soil could result from acidification of PC inorganic C, but could also result from the effect of pH on microbial communities. Soil pH is the major driver of microbial community structure, (Gleixner, 2009; Griffiths *et al*., 2011; Thoms *et al*., 2010) and microbial communities in spruce forests are specialized to degrade complex and aromatic compounds (Carletti *et al*., 2009) whose growth might be initially stimulated by the addition of PC (Luo *et al*., 2013) .

Both char amendments impacted native soil carbon mineralization rates, the effect known as priming. The potential for priming is of considerable interest in bio-char research (Keith *et al*., 2011). However, it is still unclear which basic processes might explain the role of char in priming, and this makes it is difficult to predict how different types of char will behave in a range of soils. If we relate priming (short term acceleration or decline of native carbon decomposition (Kuzyakov, 2010) to the presence of a labile pool in heterogeneous pyrolysed biomass, we would predict that the char with the highest amount of labile material would enhance degradation not only of SOM but also of recalcitrant components of the char itself. The HTC char used in this study had high amounts of DOC and volatile constitutes (more than 50%, based on TGA), and its high degree of decomposability could be easily observed in the incubation results (Fig 5.4). A decrease in SOC -derived respiration compared to the unamended control (negative priming) was observed initially when HTC was added (data not shown). This initial negative impact of HTC char on SOC mineralization might be related to shift of soil microbes from less available SOC to more labile HTC. Several studies showed phytotoxic effects of HTC due to higher PAH content and presence or emission of toxic volatiles (Busch *et al*., 2012; Jandl *et al*., 2013; Rogovska *et al*., 2012; Schimmelpfennig and Glaser, 2012) but there is no study related to toxic effect of HTC on soil micro-organisms. However, later in the incubation, SOM mineralization was enhanced by HTC amendment (positive priming), and this increase was greatest in the AG soil (Fig. 5.4A). However, this positive priming was not measureable in bulk soil carbon, which could be due to either to our assumption that the $^{13}CO_2$ from HTC char reflects its bulk $\delta^{13}C$ content, or to the fact that the C overall was greatest (and

therefore the HTC addition proportionally the smallest) in the AG soil. On the contrary, bulk soil carbon measurements confirmed the considerable negative impact of HTC on AG native soil carbon mineralization (p<0.05) (Fig. 5.4B). To our knowledge there is no previous report on HTC addition on native soil carbon mineralization.

The impact of PC amendment on native soil was overall smaller, inconsistent and varied with soil type. These inconsistencies may reflect the practical limitation of two pool model used in this study where one end member was assumed (PC $\delta^{13}C$ was assumed not to fractionate on decomposition). The AG soil, the most carbon rich soil in our experiment, respired less native soil-CO_2 when amended with PC, but no effect was found in forests soils. In summary, the priming effect of HTC and PC in forest soils was not consistent and it was so small in size that we were not able to measure it in bulk soil carbon (method 2). Our results agree with other field and incubation studies that showed PC amendment had either no effect or negative priming (Major *et al*., 2010; Zimmerman *et al*., 2011).

5.4.3 Effect of Char amendments on non-CO_2 greenhouse gases

All three native soils produced nitrous oxide. Nitrous oxide fluxes were significantly higher from SF soil compared to DF soil (Fig.5.5). These results are in contrast with studies that showed lower N_2O emissions from spruce forest soils compared to deciduous forest soils (Ambus *et al*., 2006) Application of bio-char can affect the fate and transformation of N in soils (Singh *et al*., 2010b; van Zwieten *et al*., 2010) and hence the emission of N_2O. Rondon *et al*. (2006) reported a 50 to 80% reduction in N_2O emissions following bio-char addition to tropical soils. We found similar results, but observed greater suppression of N_2O emissions with HTC compared to PC (Fig.5.5). This suppression in N_2O flux with HTC amendment was most prominent in the AG soil to which N-fertilizer was applied. Kammann *et al* (2012) also found that HTC amendment suppressed N_2O emissions, but this effect was short-lived and opposite when nitrogenous fertilizer was applied. Increased N_2O emission from HTC treatment of DF soil might be due to its role on water holding capacity of clay rich soil. Higher hydrophilicity of HTC was recorded compared to PC (Schimmelpfennig and Glaser, 2012) and this may resulted in water rich microsites in DF soil. Amendment with PC always decreased N_2O efflux regardless of soil type or fertilization, in agreement with other studies (Kammann *et al*., 2012; Libra *et al*., 2010).

Since the incubation system used air with no methane as the inlet gas, it was not possible to measure any impact of char on methane uptake in our soils. In our unamended control soils, the AG soil was the only one to emit CH_4 while no emissions were observed from either forest soils (Fig. 5.6). Globally, forest soils are considered to be natural sinks for atmospheric CH_4, while agricultural practices tend to reduce the soils' ability to take up CH_4 (Guckland *et al*., 2009; Mancinelli, 1995). However, with the addition of char we found measureable methane production from certain treatments. The addition of HTC resulted significant CH_4 emission (p<0.05) from HTC treatments of DF and SF soils, and stimulated CH_4 emissions (p<0.01) from the AG soil (Fig. 5.6). The increased in CH_4 emission could result from single or combined factors, including water content, soil type and char type. Although we kept the total moisture content constant, it was practically impossible to distribute moisture evenly throughout the soil column and anaerobic microsites must have been produced in the soils. Addition of the relatively hydrophobic HTC char could enhance anaerobicity and increase CH_4 emissions. In a field study,

HTC char amendment was observed to increase CH_4 emission (Kammann *et al*., 2012). The net emission of CH_4 includes the balance of methanogenesis and methanotrophy and our experimental set-up was not well equipped to address this balance. This study was able to demonstrate that char amendment influences not only C sequestration but other greenhouse gas fluxes. Further research will be needed to fully investigate the mechanisms involved in the role of char amendment on N_2O and CH_4 emissions.

5.5 Conclusion

Slow pyrolysis and hydrothermal carbonization are two of the most efficient methods to produce bio-char in terms of carbon yield and utility to a broad range of feedstock. The HTC method especially is proposed as a useful way to increase soil C sequestration as it uses less energy than PC production and can use unconventional wet biomass sources such as sewage sludge, city wastes, animal and human excreta without requiring additional pretreatment such as drying. Both PC and HTC have been suggested as mitigation options for carbon sequestration to help offset increasing atmospheric concentrations of CO_2. Hydrothermal char decomposes rapidly (50% in 100 days regardless of soil type) and can stimulate emissions of GHG like CH_4 and CO_2 derived from priming of native organic matter. Slow pyrolysis char is more stable in soil and had consistent effect on GHG emissions in this study. Therefore PC would seem to have the greatest climate mitigation potential, though its overall emissions during production are potentially greater because of higher temperature combustion and the need for drying feedstock also need to be taken into account. Properties of the amended soils seem to be less important than the method of char production, though they are not insignificant. Because much of the bio-char mitigation is suggested as an amendment for agriculture, the observed reduction in decomposition rates of native OM needs careful consideration as it may impact the supply of nutrients to plants.

CHAPTER 6

General Discussion

The processes determining the production and/or consumption of atmospheric greenhouse gases in soils are mostly controlled by soil microorganisms (Conrad, 1996) and regulated by soil physical and environmental variables. There is a wealth of literature on the effects of environmental factors such as moisture and temperature on the net rates of exchange of gases such as CO_2, CH_4 and N_2O between soils and the atmosphere. Such relationships include information about direct influences of these factors on microbial metabolic activity as well as the physical exchange of gases that in turn can influence microbial communities. For example, increased moisture contents limit the rates of gas exchange with the soil atmosphere. It has been widely observed that net soil CH_4 oxidation rates are negatively correlated with soil moisture content. However, it is not clear whether this relationship reflects a decrease in CH_4 supply from the atmosphere (i.e. a direct diffusion limitation), or a relative increase in methane production compared to oxidation due to decreased O_2 concentrations (an indirect consequence of lower diffusion rates). The main objective of this study was to use highly controlled incubation experiments to help unravel the direct and indirect influences by which environmental factors affect the production and consumption of greenhouse gases from a range of soil types. These studies were conducted using a unique flow through incubation system that ensured constant gas exchange rates for the soil column, which minimized the influence of physical diffusion process on soil reactions. Three experiments were performed targeting role of O_2 supply (independent of gas diffusion rates), substrate supply (using thermally carbonized biomass) and CH_4 supply on the net flux of CO_2, CH_4 and N_2O from soil samples that represented a range of soils with different properties and land use (agriculture, forest and grassland). In this chapter, I highlight some of the most important outcomes of this study, and also make some general comparisons across the experiments.

6.1 Factors related to variable CO_2 emission rates among contrasting land uses

My main intentions were not just to measure CO_2 emission rates from different soil types but to understand what factors drive microbial activity and biomass that are the principal source of CO_2 production in soils. Microbes are the primary determinant of CO_2 emission rates from soils and are also linked with stabilization of soil organic matter, as fresh biomass must pass through the microbial 'funnel' before entering soil carbon pools (Wickings *et al.*, 2012).

I observed a direct correlation between microbial biomass and CO_2 emission rates across all my incubation studies (Chap 2, 3, 5). This relationship emphasizes the importance of factors that directly or indirectly influence soil microbial biomass. In general, soil microbial life is

constrained by the availability of soluble organic matter as an available energy source, a continuous supply of O_2 or other electron acceptor for heterotrophic decomposition, conditions of optimum moisture for biofilm formation, and temperatures in the range required for metabolic activities (Don, 2013). In this regard my thesis targets two aspects: i) the role of soil organic carbon (SOC) and its relationship with microbial biomass, ii) the role of labile carbon, O_2 concentration and moisture on microbial metabolic activities.

Differences in microbial biomass are often linked with land use and management activities (Iqbal *et al.*, 2010). Across the different land uses represented by the soils studied in this work, microbial biomass correlated positively with soil C and N (Table 6.1). However, this relation was statistically significant ($p<0.001$. R^2, 0.95 and 0.89 for C and N respectively) only in samples that were thoroughly cleaned from all plant fragments prior to incubation. Soil samples that were amended with hydrochar or had remnant fine plant fragments had a relatively weaker relationship of microbial biomass with total C and N contents (Table 6.1). It could be due to differences in quality (C: N) of organic matter under different land use which influence carbon use efficiency of soil microorganisms (Kögel-Knabner, 2002; Yang *et al.*, 2010).

These results are in consistent with other studies that performed meta-analysis of data reported for several hundred sites under different land use and found positive correlation between two variables; microbial biomass and SOC (Hartman and Richardson, 2013; Xu *et al.*, 2013). In addition, correlations between microbial biomass and total nitrogen have also been observed in natural soils (Waldrop *et al.*, 2004). Since microbial biomass is the source of SOC and the major means of its decomposition, the fact that they are related in a nearly constant ratio across a wide range of soil types means that there must be compensating feedbacks - i.e. if microbial biomass increases, it increases both the source of SOC (since that is dead microbial biomass) and the sink (since more microbes mean more decomposition).

Table 6.1: Correlation between microbial biomass (MBC) with other variables as observed in different incubation studies.

x	y	R^2	n	Study	Land use types
MB	CO_2●	0.95	15	Chap 2	AG1,AG2,DF,GR,SF
		0.74	11	Chap 3	AG,DF,SF
C:N	MBC	0.68	15	Chap 2	AG1,AG2,DF,GR,SF
		0.60	11	Chap 3	AG,DF,SF
N	MBC	0.89	15	Chap 2	AG1,AG2,DF,GR,SF
		0.04	11	Chap 3	AG,DF,SF
C	MBC	0.95	15	Chap 2	AG1,AG2,DF,GR,SF
		0.28	11	Chap 3	AG,DF,SF

●Microbial biomass (ug-C g^{-1} soil), CO_2 emission rates (ug-CO_2-C g^{-1} soil. day), C and N contents in (mg-C or N g^{-1} soil)

Ecophysiological indices or "quotients" have been extensively used as indicator for change in microbial community response to shift in environmental conditions ('disturbances') (Anderson,

2003). These ecophysiological indices are calculated using physiological variables (CO_2 emission or respiration rates, carbon assimilation rates, or cells growth/death) against microbial carbon or soil carbon content. Two main variables have been extensively used to determine differences in response of microbial communities present in contrasting land uses and are termed as metabolic quotient (microbial community respiration per unit biomass; qCO_2) and microbial quotient (the ratio of biomass carbon to soil carbon).

