An investigation into the degradation of biochar and its interactions with plants and soil microbial community

By

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DECLARATION

By submitting this thesis electronically, I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any other university for obtaining a degree.

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C.F. Olivier Date

December 2011

ABSTRACT

Biochar (charcoal) is lauded by many scientists as an effective way to remove carbon dioxide from the atmosphere and storing it in a very stable form in the soil for hundreds to thousands of years, whilst promoting soil fertility and productivity. Considering that no significant amounts of charcoal are presently accumulating in the environment, despite considerable amounts produced globally in natural and man-made fires, this study focuses on understanding the degradation of biochar and its interactions with plants and soil organisms. The following experiments were conducted to achieve this goal.

Controlled chemical oxidation of biochar, using different concentrations of hydrogen peroxide, was conducted in an attempt to mimic the enzymatic degradation of biochar by basidiomycetes. The changes occurring in biochars structure and chemistry were assessed afterwards. Furthermore, aerobic and anaerobic digestion of biochar was conducted *in vitro,* and *in vivo* to investigate the changes occurring in biochar‘s elemental composition and chemistry during oxidation and factors that play a determining role in the rate of biochar degradation. The influence of biochar in soil on free-living and symbiotic microbial communities as well as its impact on total plant biomass production and root development was assessed in three greenhouse pot trials using wheat and green beans as test plants

It was proven that biochar is almost fully H_2O_2-degradable, mostly through hydroxylation and carboxylation reactions which led to the formation of various short chained carboxylic acids, surface saturation with acidic functional groups as determined by the surface acidity measurements and proven by the increase in the intensity of FT-IR peaks associated with carboxyl and phenolic C-O groups. Furthermore, hydrogen peroxide treatment resulted in preferential removal of volatile organic carbons and led to the purification of biochar as evident by the new, more intense and sharper peaks in the region of 1600-1000 cm^{-1}. These FT-IR peaks are considered as the more recalcitrant fraction of biochar and were shown to be mostly associated with transformation products of lignin and cellulose formed during pyrolysis.

The incubation trial confirmed that biochar cannot be utilized as a sole carbon source without the addition of nutrients or glucose, to activate microbial activity within the columns. Furthermore, abiotic oxidation can be facilitated by oxidative soil minerals such as birnessite, but oxidation

with atmospheric oxygen did not result in the evolution of CO_2 from biochar. The average CO_2 production in pot trials without plants in both the fertilized and unfertilized treatments increased linearly (R^2= 0.80; 0.79 respectively) with increasing biochar application rates when biochar was the main carbon sources.

Anaerobic degradation of biochar by a methanogenic consortium was much more efficient in utilizing biochar as a carbon source, compared to aerobic digestion. The anaerobic digesters maintained a chemical oxygen demand (COD) removal efficiency of 30% per week with continuous production of CO_2, whilst methane production was very erratic. We proposed that better control over pH and alkalinity as well as an increase in hydraulic retention time would improve both the COD removal efficiency and methane production.

Field incubations resulted in various degrees of oxidation at different incubation sites. An increase in the oxygen content and a decreased in the carbon content of biochar‘s elemental composition and also an increase in the surface acidity due to a larger amount of carboxyl acid groups on the surface as seen in the increase in the FT-IR peak at 1700 cm^{-1} confirmed that biochar are susceptible to oxidation under field conditions. We came to the conclusion that oxidation and mineralization of biochar in this trial occurred at a faster rate in soils with a higher microbial activity.

The pot trials, confirmed that biochar does not serve as a fertilizer even though it did increase total biomass production between biochar application rates of 0.05-2.5 % (w/w). For agricultural purposes the addition of biochar should always be applied together with NPK fertilizer. In both the wheat and green bean trials it was confirmed that biochar application rates of 0.05-0.5% (w/w) on the sandy, slightly acidic soil used in this trial resulted in the greatest biomass production and fertilizer use efficiency.

Biochar additions resulted in considerable increases in soil pH and C/N ratios which were considered as the main reasons for the decrease in microbial biomass in the unfertilized green bean treatments as it made the uptake of N more limited. The addition of fertilizer however, alleviated N-supply constraints and as a result promoted microbial growth at all biochar application rates of pot trial 1. However, biochar did not promote mycorrhyzal colonization and caused a decrease in the mycorrhizal colonization of roots with increasing biochar application rates and within biochar layers. Biological nitrogen fixation, however, reacted positively to the

addition of biochar. High biochar application rates significantly enhanced the plants reliance on these symbiotic relationships. We hypothesized that biochar physically immobilized N into its microvoids through capillary suction and then served as a physical barrier between plant roots and absorbed N. However, immobilzation of N by microbes could also have contributed to the decrease in N uptake if one takes into account that microbial activity was higher (respiration data) at the higher biochar application rates. Further investigations are needed to warrant this hypothesizes.