I calculated qCO_2 and microbial quotient from results of all incubation experiments to attempt to address two questions. First, how did qCO_2 and microbial quotient differ among land uses for samples incubated under identical conditions? Second, how does O_2 availability affect microbial activity and qCO_2 across the different soils/land uses? One issue with incubation-based qCO_2 values is the potential contribution of a dormant microbial community which on one hand expresses itself in basal respiration rates but on other hand is not included in MB which only shows active communities (Anderson and Domsch, 1985).

The degree to which soil samples contained fresh plant litter of highly labile substrate clearly impacted the measured qCO_2. The five different soils assess in the first experiment, from which fresh litter was carefully hand-picked, had the same qCO_2 values (Chap 2; Table 6.2). Similar soils were used in the second study but fine plant material was not removed. In this case (Chapter 3), qCO_2 values showed that the agricultural soil had the highest metabolic activity followed by the two forest soils (DF and SF) (Chap 3). Other studies that determined qCO_2 values in soils from different land uses reported similar outcomes. For instance, Anderson and Domsch (2010) recorded similar qCO_2 values in three contrasting soils, Cambisol, Chernozym and Rendzina, under agricultural and forest land use (Anderson and Domsch, 2010). The same study also measured differences in qCO_2 among soils when samples were amended with glucose, highlighting the role of labile carbon and land use, agricultural soils showed high ability to take advantage of labile C addition.

The metabolic quotient (qCO_2) also varies among soil types in when environmental variables (pH, O_2 availability) change, or in disturbed soils. The different responses of contrasting soils to a similar change could be a function of microbial community structure. For example, in Chapter 2, qCO_2 values for five different soils did not respond in the same way to shifts in O_2 availability. While Wardle and Ghani (1995) reported increase in qCO_2 values with external stress, fertilizer application (Wardle and Ghani, 1995), qCO_2 values in our study were significantly lower at sub-oxic conditions (5 & 1% O_2). The degree of decrease in metabolic quotient for a given shift in O_2 availability varied among land use types, with agricultural soils having the largest decrease in qCO_2 values at 1% O_2. It is unlikely that a decrease in labile carbon sources was the main driver for the shift in qCO_2 values, as all soil samples had similar qCO_2 at the start and end of experiment, when the O_2 level was 20.5%. (Chap 2).

The agricultural soil demonstrated stronger responses to changes in O_2 concentrations compared to the forest soils. This perhaps reflects the greater heterogeneity in forest soil microbial communities on small spatial scales, and the potential for low O_2 to be a common phenomenon in forest soils. In contrast, agricultural soils are extensively vertically mixed by plowing, which leads to homogenization of microbial community and better soil aeration. Another incubation study applying constant temperature and O_2 but different moisture contents for the DF soil

showed a significant increase in qCO_2, approximately 56%, at 60% WFPS compared to samples with 45% WFPS (Unpublished data). These two separate studies reveal that at high moisture conditions the decrease in CO_2 emissions are primarily due to O_2 limitation, which suppress the positive role of soil moisture.

As with qCO_2, the microbial quotients differ for soils with and without prior cleaning, indicating a direct impact of soil carbon substrates on microbial biomass (Don, 2013). When soil was pre-cleaned (picked for litter fragments), all soils had 2-3% microbial to soil carbon ratio regardless of land use (Table 6.2). These results are similar to those reported elsewhere; including those reported by Wardle (1992) (1–3%), Hartman and Richardson (2013) (2.01%). These results suggest that SOC quality, defined as the availability of SOC to microbial mineralization, is similar across these very different soil types, for example because much of it is derived from microbial C sources (Grandy and Neff, 2008; Grandy *et al.*, 2009). Moreover, these land use share similar abiotic characteristics that control SOC availability. Alternatively, different C inputs may not necessarily lead to high –or low -quality SOC pools because C quality may partly be a function of the resource input (Grandy *et al.*, 2009). Altogether, SOC may simply be a better predictor of microbial biomass than other site characteristics because it provides an integrated measure of the biotic and abiotic factors that regulate the size of the microbial biomass pool.

Table 6.2: Values of metabolic and microbial quotients across different land use soils determined in incubation studies and role of environmental factors.

Study	Conditions	Land use	qCO_2 mg CO_2-C mg $MB^{-1}{\cdot}h^{-1}$	Microbial quotient (%)
Chap 2	T, 20°C; Moisture, 50% WFPS; O_2, 20%	AG1	0.41±0.02	2.84±0.04
		AG2	0.40±0.01	2.87±0.34
		DF	0.35±0.06	2.49±0.42
		GR	0.38±0.07	4.08±0.46*
		SF	0.40±0.03	2.38±0.06
Chap 3	T, 25°C; Moisture, 50% WFPS; O_2, 20%	AG1	0.90 ± 0.17^{a}	0.56 ± 0.05^{a}
		DF	0.69 ± 0.17^{ab}	1.72 ± 0.16^{b}
		SF	0.47 ± 0.08^{b}	1.15 ± 0.23^{c}
Chap 5	T, 20°C, Moisture, 60% WHC; O_2, 20%	•AG	0.60±0.2	-
		DF	0.66±0.05	-
		SF	0.77±0.14	-

•This soil does not truly represent agricultural land use as field was established with forest soil.

Different letters in superscript represent statistical significance (Tukey α 0.05)

6.2 Microbial control of atmospheric CH_4 oxidation in upland soils

The work presented in this thesis also gives information on several aspects of atmospheric CH_4 oxidation by upland soils, including the role of microbial community size and structure. Major conclusions from the various experiments include: 1) The variability in the capacity of different

land uses/soils to oxidize atmospheric CH_4 is driven by microbial community structure (Chap 3); 2) Linear relation between CH_4 oxidation rates and CH_4 concentrations (Chap 3) while insensitivity with respect to change in O_2 concentrations (Chap 2) indicate low CH_4 oxidation rates at higher moisture content are probably low supply of CH_4. 3) No sensitivity to change in O_2 concentrations also indicate that methanotrophs successfully compete with other heterotrophs (Chap 2). 4) At last but not least, no increase in methanotrophic community abundance or population size was evident among contrasting CH_4 treatments highlighting the role of other simple compounds such as acetate for maintaining the growth of methanotrophic community (Chap 3).

Global models for estimating the terrestrial CH_4 sink identify two main factors, soil diffusivity and microbial activity, as the main controls on the capacity of soils to oxidize atmospheric CH_4 (Curry, 2007; Curry, 2009; Hashimoto, 2012). The role of soil diffusivity is rather simple and evident from field data as soils with high diffusivity ensure a continuous supply of CH_4 (Smith *et al.,* 2003). However, microbial activity or the microbial control over intrinsic CH_4 oxidation capacity of soil is complex and it involves number of parameters. The majority of field based studies linked microbial activity with NH_4^+, pH, and vegetation type (Bender and Conrad, 1993; Dobbie and Smith, 1996; Luo *et al.,* 2013). However, in recent studies two main variables are extensively discussed to be responsible for different atmospheric sink capacity of contrasting land use; methanotrophic community structure and abundance and proposed indirect impact of soil physicochemical characteristics.

The role of methanotrophic community structure was evident in this study as terminal restriction fragment length polymorphism (T-RFLP) analysis showed clear differences in dominant methanotrophic taxa between forest and agricultural land use types. T-RFs associated with upland soil cluster (*USCα*) were dominant in soils (SF, DF) that showed significantly higher intrinsic CH_4 oxidation. Interestingly, a decrease in CH_4 oxidation rates was also measured in both soils at 1% O_2 suggesting strict aerobic nature of *USCα*. On other hand, agricultural soils which had significantly low capacity to oxidize atmospheric CH_4 and was insensitive to O_2 concentrations, was abundant in T-RF associated with *Type 1a* methanotrophs. Interestingly, the abundance of *USCα* related *pmoA* gene was below detection limit in AG soil. These outcomes proposed a direct role of *USCα* in regulating the CH_4 oxidation rates in forest soils and are in consistent with other studies (Kolb, 2009; Nazaries, 2011, 2013). In addition, it also suggests a direct role of type 1a methanotrophs in atmospheric CH_4 oxidation in agricultural soils.

The main factor responsible for high abundance of type 1 methanotrophs in agricultural land use could be physical disturbance as evident from other studies that also reported its presence on perturbed soils (Abell et al 2013). In my opinion, main governing factors behind the successful survival of *Type 1* methanotrophs in agricultural soils could be related to their capability to exploit low O_2 niches (Reim et al 2012) and other carbon sources (Benstead et al 1998, Pratscher et al., 2011). However, latter may also be true for *USCα.*

The strategy used by methanotrophs to survive in upland soils has been debated since the discovery of the process, as concentrations of CH_4 are apparently too low to provide energy needed to fulfill the growth requirements of microbial cells. Three possible survival strategies of have been proposed: (i) High affinity methanotrophs solely depend on atmospheric concentration

of CH_4 (Kolb *et al.*, 2005); (ii) Methanotrophs possess the capability to utilize alternate, chemically simple, substrates such as acetate, methanol, formate, formaldehyde (Benstead *et al.*, 1998; Pratscher *et al.*, 2011); (iii) Periodic production of CH_4 from anoxic microsites/microzones provide an extra source of energy and the methanotrophs' abilities to acclimate to a feast-feminine cycle help them to survive in upland soils (Kammann *et al.*, 2009; von Fischer *et al.*, 2009). Outcomes of the incubation study that included 4 different CH_4 concentrations (Chap 3) highlight presence of 2nd scenario. No differences in methanotrophic population size, determined with qPCR, in spite of 30x time more available CH_4 and 20 times high CH_4 oxidation rates suggest involvement of additional carbon sources (other than CH_4). However, role of other limiting factor i.e. nutrient cannot be ruled out and require further investigations.

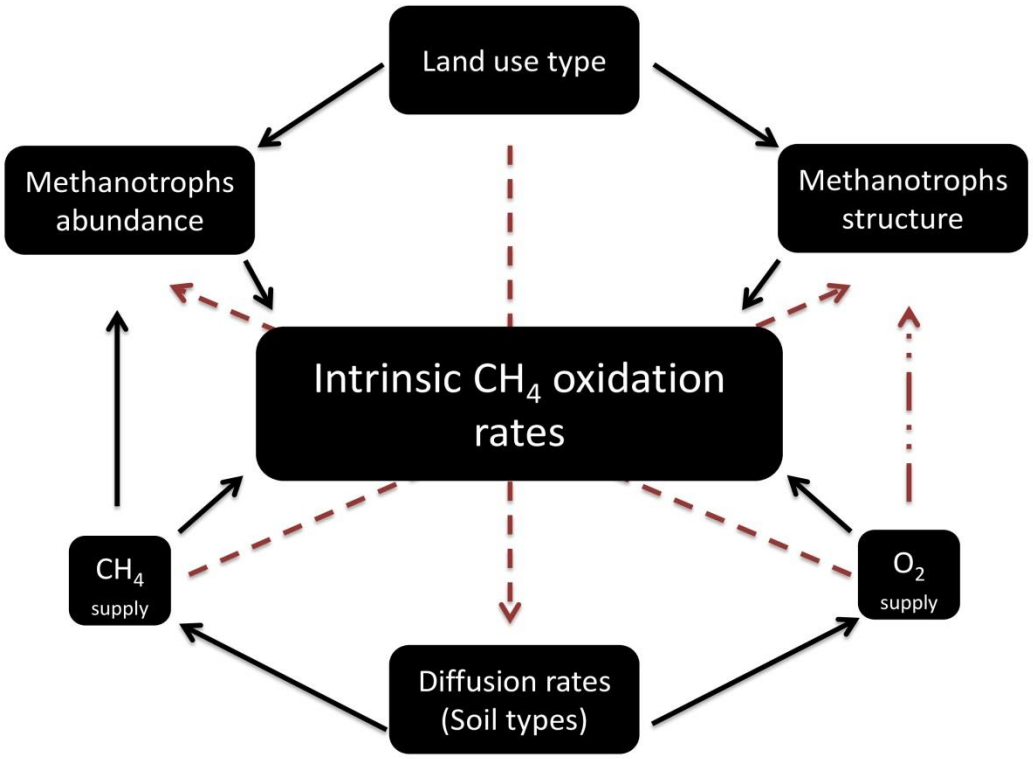

Figure 6.1: A schematic diagram of principal factors that regulate intrinsic CH_4 oxidation capacity of upland soils. This study highlights the direct role of O_2 and CH_4 supply availability rates of the process. In addition effect of latter was also determined on microbial variables (methanotrophic abundance and methanotrophs structure). Altogether, it was evident that all factors co-drive the CH_4 oxidation rates and best combination of all results into high potential of soil to sequester atmospheric CH_4.

6.3 Large fluxes of N_2O under anoxia and variability among contrasting soils sampled from contrasting land uses

This study highlights two main aspects of soil N_2O production, including role of O_2 supply and the kind of soil/land use type. In general, O_2 plays a dual role; it acts as electron acceptor for the nitrification process led by autotrophic ammonia oxidizing bacteria, while, it is an inhibitor for denitrifying bacteria (Arp *et al.*, 2002; Khalil *et al.*, 2004; Morley and Baggs, 2010; Huang *et al.*, 2014). Although processes, nitrification and denitrification, can act as sources for N_2O in soils, denitrification typically is has the higher potential for N_2O production and is responsible for higher fluxes than nitrification (Ussiri, 2013). Large diverse group of denitrifiers are divided into three sub-groups based on their sensitivity to O_2 supply and their ability to carry out denitrification (Cavigelli and Robertson, 2001; Morley and Baggs, 2010; Blagodatskaya *et al.*, 2014). Soil incubation studies have reported contrasting results on the influence of sub-oxic conditions on N_2O emission rates. Morley and Baggs (2010) observed significantly enhanced rates of denitrification at 2% O_2. In contrast, Khalil et al (2004) reported high sensitivity of denitrification to very low O_2 levels and recorded N_2O (denitrification) only under complete anoxia. My results were consistent with Khalil et al (2004) as N_2O fluxes from all soils were 4-100 fold higher in the absence of O_2 (Fig 6, Chap 4), while nitrification rates were rather insensitive to O_2 levels (Fig 5s, Chap 4).

The results of incubations done for this study indicate that forest soils could act as a large source of N_2O, and show that differences in N_2O production rates for the various soils were related with NO_3^- and soil carbon contents. These factors have been proposed to play an important role in determining rate of denitrification (Goodroad and Keeney, 1984; Morley and Baggs, 2010; Blagodatskaya *et al.*, 2014). However, this study only provides preliminary information for future studies, though we infer that the temperate forest soils we studied have the potential to contribute larger fluxes of N_2O than have been generally reported.

6.4 Potential of hydrochar for carbon sequestration

Materials recommended for carbon sequestration in soils must possess characteristics such as stability or long residence times. Chemical composition and the ability to interact strongly with soil minerals are two important variables that are known to determine organic matter stability in soils (Marschner *et al.*, 2008; Schmidt *et al.*, 2011). Black carbon in soils is the best example of compounds that not only possess these characteristics but have the ability to alter microbial diversity (Glaser, 2007; Khodadad *et al.*, 2011). As agricultural soils are the main target sites for carbon sequestration, the potential of a soil amendment to have a positive influence on crop production is pre-requisite quality of such materials. Ideally the amendment materials should also suppress production of non-CO_2 greenhouse gases or at least must not promote their emissions. As already mentioned in the introductory chapter, the proposed use of hydrochar for carbon sequestration is relatively new idea (Titirici *et al.*, 2007; Titirici *et al.*, 2012) and is still not well tested. As the final part of this thesis, I designed two experiments that used incubations and field tests to test almost all above-mentioned aspect of hydrochar. In addition, I also compared hydrochar with the more extensively studied slow pyrolysis char.

Compared to slow pyrolysis char, hydrochar was unstable and its decomposition rates were independent of soil type. The main route of hydrochar carbon loss was via soil respiration and

approximately 35-60% of hydrochar carbon was decomposed in a period of 100 days (Fig 7, Chap 4; Fig 4, Chap 5). A bi-phase decomposition rate was recorded in both studies, initially large proportion (approximately 90% of total loss) of added hydrochar was decomposed in a relatively short period of time (90 days for field study, 60 days for incubation study) and during later stages decomposition was so slow it required a $\delta^{13}C$ label to be quantifiable (Fig 2, Chap 2; Fig 3, Chap 3). Using a two pool nonlinear regression model, the half-life of this more slowly decomposing pool of hydrochar was estimated to be approximately 19 years, which is relatively longer than non-altered biomass (Qayyum *et al.*, 2012). Compared to hydrochar, very low to zero decomposition was observed in pyro-char, confirming its inert nature on time scales of decades (Fig 4a, Chap 5).

The higher decomposability of hydrochar is related to its chemical characteristics (Fuertes *et al.*, 2010; Libra *et al.*, 2011; Kalderis *et al.*, 2014). One of the main drawbacks of the hydrothermal process is the dissolution of large amounts of structural components in reaction water which act as a labile carbon pool (Sevilla *et al.*, 2011). Recently, it has been recommended to separate and remove water and water soluble materials from the solid phases in hydrochar either by drying or composting (Libra *et al.*, 2011; Busch *et al.*, 2013). However, in both of the studies reported here, I used hydrochar slurry without any pre-treatment, and the presence of approximately 50% of labile carbon was evident from TGA analysis of the wet amendment material (Fig1, Chap 5). Contrary to my results some incubation studies observed very low decomposition rates in hydrochar, approximately 10% during a period of 1 year. We attribute these differences to the variations in feedstock materials and different process conditions (temp, catalyst) that can give rise to compositional differences (Libra *et al.*, 2011) between hydrochar used in this and previous studies (Steinbeiss *et al.*, 2009; Qayyum *et al.*, 2012).

The effect of hydrochar on native soil carbon is particularly interesting as it is well known that addition of highly labile carbon substrates to soil can enhance mineralization of native carbon stocks, a process known as positive priming (Kuzyakov, 2010). Both incubation and field studies recorded positive as well as negative priming by hydrochar amendment, though these effects were observed on different time scales after application. Immediately after hydrochar application, positive priming was recorded resulting accelerated native soil carbon mineralization but this effect was reversed after this initial decomposition pulse. The cumulative effect of hydrochar carbon on SOC was positive as both the field and incubation study recorded higher native soil carbon in hydrochar treated soils compared to untreated soil samples that were allowed to decompose under the same environmental conditions for the same amount of time. Nonetheless, this effect was soil dependent and was more prominent in clay rich soils (Table 2, Chap 4). This protective role of hydrochar towards native soil carbon is proposed to be related to enhanced aggregation, as extensive fungal growth was observed in hydrochar-amended soils, and promotes soil aggregate formation (Libra *et al.*, 2011). This positive influence was also visible from soil moisture contents, as hydrochar amended plots had considerably larger water content compared to control plots for almost 10 months following hydrochar addition (Fig 8, Chap 5).

Regarding the influence of hydrochar on emissions of non-CO_2 greenhouse gases and on plant growth, the results of this study are insufficient to give conclusive remarks. Hydrochar addition is reported to enhance CH_4 and N_2O emissions from soil but this could only be observed in field conditions as both processes prefer complete anoxia. In contrast, my incubation system was

aerobic for these experiments, and as soil columns were continuously flushed with synthetic air, there were fewer chances for anaerobic microsites to form and provide regions of methanogenesis and denitrification. Negative influences of hydrochar on plant growth were observed in our experiments, but there was no supporting evidence for whether these influences were related to volatiles or labile portions of hydrochar or were an effect of N immobilization as reported in some studies (Busch *et al.*, 2012; Busch *et al.*, 2013; Bargmann *et al.*, 2014).

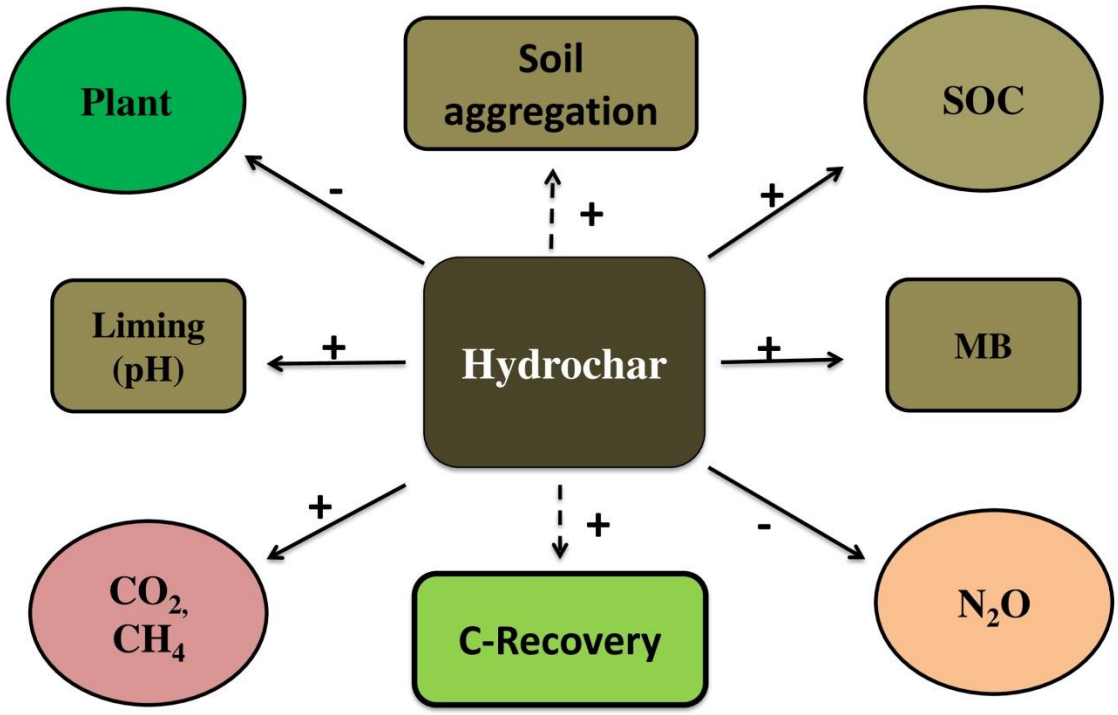

Figure 6.2: A schematic diagram of influences of hydrochar on different soil functions related to carbon sequestration, dotted lines represent observations based on the literature. The sign (+ & -) indicate type of influence of hydrochar on different variables. (MB, microbial biomass; SOC, native soil organic carbon; C, carbon).

In my opinion, this study is still not sufficient to draw a final conclusion on the potential of hydrochar for carbon sequestration. Quantitative estimates for carbon recovery during the hydrothermal process were not available since we purchased the hydrochar commercially. Nor could we quantify the positive or negative influences on non-GHGs and on plant growth in terms of equivalent carbon units. Considering only the stability of char and related priming effects, I hypothesize that hydrochar can successfully sequester at least 10-20% biomass carbon for a long term (decades), excluding energy equivalent carbon emissions and thus representing a potential carbon sequestration tool. However, this method should be limited to usage of unconventional biomass as pyrolysis technique could be the best available technique for carbonization of dry conventional biomass. Furthermore, the direct application of hydrochar on agricultural soils may

require pre-treatments, such as drying or composting, both of which have been reported to be beneficial (Libra *et al.*, 2011; Busch *et al.*, 2013).

6.5 Conclusion and future outlook

This work will contribute in understanding a number of aspects of the processes controlling atmospheric trace gas production and consumption in soils. Most of the aspects have already been discussed in individual discussion and conclusion sections of each chapter here at this point the main idea is to highlight some future ideas to further enhance our understanding.

Although the study regarding the role of O_2 was incomplete and had several drawbacks it indicated several important parameters that are worth including in ecosystem studies. It is known that O_2 is not equally distributed within the soil matrix, and most probably exhibits strong gradients within the scale of a soil aggregate but there is not much data to quantify O_2 availability in soil. This work demonstrated a direct dependence of microbial activity on O_2 concentrations as observed in CO_2 emission rates, highlighting a decisive role of such sites depending on their proportion to total soil volume. In this regard, more research is needed, especially using advanced O_2-sensing technologies and mapping techniques to show the distribution of O_2 gradients across soil profiles.