OPSOMMING

Biochar (houtskool) is deur talle wetenskaplikes die lof toegeswaai as ‘n doeltreffende manier om koolstofdioksied uit die atmosfeer te verwyder en in ‘n baie stabiele vorm in die grond vir honderde tot duisende jare te stoor, terwyl dit die grondvrugbaarheid en produktiwiteit bevorder. As daar in ag geneem word dat geen beduidende hoeveelheid houtskool in die omgewing opgaar nie ondanks groot hoeveelhede wat wêreldwyd deur natuurlike en mensgemaakte brande gevorm word, is die doel van hierdie studie om die afbraak en die interaksie van *biochar* met plante en grondmikrobes beter te verstaan. Om hierdie doel te bereik is die volgende eksperimente uitgevoer:

Beheerde chemiese oksidasie is op die *biochar* toegepas deur gebruik te maak van verskillende konsentrasies waterstofperoksied in ‗n poging om die ensiematiese afbraak van *biochar* deur basidiomysete na te maak. Die veranderinge wat plaasvind in die struktuur en chemie van biochar is daarna bestudeer. Daarbenewens is die aerobiese and anearobiese afbraak van biochar toegepas beide *in vitro*- en *in vivo*-, om die veranderinge wat in biochar se elementele samestelling en chemie plaasvind gedurende oksidasie en ook die faktore wat ‗n bepalende rol in die tempo waarteen biochar afbreek, te ondersoek. Die invloed van biochar in die grond op vrylewende en simbiotiese mikrobiese populasies, sowel as die impak daarvan op die totale plant biomassa produksie en ontwikkeling van plantwortels, is vasgestel tydens drie groeitonnel potproewe waarby koring en boontjies as planttoetsspesies gebruik is

Dit is bewys dat biochar byna volledig deur H_2O_2 afgebreek kan word, meestal deur hidroksilasie en karboksilasie reaksies wat gelei het tot die vorming van ‗n verskeidenheid kort ketting karboksielsure, ‗n biochar oppervlak versadig met suurvormende funksionele groepe soos bepaal en bewys deur die toename in intensiteit van die FT-IR (Fourier Transvorm Infrarooi Spektroskopie) pieke geassosieer met karboksiel en fenoliese C-O groepe. Die behandeling van biochar met H_2O_2 het by voorkeur die vlugtige organise koolstof verwyder wat gelei het tot suiwering van die biochar, wat bevestig is deur die nuwe, meer intens en skerper FT-IR pieke in die area tussen 1600-1000 cm^{-1}. Die FT-IR pieke word beskou as die meer weerstandbiedende fraksie van biochar en daar is bewys dat die pieke meestal met getransformeerde produkte van lignien en sellulose wat tydens pirolise gevorm is, geassosieer word.

Die inkubasie proef het bevestig dat biochar nie deur mikrobes benut kan word as enigste bron van koolstof sonder die byvoeging van nutriente of glukose nie, om die mikrobes binne die inkubasie kolom te aktiveer. Daarbenewens kan abiotiese oksidasie van biochar deur oksidatiewe grondminerale soos birnessite (δ-MnO2) gefasiliteer word, terwyl oksidasie van biochar deur atmosferiese suurstof nie tot enige CO_2 produksie gelei het nie. Nogtans het die gemiddelde CO_2 produksie in die boontjie potproef, sonder die plante, in beide die onbemeste en bemeste behandelings linieêr toegeneem (R^2= 0.80; 0.79 onderskeidelik) met toenemende aanwendingskoers van biochar, toe biochar die dominante bron van koolstof was.

Anaerobiese afbraak van biochar deur ‗n metanogeniese konsortium was heelwat meer effektief in die benutting van biochar as enigste koolstofbron in vergelyking met aerobiese afbraak. Die anaerobiese verteertoestel het konstant 30% van die chemiese suurstof behoefte (CSB) weekliks verwyder, gepaardegaande met die voortdurende produksie van CO_2, terwyl metaangasproduksie baie onegalig was. Dit word voorgestel dat met beter beheer oor pH en alkaliniteit en ook ‗n langer hidrouliese retensie tyd, kan beide die CSB verwyderingseffektiwiteit en metaangasproduksie verbeter kan word.