A large amount of research is needed to fulfill the knowledge gap in the area of understanding of atmospheric CH_4 oxidation in upland soils. This study revealed that capacity of upland soils to oxidize atmospheric CH_4 depends critically on the methanotrophic community structure. However, it is still unclear whether all methanotrophs that are extracted from soil are actively participating in the methane oxidation. In this regard, SIP-RNA technique -would be the best option to identify active methanotrophs. As I did not observe a community shift, but did observe a uptake rates proportional to the CH_4 concentration in supply air, the use of 60 ppm of ^{13}C-labeled CH_4 to enhance the turnover time of methanotrophic community and to label C in active high affinity methanotrophs. Apparently the upland soil cluster (*USCα*) seems to be the best candidate as evident from their high abundance in soils exhibiting higher CH_4 oxidation rates. However, *USCα* absence from the CH_4 oxidizing agricultural soil indicates activity of some other methanotrophic groups.

I included certain factors in this study to observe whether they enhance methanotrophic activity in soils or not. Unfortunately, none of following factors affected methanotrophic activity: low O_2 (it was expected that methanotrophs are aerophiles and perform better at sub-oxic conditions (Henckel *et al.*, 2000), increased CH_4 sources (expected in the presence of intrinsic CH_4 production performance to also increase methanotrophy (Kammann *et al.*, 2009)), or pyrochar had positive influence on methanotrophic activity. The only positive parameter that was linked with high CH_4 oxidation rates was methanotrophic community structure. To this end, it is important to reveal which factors other than agricultural practices shape methanotrophic community structure in upland soils.

SUMMARY

This work is comprised of two parts, both of which use controlled experiments to understand how soil microbes influence greenhouse gas production and consumption for soils selected to represent a range of different properties or land use characteristics. The first part explores how methane (CH_4) and nitrous oxide (N_2O) fluxes respond to factors that have the potential to alter microbial activity or community structure, including soil moisture, O_2 supply and CH_4 fertilization. Experiments were conducted in laboratory using a continuous flow through system designed to flush chambers continuously with inlet air (30 ml/min) of variable composition, and that mixes air within the chamber thoroughly, minimizing effects of diffusion

Across all experiments and soils we performed, the emissions of carbon dioxide (CO_2) were directly correlated with microbial biomass, and microbial biomass represented a nearly constant fraction of the soil organic carbon. Overall, forest soils had significantly higher soil microbial biomass, soil organic C concentrations, and the highest CO_2 emission rates compared to agricultural soils. The constant ratio of CO_2 emission per unit microbial biomass (metabolic quotient) highlights the role played by soil organic matter in controlling microbial community size and therefore its own rate of decomposition. In the presence of plant litter or biomass amendments, this ratio was not conserved, indicating that fresh organic matter of different quality exerts a different kind of control on decomposition rates.

N_2O production rates were found to be insensitive to shifts between 20.5% and 1% in O_2 levels in soil inlet air across five very different soils that represented a range of land uses, C contents and NO_3^- availabilities. Interestingly, the same soils produced 4-100 times more N_2O under complete anoxia. Under anoxic conditions required for denitrification, the N_2O fluxes were highest in forest soils followed by grassland and agricultural soils and were correlated with soil NO_3^- contents.

CH_4 oxidation in upland soils plays an important role as a sink for atmospheric methane. A detailed experiment that manipulated the supply of CH_4 demonstrated that the capacity of different soils to oxidize atmospheric CH_4 was related to methanotrophic community composition rather than other factors that influence microbial activity. The methanotrophic community composition results revealed the presence of upland soil cluster (*USCα*) and other high affinity methanotrophs in forest soils. In an agricultural with lower CH_4 oxidation rates, however, the soil microbial community was dominated by *Type 1a* methanotrophs which are generally observed in perturbed or disturbed soils.

A DNA based QPCR assay revealed no significant shift in *pmoA* gene copy number in two forests and one agricultural soil with sustained differences in the supply of CH_4 substrate, even after exposure to 60 ppm of CH_4 for 24 connective days. This supports the hypothesis that CH_4 is not limiting for these microbial communities, and that other carbon sources must be involved in maintaining methanotrophic community size. The only exception, where higher CH_4 concentrations were reflected in *USCα* related *pmoA* gene copy numbers, occurred in the forest soils but the increase in *USCα*-related methanotroph community was small compare to change in

CH_4 oxidation rates. This outcome indicated that the proportion of CH_4-C assimilated by *USCα* was lower than the 30-50% of CH_4 oxidized that has been proposed previously.

The second part of this work investigates the carbon sequestration potential of char produced by hydrothermal carbonization. Hydrothermal carbonization is a low temperature technique which carbonizes biomass in the presence of water. Two experiments showed decomposition of hydrochar was comprised of two components, a labile component that was rapidly decomposed and a sustained phase that persisted for longer times (turnover time of ~19 years) in soils. These decomposition dynamics were only little influenced by the properties of the soil to which hydrochar was added. In, both field and laboratory experiments, the addition of hydrochar reduced the decomposition rate of native soil carbon, even though positive priming was also recorded during initial stages of decomposition. Other positive influences of hydrochar included the suppression of N_2O production, most probably due to NH_4^+-immobilization. Negative effects of hydrochar addition included significantly lowered plant growth during the first cropping season after amendment, and increases in CH_4 production by soils. In contrast to hydrochar, pyro-char was immediately more stable in soil and reduced N_2O production rates. To reduce the negative impacts associated with direct application, hydrochar should be pre-treated by removing the soluble phase prior to addition to soils.

ZUSAMMENFASSUNG

Die vorliegende Arbeit besteht in ihrer Gesamtheit aus zwei Teilen, wobei in beiden Teilen der Anspruch vorlag, mithilfe von Experimenten und unter kontrollierten Bedingungen ein besseres Verständnis mikrobieller Prozessen zu erlangen, die verantwortlich für Bildung und Verbrauch von Treibhausgasen in Böden sind. Hierbei repräsentieren die ausgewählten Böden eine Reihe von verschiedenen ökologischen sowie Landnutzungseigenschaften. Im ersten Teil der Arbeit wird die Reaktion von Methan (CH_4) und N_2O Flüssen auf sich ändernde Umweltparameter wie Bodenfeuchte, Sauerstoffzufuhr und CH_4 Düngung untersucht, da diese Parameter das Potential haben, die mikrobielle Aktivität sowie die mikrobielle Gemeinschaft zu verändern. Versuche wurden unter Labor-Bedingungen mit einem "continuous flow" System durchgeführt, dass entwickelt wurde, um Inkubationskammern mit einem permanenten Luftstrom unterschiedlicher Konzentration zu versorgen sowie eine vollständige Durchmischung der Kammern zu gewährleisten und Diffusionsprozesse zu vermeiden.

Emissionen von Kohlendioxid erwiesen sich in sämtlichen untersuchten Böden und unter allen experimentellen Bedingungen als direkt proportional zur mikrobiellen Biomasse, wobei die mikrobielle Biomasse stets einen konstanten Anteil des Bodenorganischen Materials einnahm. Waldböden wiesen im Allgemeinen eine signifikant höhere mikrobielle Biomasse, einen höheren Kohlenstoffgehalt und höhere CO_2 Emissionsraten im Vergleich zu Ackerböden auf. Das konstante Verhältnis von CO_2 Freisetzung zu Einheit mikrobieller Biomasse (metabolischer Quotient) spiegelt die Bedeutung des bodenorganischen Materials für die mikrobielle Biomasse und somit für dessen eigene Abbaurate wider. In Anwesenheit von Streu oder unter Zufuhr von Biomasse wurde der metabolische Quotient nicht beibehalten, was darauf hinweist, dass neu eingebrachtes organisches Material von unterschiedlicher Güte unterschiedlichen Einfluss auf Abbauraten hat.

N_2O Bildungsraten in fünf verschiedenen Böden wiesen bei unterschiedlichen Sauerstoff Konzentrationen keine Änderungen auf, wobei der Sauerstoffgehalt der einströmenden Luft zwischen 20.5 % und 1 % schwankte. Die ausgewählten Böden umspannten ein breites Spektrum von Landnutzung, Kohlenstoffgehalt und NO_3^- Verfügbarkeit. Bemerkenswerterweise produzierten die gleichen Böden unter gänzlich anoxischen Bedingungen 4 bis 100 mal mehr N_2O. Unter anoxischen Bedingungen, einer Grundvoraussetzung für Denitrifikation, erwiesen sich N_2O Flussgrößen in Waldböden am höchsten, gefolgt von Grünland und Ackerböden. Sie korrelierten dabei immer mit NO_3^- gehalten.

CH_4 Oxidation in Oberland Böden ist eine bedeutende Methan Senke. Ein ausführliches Experiment, bei dem die Zufuhr von CH_4 reguliert wurde, veranschaulichte deutlich, dass die Methanoxidationskapazität verschiedener Böden weniger mit Faktoren, welche die Aktivität der mikrobiellen Gemeinschaft kontrollieren, sondern vielmehr mit deren Struktur zusammenhängt. Ergebnisse der Charakterisierung der mikrobiellen Gemeinschaft deuten in Waldböden klar auf die Präsenz von Oberland Boden Cluster (*USCα*) und anderer hoch affiner methanotropher Mikroorganismen hin, wohingegen *Typ 1a* Methanotrophe einen Ackerboden mit geringeren CH_4 Oxidationsraten dominierten. Diese Gruppe kommt vor allem in gestörten Böden vor.

DNA und qPCR Untersuchungen wiesen keine signifikante Änderung in der Expression von pmoA Genen in zwei Waldböden und einem Ackerboden bei andauernd unterschiedlicher Zufuhr von CH_4 auf, selbst nach 24 Tagen sowie unter einer Atmosphäre von 60 ppm CH_4. Die Ergebnisse stützen somit die Hypothese, dass CH_4 nicht der limitierende Faktor für das Wachstum dieser Organismen ist und andere Kohlenstoff Quellen von Bedeutung sein müssen. Lediglich in Waldböden spiegelte die Expression von *USCα* zugehörigen *pmoA* Genen CH_4 Konzentrationen wider. Allerdings war die Änderung der *USCα* Gemeinschaft klein verglichen mit der Änderung der CH_4 Oxidationsrate. Diese Ergebnisse weisen darauf hin, dass der Anteil von CH_4 assimiliertem Kohlenstoff durch *USCα* geringer ist als 30-50%, wie bisher angenommen.

Im zweiten Teil der Dissertation wird das Kohlenstoffspeicherungspotential hydrothermaler Biokohle untersucht. Hydrothermale Durchkohlung ist ein niedrig thermaler Prozess, bei dem Biomasse in Anwesenheit von Wasser inkohlt wird. In zwei Experimenten wurde veranschaulicht, dass der Abbau von Hydrokohle aus zwei Komponenten besteht. Eine labile Komponente wurde schnell abgebaut, im Gegensatz zu einer schwer abbaubaren Komponente, die eine längeren Verweilzeit im Boden aufwies (Umsatzrate = 19 Jahre). Die Abbau Dynamik wurde nur geringfügig von den Bodenparametern beeinflusst. Sowohl in Feld-, als auch in Laborversuchen verringerte die Zugabe von Biokohle die Abbaurate des bodenorganischen Materials, obgleich positives Priming auch im Anfangsstadium des Abbaus beobachtet wurde. Eine weitere positive Auswirkung von Biokohle war Unterdrückung von N_2O Produktion, wahrscheinlich durch NH_4^+ Immobilisierung. Negative Folgen der Biokohle Zugabe waren ein stark vermindertes Pflanzenwachstum in der ersten Wachstumsperiode sowie eine Zunahme der CH_4 Produktion in den Böden. Im Gegensatz zu hydrokohle erwies sich Pyrokohle im Boden als wesentlich stabiler und verringerte die N_2O Produktion. Um Negativfolgen der Biokohle Zugabe zu verringern, sollte Hydrokohle vorgetestet werden, indem lösliche Phasen vor Zugabe in Böden entfernt werden.

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APPENDIX

Appendix A1

(For Chapter 2)

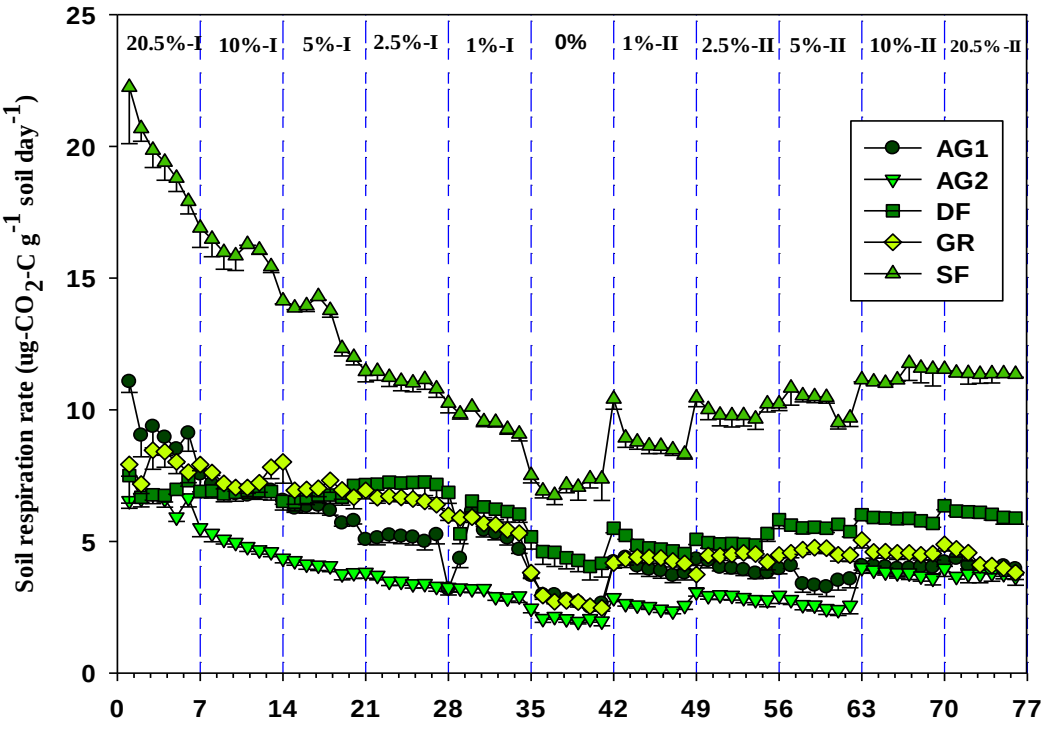

Fig A1.1: Mean respiration rates measured from different soils incubated in the continuous flow system at constant temperature and moisture (20°C temperature and 50% WFPS).Treatments began on the 4th day of incubation. Mean±standard error (n=4).

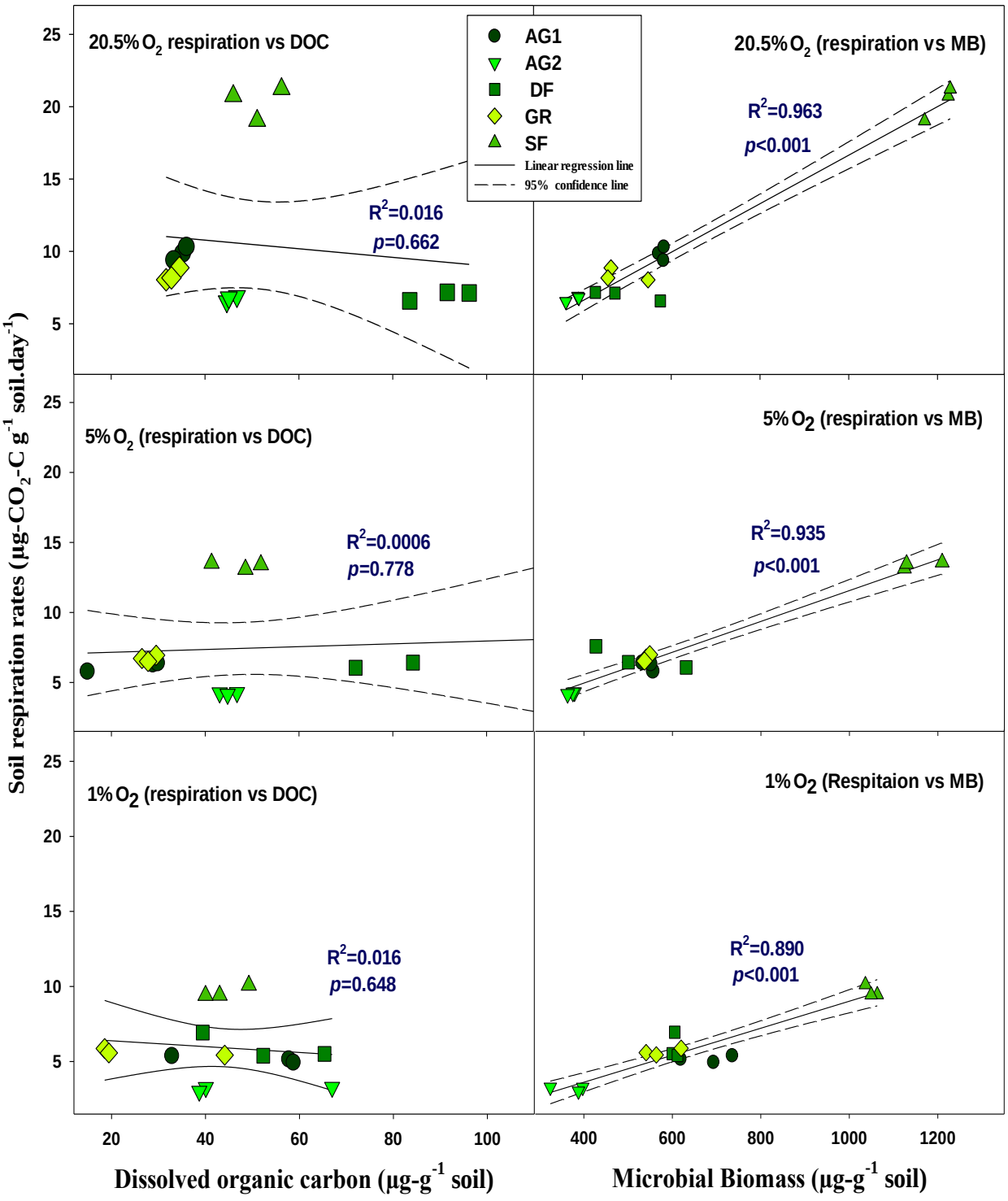

Figure A1.2: Linear regression of respiration rates in different soils with dissolved organic carbon (DOC) and microbial biomass (MB) (n=15).

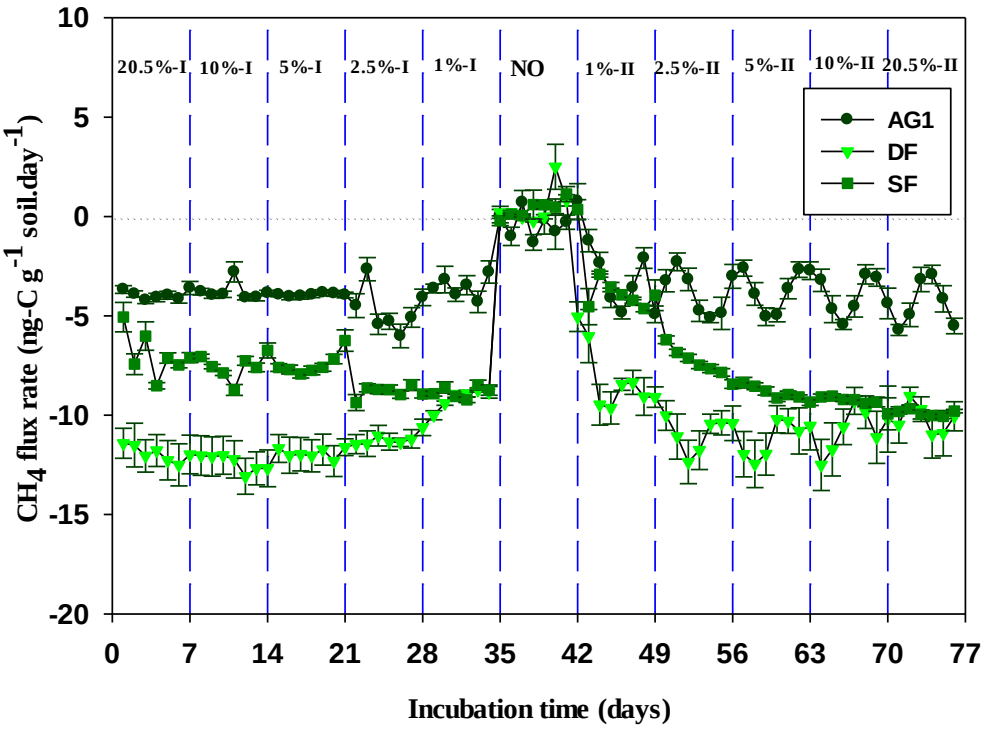

Figure A1.3: Daily CH_4 fluxes measured in three contrasting soils. Mean± standard deviation (n=4).

The isotopic values of CH_4 were determined by stable isotope ratio mass spectrometry (GC-C-IRMS). The analyses were carried out in laboratories at MPI-BGC (Sperlich, 2013). While, The isotopic fractionation factor (α) for carbon (^{13}C) and Hydrogen (D) isotopes between inlet and out air was determined using Rayleigh equation that best explain changes in CH_4 concentrations and isotopes values in closed chamber systems (Snover and Quay, 2000)

$$\frac{1}{\alpha_{soil}} = \left[\frac{Ln\left\{\frac{(\delta_{oulet} + 1000\ )}{(\delta_{inlet} + 1000)}\right\}}{Ln\left(\frac{C_{outlet}}{C_{intlet}}\right)}\right] + 1$$

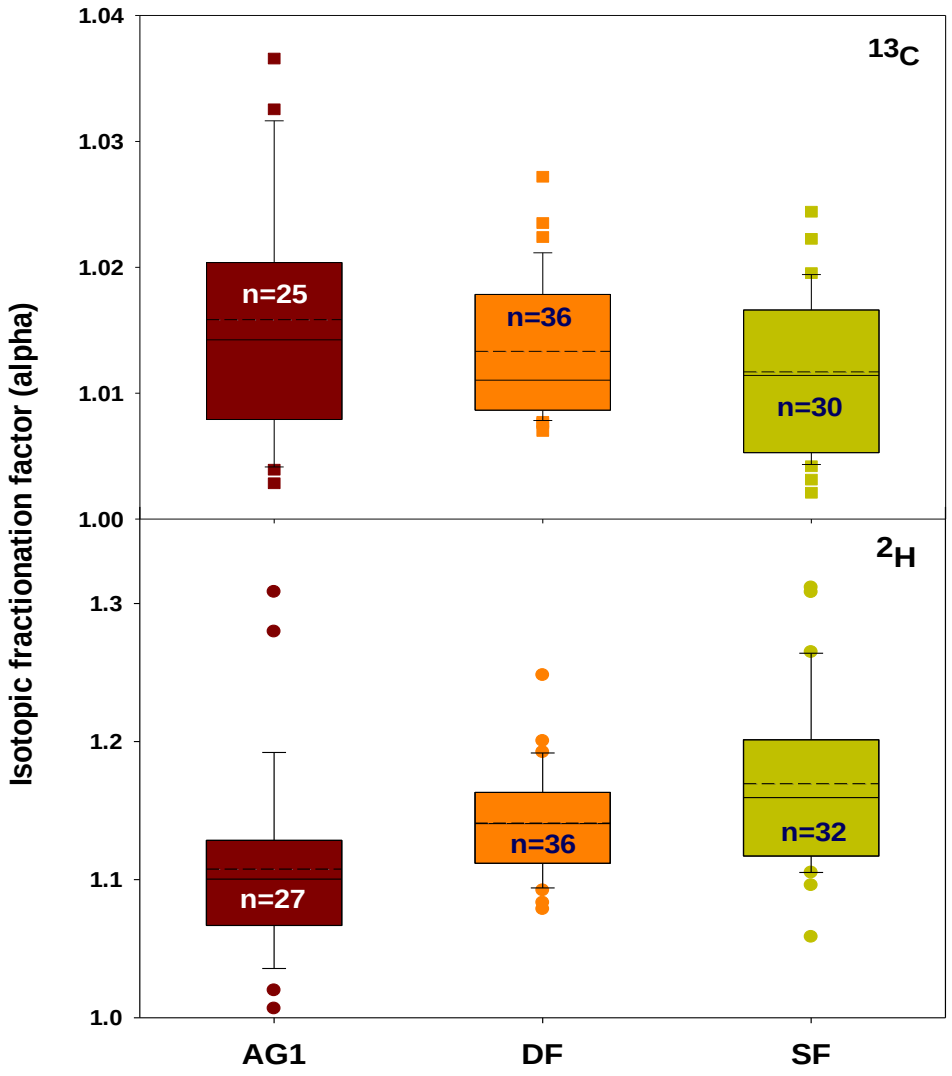

Figure A1.4: Isotopic fractionation factors (α) for stable isotope of CH_4 ($\delta^{13}C$ and δD) recorded in three different soils in the presence of O_2. The number written on each box represent total measurements of each soil type at all O_2 levels.