Veld inkubasies het verskeie mates van oksidasie meegebring tussen die verskillende inkubasie liggings. ‗n Toename in die suurstofinhoud en ‗n afname in die koolstof inhoud van biochar se elementele samestelling sowel as ‗n toename in die oppervlak suurheid weens die groter hoeveelheid karboksielsure aan die oppervlak soos blyk uit die FT-IR piek by 1700 cm^{-1}, het bevestig dat biochar wel vatbaar is vir oksidasie onder veld kondisies. Die gevolgtrekking was dat biochar oksidasie en mineralisasie in hierdie proef teen ‗n vinniger tempo plaasgevind het in die gronde met hoër mikrobiese aktiwiteit.

Die potproewe het bevestig dat biochar nie as bemestingsstof sal dien nie, alhoewel dit tot ‗n toename in die biomassa produksie gelei het tussen die biochar aanwendingskoerse van 0.05-2.5% (w/w). Vir landbou doeleindes moet die aanwending van biochar altyd gepaardgaan met NPK bemesting. Beide die koring- en boontjie proewe het bevestig dat die biochar aanwendingskoerse tussen 0.05-0.5% (w/w) op die sanderig, effens suur grond wat gebruik is in die proef, gelei het tot die hoogste biomassa produksie en bemestingseffektiwiteit.

Die toediening van biochar het gelei tot merkbare toenames in grond pH en C/N verhoudings en hierdie toestande was beskou as die hoof redes vir die afname in mikrobiese biomassa in die

onbemeste boontjie behandelings omdat dit die opname van N meer beperk. Die toediening van bemesting het egter die beperkings op N voorsiening opgehef en as gevolg hiervan die mikrobiese biomassa bevorder by alle biochar aanwendingskoerse. Biochar het egter nie mikorrisa kolonisasie bevorder nie en het gelei tot _n afname in die mikorrisa kolonisasie van die wortels met toenemende biochar aanwendingskoerse en binne in die biochar lae van potproef 1. Biologiese stikstof vaslegging het egter positief reageer op die toediening van biochar. Hoë biochar aanwendingskoerse het beduidend die plant se afhanklikheid op hierdie simbiotiese verhouding verhoog. Ons hipotese is dat die biochar fisies N immobiliseer binne in die mikro-ruimtes deur kapillêre suigaksie en dan as _n fisiese versperring dien tussen die plantwortels en die geabsorbeerde N. Die immobilisasie van minerale N deur mikrobes kon egter ook grootliks bygedra het tot die afname in N opname as daar in ag geneem word dat mikrobiese aktiwiteit (respirasie data) hoër was by die hoër biochar aanwendingskoerse. Verdere ondersoeke moet daarom uitgevoer word om hierdie hipotese te bevestig.

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INTRODUCTION

In a world where atmospheric CO_2 concentrations are increasing, agricultural land is degraded (due to pollution, poor managing strategies and practices), and continuous cultivation causes a decrease in soil fertility and depletes soil biodiversity, biochar could serve as one of the tools in the arsenal developed to address these problems The addition of biochar to soils is an ancient practice that has only recently attracted the attention of scientists and is strongly promoted by many as a way to sequester carbon whilst improving soil properties.

Biochar refers to organic biomass which structure has been thermally altered during the process of pyrolyses, presenting a complex mixture of aromatic structures. In coal these aromatic structures consist primarily of benzene-like rings linked together in a complex heterogeneous structure. The aromaticity of biochar gives it a high degree of stability compared to the original biomass and renders it less reactive to reactions occurring within the soil system including resistance to microbial mineralization.

On the other hand, soil organic carbon (SOC) is readily mineralized by microbes and cycled at a high tempo through the soil system to provide nutrients to plants and the microbial population of the soil. The pyrolysis process provides a way to divert carbon from the rapid biological C cycle into a slow geological C cycle (Kuhlbusch and Crutzen, 1995) and storing it within the soil in a stable form, whilst at the same time causing an increase in the black carbon pool of the soil itself.

The use of biochar in agriculture has attracted a lot of attention worldwide as it can possibly create soil conditions similar to the Terre Preta de Indio (Amazonian Dark Earths) soils produced by small farmers 500-2500 years ago. However, it is questionable whether biochar would be able to promote soil fertility by increasing microbial activity, since the princible carbon source of this material forms part of the slow geological carbon cycle rather than the rapid biological carbon cycle (Kuhlbusch and Crutzen, 1995).