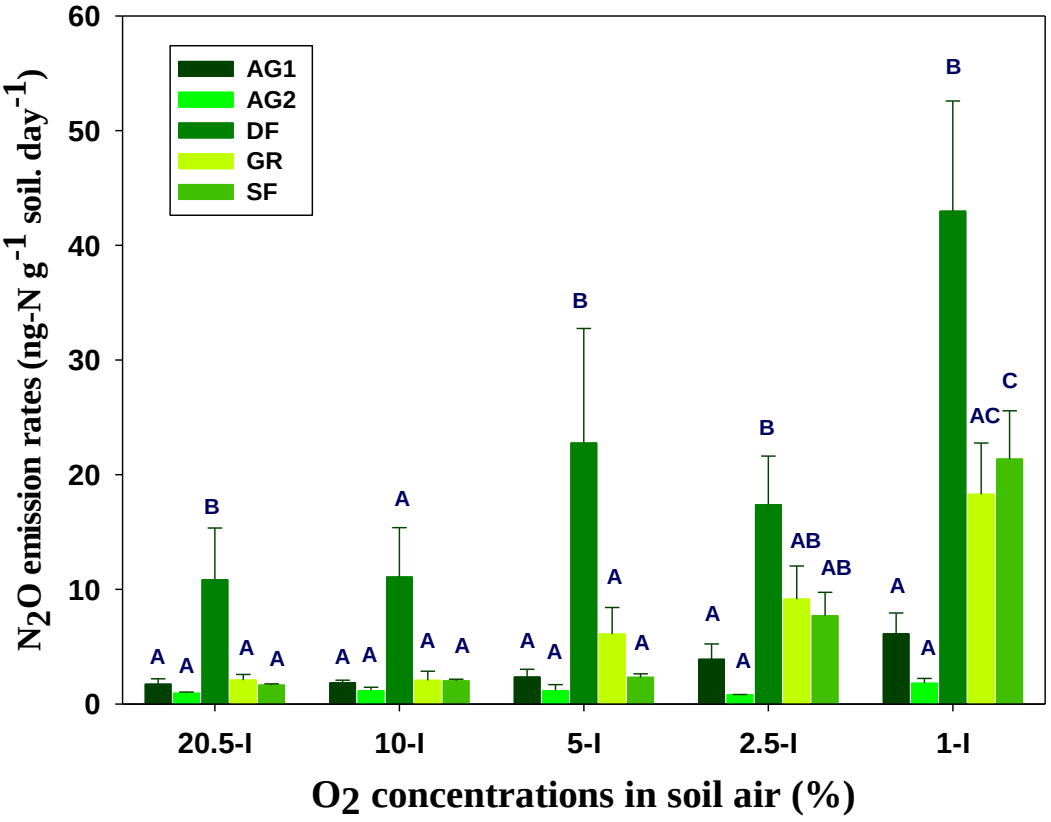

Figure A1.5: N_2O emission rates from different upland soils incubated in continuous airflow system. O_2 concentrations in inlet air (soil air) were shifted weekly and N_2O fluxes were measured on 5^{th} day of each shift. Difference uppercase letters represent statistical significance between soil types.

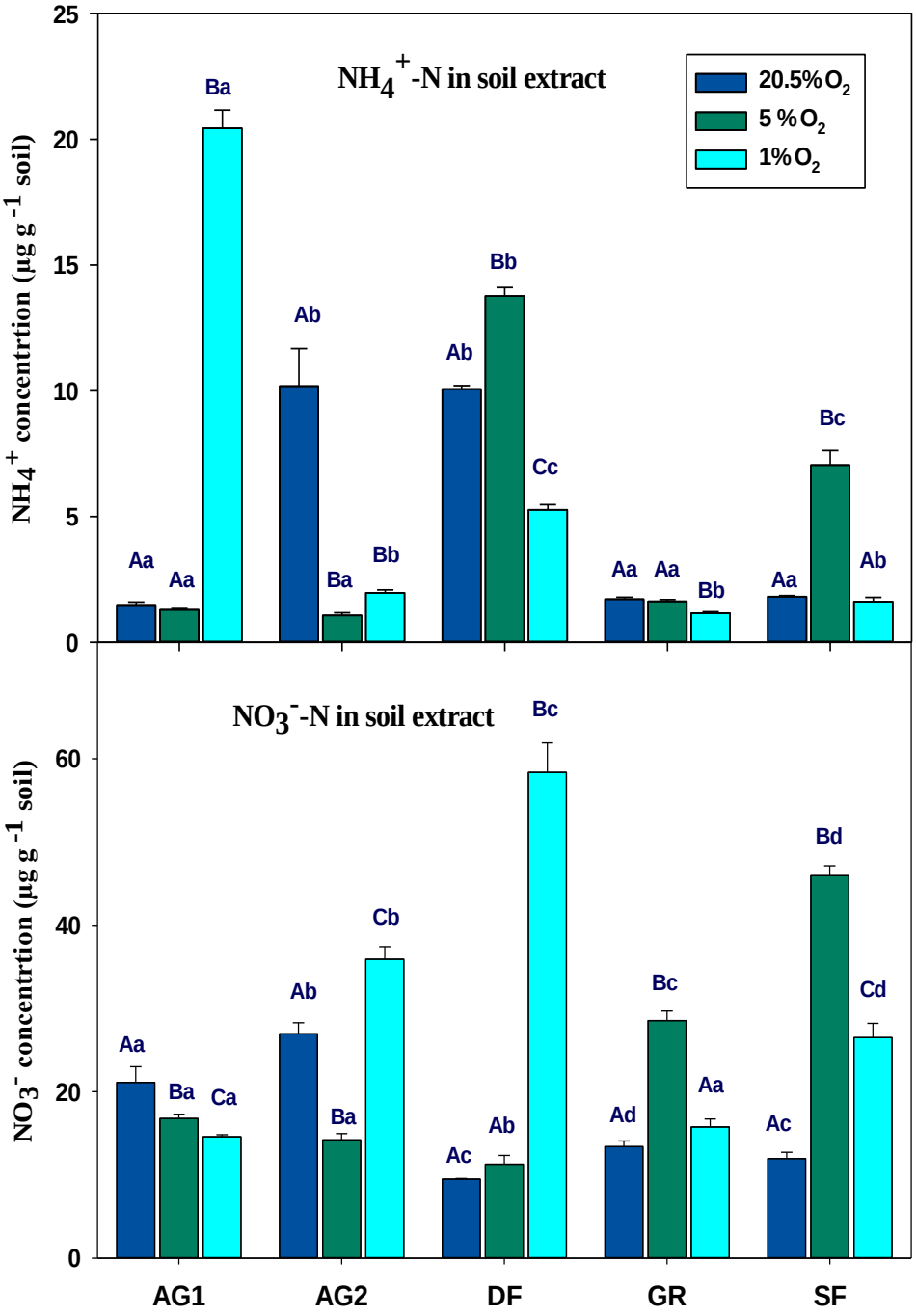

Figure A1.6: Concentration of mineral nitrogen, NH_4^+ and NO_3^- determined after different O_2 treatments. Different uppercase letters represent statistical significance among O_2 levels in one soil type whereas, lowercase letters represent significant differences among soil types in each O_2 level.

Appendix A2

For Chapter 3

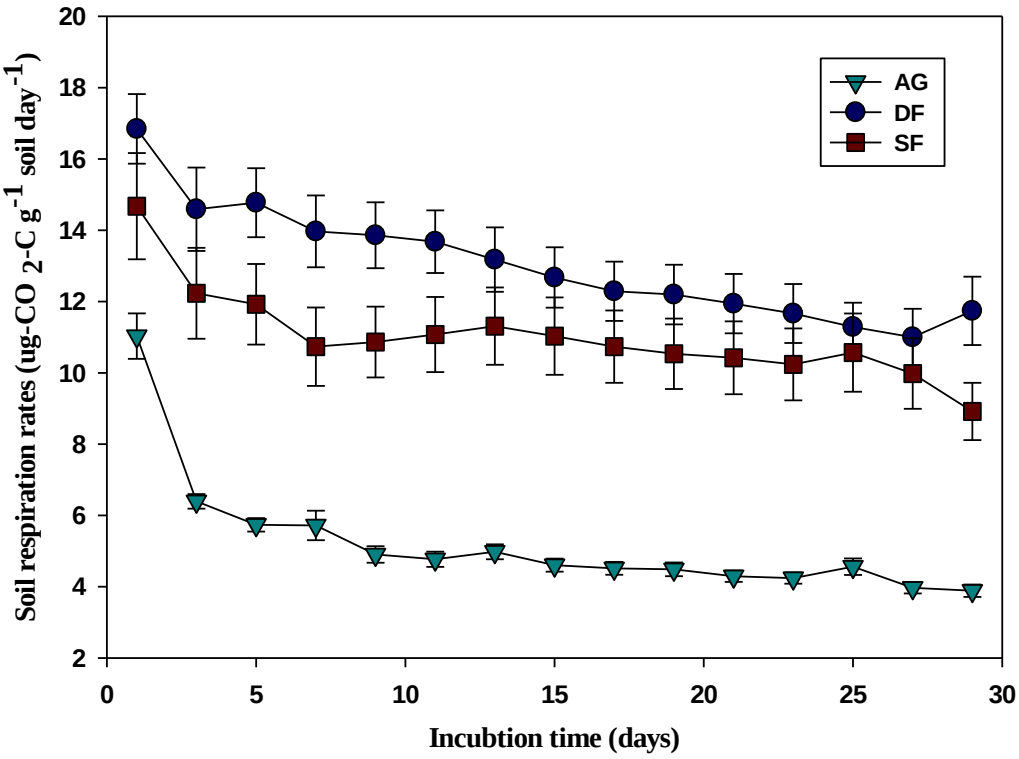

Figure.A2.1: Average soil respiration rates of three different soils incubated at constant temperature (25℃) and moisture (50% WFPS). Each symbol represent Mean±S.E (n=15)

Table A2.1: Detail about reaction mixture and thermal profile used for Real Time PCR assays

Gene	Reaction Mixture	Thermal profile
16S-rRNA gene (Degelmann et al., 2010)	20 µl {10 µl l EvaGreen$^{(R)}$ Supermix (Bio-Rad), 0.08 µl each primer (100 µM), 0.2 µl BSA (50 mg/ml), 3.2µl $MgCl_2$(25 mM), H_2O^a, 5 µl DNA template (1:500 diluted)	Denaturing (94°C, 20s), Annealing (50°C, 20s), elongation (72°C, 50s), Cycles (50), fluorescence data acquisition (75°C, 6s)

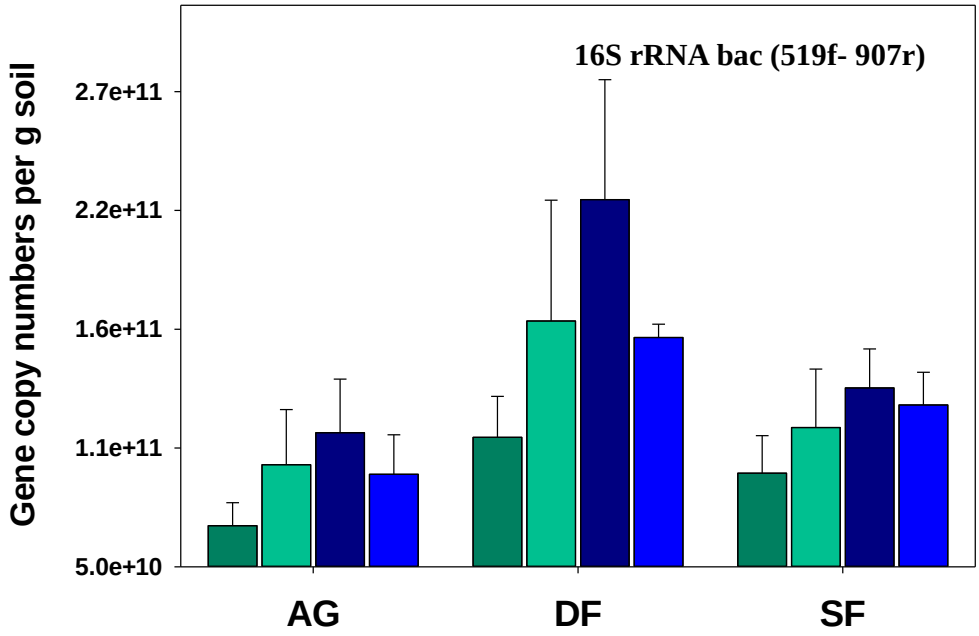

Figure A2.2: The 16S rRNA gene copy numbers in soils representing contrasting land use types. Mean±standard error (n=4)

Table A2.2: Details about primers and thermal profile used for PCR cycle

Primer Pair	Sequence (5'–3')	Thermal profile [a]	Molecular Analysis
27f-FAM* 907r	AGAGTTTGATCCTGGCTCAG CCGTCAATTCMTTTRAGTT	Denaturing (94°C, 45s), Annealing (52°C, 45s), elongation (72°C, 90s), Cycles (25),Final elongation (72°C, 7min)	T-RFLP 16S.Bac

* 5-carboxyfluorescein.

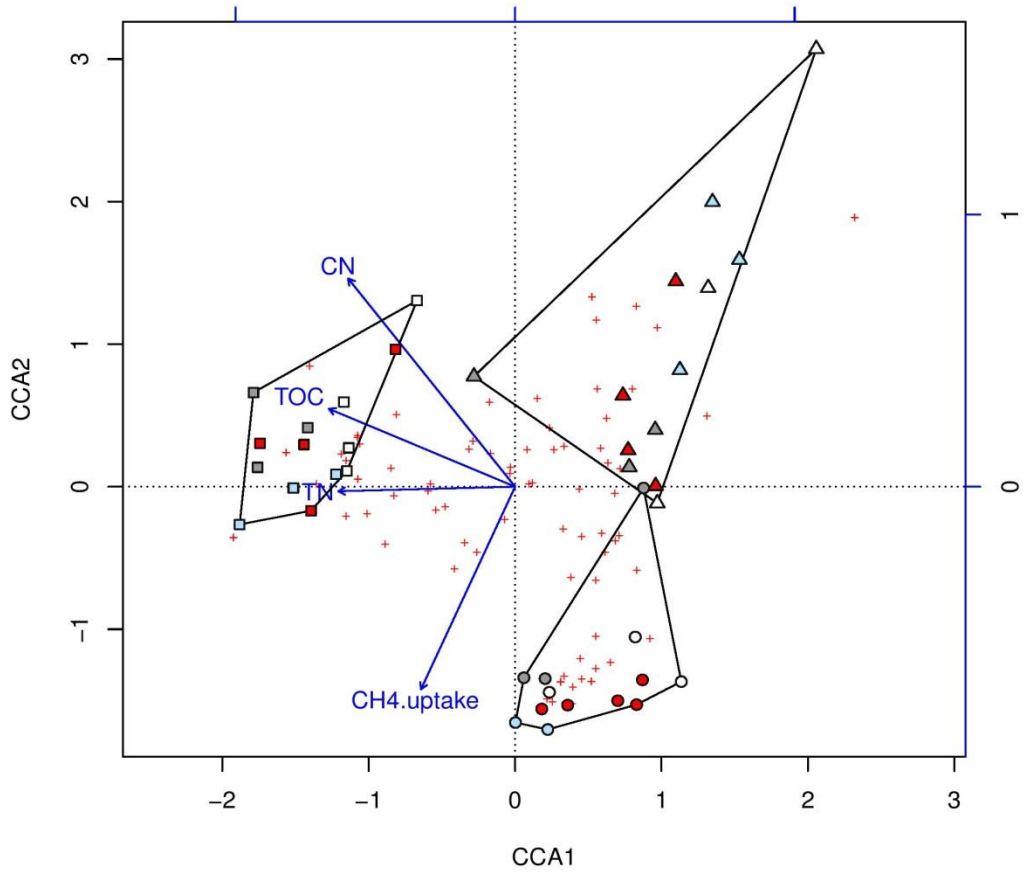

Figure 2A.3: Canonical correspondence analysis of 16 S rDNA gene based T-RFLP data sets and environmental data in different land-use types. (■) AG (●) DF (▲) SF, Different color of symbols represents CH_4 treatments. Environmental variables were chosen based on significance (P<0.001).

Appendix A3

For Chapter 4

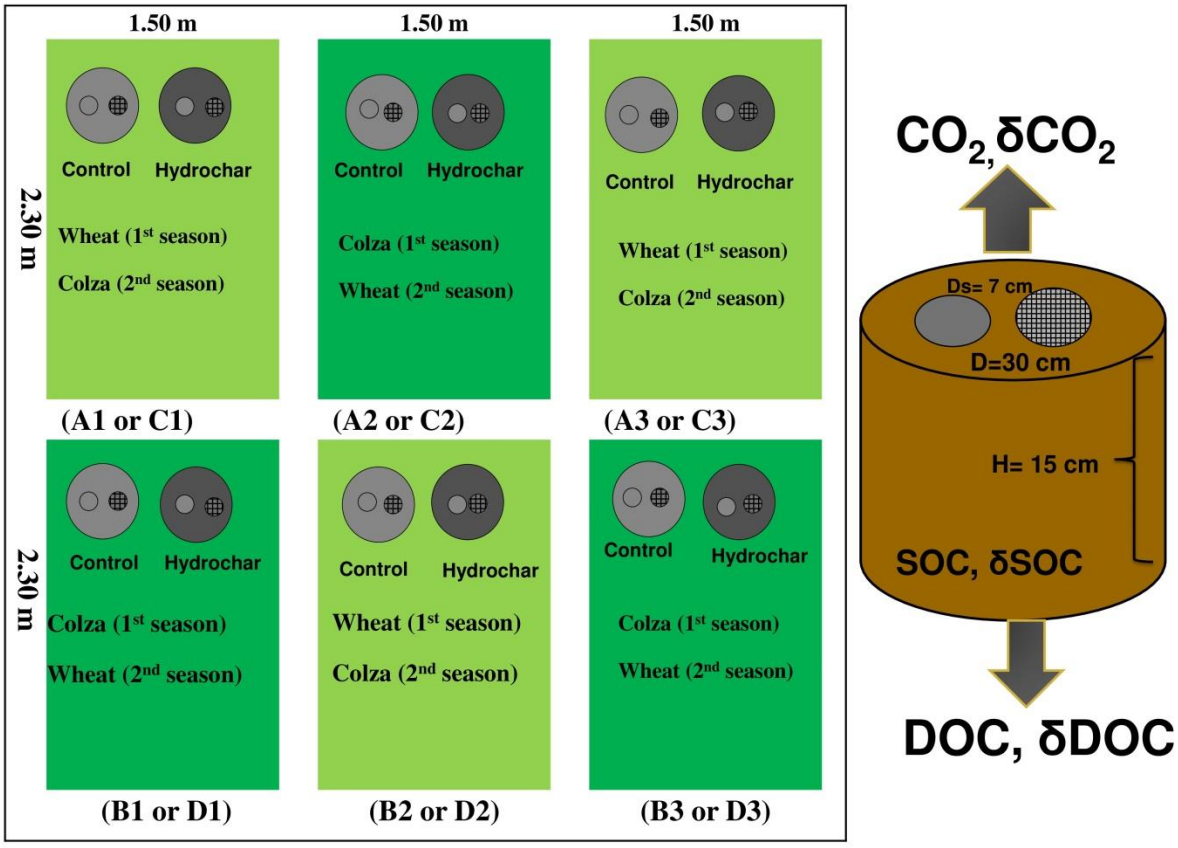

Figure A3.1: Schematic diagram of experimental set up in field and design of collar (ring). Air samples were determined directly on top of small size ring with screen in bottom to avoid any root growth. While, soil leachate was collected at the bottom of soil column via suction plates that was placed in center of large ring. Bulk soil carbon was measured 3 different time intervals, at initial, after 31/2 months and end of the experiment (after 1 year).

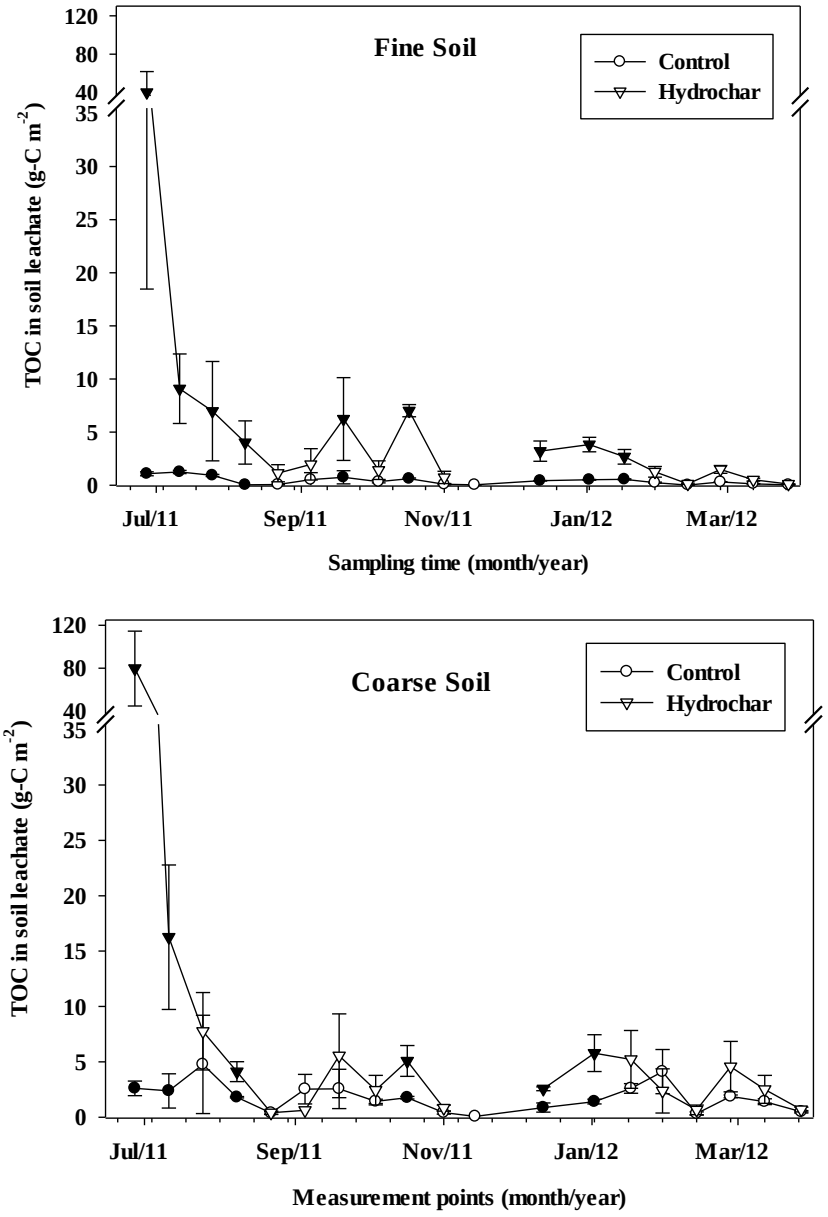

Figure A3.2: Rates of dissolved organic carbon in soil leachate collected at 15 cm depth during 1 year of field trial (A) Fine soil (B) Coarse soil. Filled symbols represents statistical significance between control and hydrochar treatments (Tukey α=0.05). (Mean±S.E, n=6)