The objective of the following review is to look at biochar dynamics within the soil system and to critically analyze all the factors influencing the degradation rate of biochar and total residence time within the soil system. The second objective is to determine the effect of biochar on soil microbial activity and on plant root-microbial interactions in soils amended with biochar.

LITERATURE REVIEW: BIOCHAR CHARACTERISTICS AND DEGRADATION

Introduction

The incomplete combustion of organic biomass in the absence of oxygen (process called pyrolysis) leads to the formation of a black charred material with a highly condensed, stable, aromatic structure, called biochar. Pyrolysis is a process through which organic biomass can be converted to a product which is highly recalcitrant to biological and chemical oxidants and can persist within the soil system for hundreds to thousands of years (Goldberg, 1985). The assumption is made that over 50% of the initial carbon within the biomass is retained in biochar after pyrolysis and after a 100 years over 40% of the initial biomass carbon would still be sequestered within the biochar. (www.biofuelwatch.org.uk)

The idea of –capturing" CO_2 and storing it in a very stable form within the soil was derived from the Terre Preta de Indio (Amazonian Dark Earths) which was produced by local farmers 500-2500 years ago. During the 16th century Francisco de Orellana, a Spanish explorer, wrote about these incredibly fertile soils he had come across in the Amazon basin. In the 19th century geologist from Canada and America discovered that the local inhabitants of the region added charred wood and leaves to the soil for hundreds of years. Thousands of years later the soils still remained highly fertile and blackened by the black carbon additions.

Certainly this new technology provokes excitement among soil scientist and environmentalists for several reasons. According to the International Biochar Initiative, biochar provides climate benefits through carbon sequestration, carbon and other greenhouse gas reductions, co-production of bio-energy, improved water quality through reduced nutrient leaching which in turn leads to a decrease in chemical fertiliser inputs, improved plant yields, enhanced water retention, waste reduction and utilisation, reduced soil erosion and degradation, agricultural intensification and the potential for distributed on-farm use. However, a lot of these benefits depend on the long term persistence of biochar and its susceptibility to microbial and abiotic degradation within the soil system.

In this review several factors influencing biochar‘s residence time and ability to promote microbial activity are considered which will help us understand the effect and role biochar will play in the soil system when used as a soil amendment.

Properties of Biochar

Biochar is a significant carbonaceous component with intricate surface and structural properties arising from the incomplete combustion of various organic precursors (Qiu et al. 2008). The chemical structure and major organic components within the original biomass directly influence the physical and chemical properties of the black carbon formed during pyrolysis. These physical and chemical properties of the biochar determine the beneficial effects of the biochar and its residence time within the soil when used as a soil amendment.

Physical properties

During the process of incomplete combustion of plant derived matter, high temperatures cause a transition within the physical structure of the matter leading to the formation of aromatic ring structures (Keiluweit et al. 2010). The black substance formed during the process is associated with a highly porous structure, coupled with a low bulk density and a high degree of aromaticity and stability due to the formation of these stable ring structures. Wu et al. (2009) described biochar as having a highly heterogeneous and disordered structure prone to changes during natural oxidation whilst Scanning Electron Microscopy done by Qui et al. (2008) revealed biochar to have an amorphous structure.

However, it is really difficult to define the physical and molecular characteristics of biochar, as it is highly dependent on the charring temperatures and the precursor used. These two factors strongly influence the rate at which the organic precursor devolatilizes, which in turn determines the pore structure and distribution, porosity, bulk density and water holding capacity of the biochar produced (Özçimen and Ersoy-Meriçboyu, 2010). Assigning specific physical characteristics to the term biochar, is therefore not possible. Furthermore, the physico-chemical properties of biochar are dependent on time, as changes occur within biochar‘s chemistry and structure during its ~~–a~~geing” process (Cheng and Lehmann, 2009).

Most commercially produced biochar has variable pore size distribution which encompasses nano- (<0.9 nm), micro- (<2 nm) and macro-pore (>50 nm) sizes. Biochar‘s pore size distribution greatly influences the role and function it plays within the soil system (Downie et al. 2009; Atkinson et al. 2010). Macro-pores can serve as a habitat to microbes and also influences soil aeration and hydrology, whilst the smaller pore fraction influences molecule adsorption and transport as it can increase the specific surface area of the soil. Biochar, therefore, has the ability to increase the water holding capacity of soils, increase nutrient retention and also the retention of contaminants within the soil profile (Glaser, 2006).