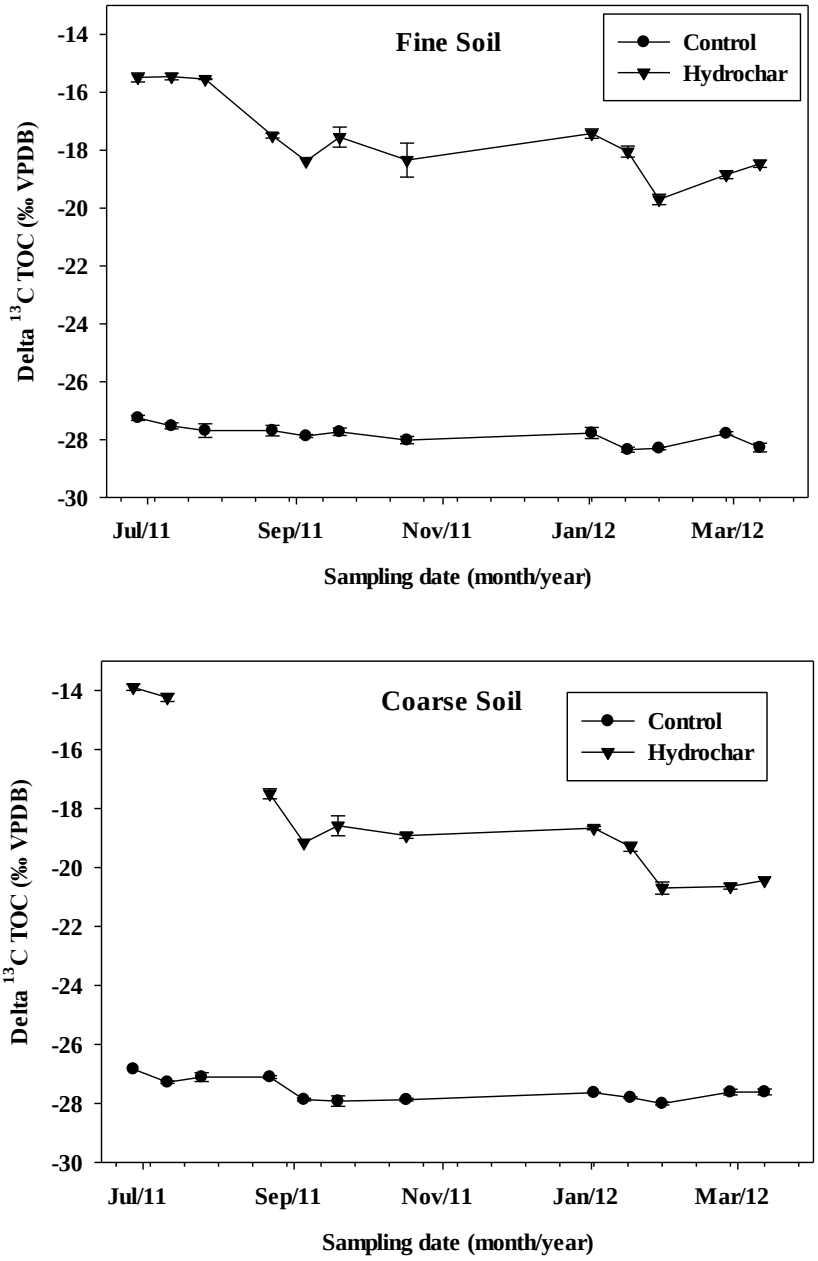

Figure A3.3: Stable isotope ratio ($\delta^{13}C$) of dissolved organic carbon in soil leachate collected from (A) Fine soil (B) Coarse soil. Samples from fine soils were treated with weak acid prior to analysis to remove effect of $CaCO_3$. Replicates with very low amount of leachate were not analysed for ($\delta^{13}C$) thus each measurement point represent Mean±S.E (n=4-6).

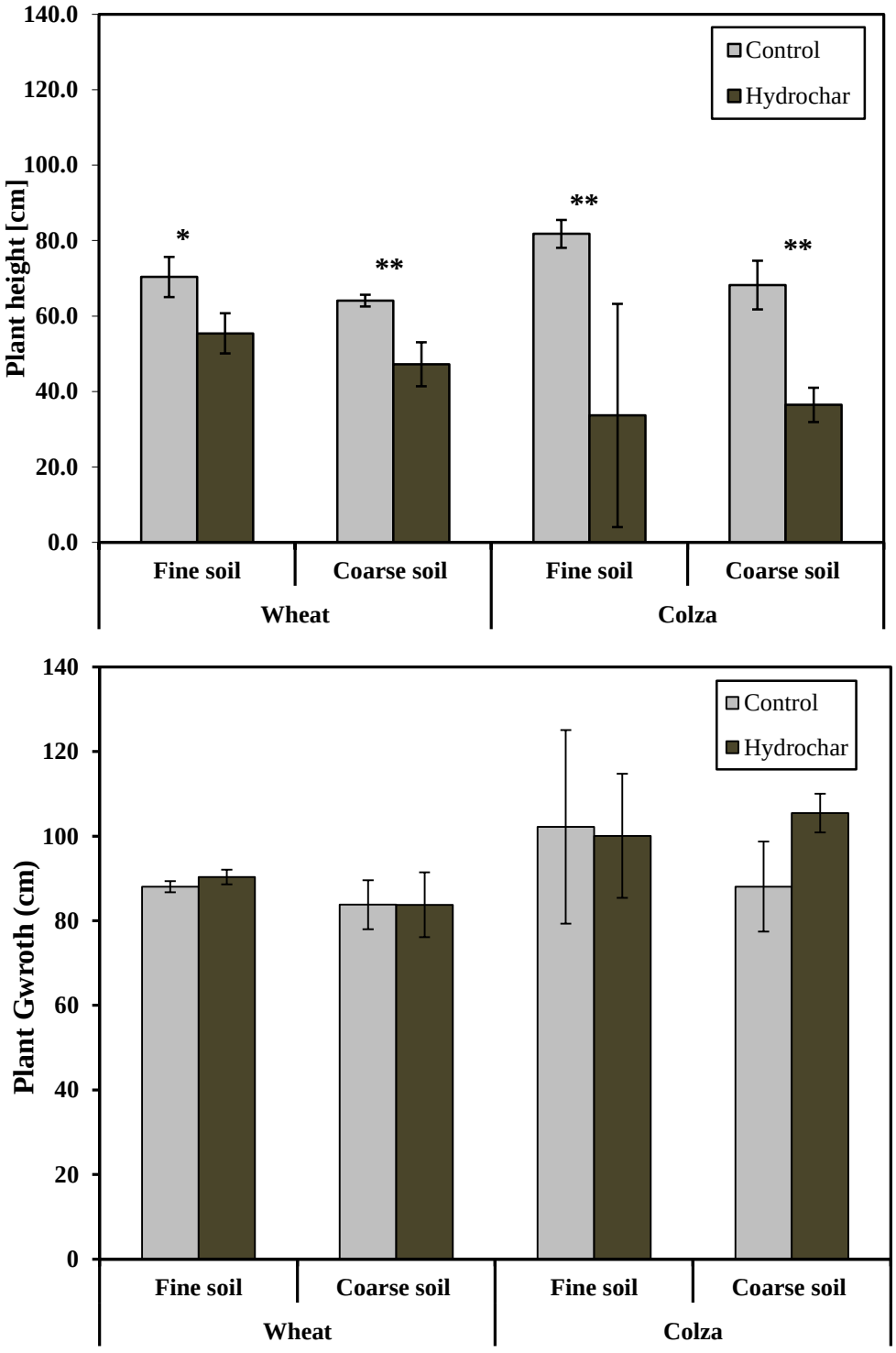

Figure A3.4: Heights of the plants (cm) measured after harvesting at (A) First cropping season (Jun-October 2011) (B) Second cropping season (Oct-April 2012). Symbol on top of the column represents statistical significance between control and hydrochar treatment of each soil type. Mean±S.E (n=3).

Appendix A4

(For Chapter 5)

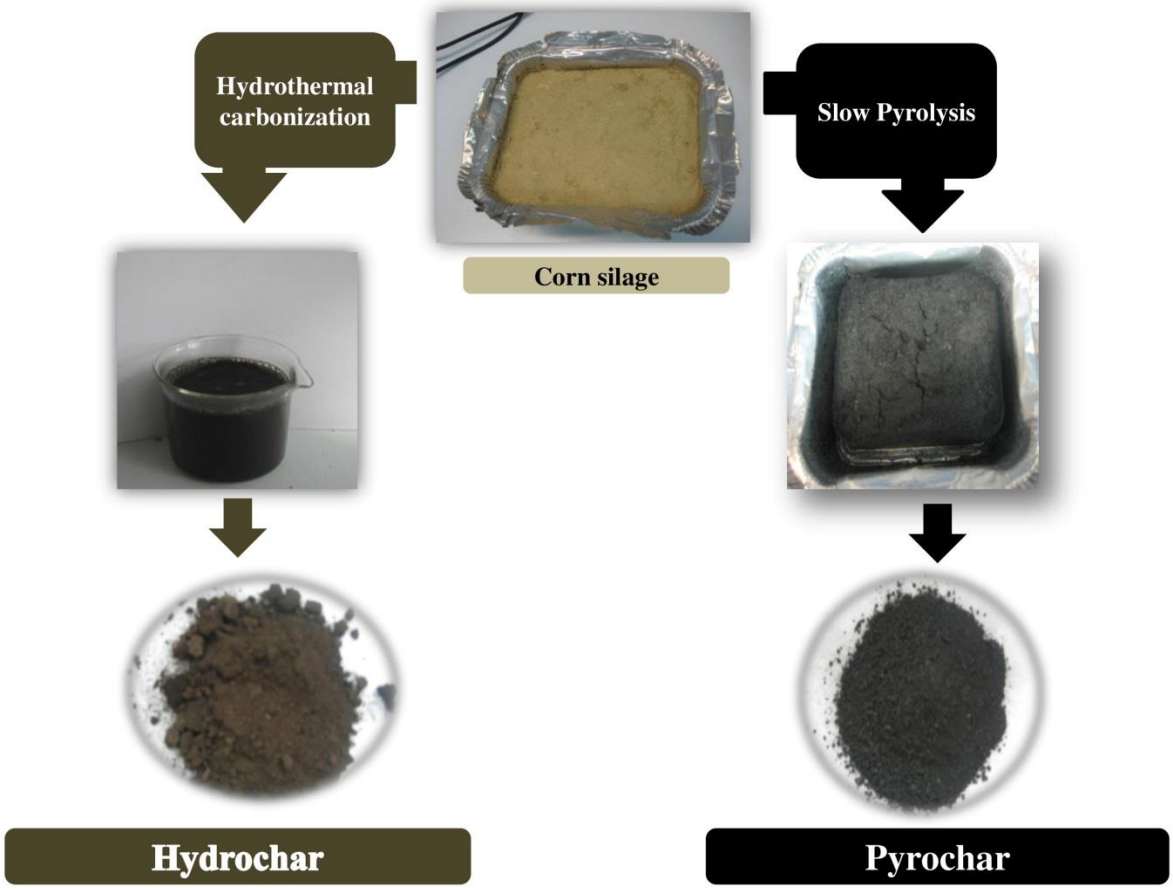

Figure A4.1: Schematic diagram to show differences in appearance between two char produced by same feedstock but contrasting thermal methods. Pyrochar is product of slow pyrolysis process and was produced at 500°C for 2 hour residence time. While, hydrochar is product of hydrothermal carbonization produced at 180-220°C under 18-20 bar pressure

Declaration of independent work

I hereby declare that this thesis was written by me under the supervision of Prof. Susan Trumbore and whatever, personal or literature help was taken for the preparation of thesis is mentioned in the acknowledgements and references sections.

Jena, 25.10.2014

Saadatull Malghani