Chemical properties

Various precursors and charring conditions can be used to produce biochar, resulting in biochars with different chemical properties, nutritional- and agriculture value when used as soil amendments. Chemically, biochar can be described as a substance low in nutritional value and exhibiting low reactivity due to its highly condensed aromatic nature (Anderton et al. 1996; Glaser, 2006). During pyrolysis the three major components of plant biomass (hemicellulose, lignin and cellulose) are thermally transformed, causing the release of gases and volatiles, leaving behind a carbon-rich material (McLaughlin et al. 2009). Novak et al. (2010) found that the biochar had a wide C/N ratio with 58% of the carbon composed in the form of highly aromatic structures. The elemental composition of biochar synthesized from Oak contained 90.8% carbon, 7.2% oxygen and 1.7% hydrogen (Cheng et al. 2008). Elemental analysis of charred rice and wheat had a carbon content of 80.7% and 80.4%, H content of 2.79% and 2.75% and O content of 9.11% and 9.03%, respectively (Qui et al. 2008).

The ability of biochar to adsorb and retain nutrients and contaminants is also dependent on the surface chemistry of the biochar. The FT-IR spectra (Fig. 1) done by Novak et al. (2010) on pecan shell derived biochar, revealed the basic functional groups found on most biochars. FT-IR spectral scans revealed the presence of oxygen containing functional groups on the surface as spectral bands at wave numbers 3435 cm^{-1} assigned to phenolic hydroxyl stretching, 1620 cm^{-1} to C=O aromatic stretching which is related to both acidic and basic groups (Chun et al. 2004a) and bands at 1090 and 795 cm^{-1} assigned to aliphatic ether (C-O) stretching and aliphatic CH_2 deformation (Qui et al. 2008). Table 1 shows spectral scans done on a variety of biochars derived from different precursors and clearly shows an abundance of aromatic functional groups within each biochar structure (Özçimen and Ersoy-Meriçboyu, 2010).

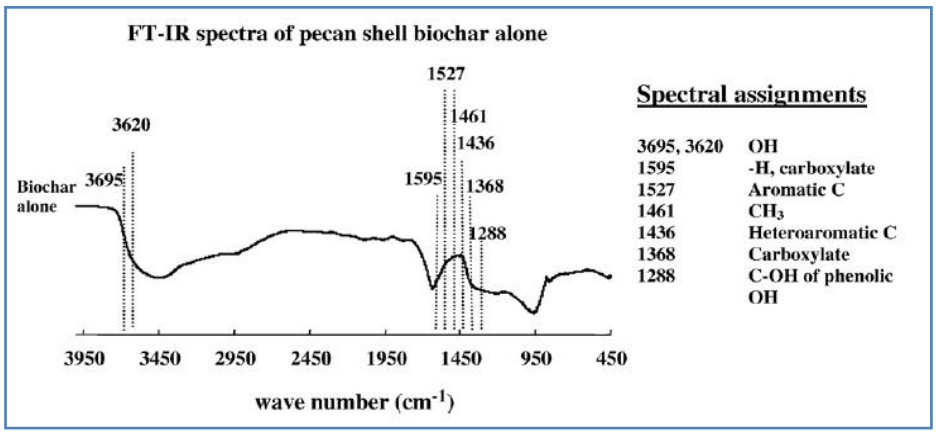

Figure 1: FT-IR spectral scan of pecan-shell biochar (Novak et al. 2010).

Kaal et al. (2008) characterised aged black carbon using pyrolysis-GC/MS and thermally assisted hydrolysis and methylation (THM) with both techniques, revealing the aged charcoal to contain a large fraction of benzene and significant amounts of other aromatics such as toluene, C_2 benzenes, benzonitrile and PAHs. THM also revealed a large fraction of aromatic methyl ethers and methyl esters within the biochar structure.

Furthermore, freshly produced biochar had a higher degree of surface positive charges than negative charges, but as ageing proceeds this will tend to be reversed as aged biochar had more negative charges than positive charges and displayed increased surface acidity and decreased basicity (Cheng and Lehmann, 2009). These results and conclusions are supported by Cheng et al. (2008) who also found that progressive oxidation led to higher amounts of surface negative charge with biochar incubated at 70˚C for twelve months having almost zero surface positive charge. The changes in surface charge are therefore strongly correlated with time and mean annual temperatures.

Table 1: FTIR analysis of various biochar samples (Özçimen and Ersoy-Meriçboyu, 2010).

Wave numbers (cm^{-1})	Functional groups
Hazelnut	
2924	Aliphatic CH stretching vibration
2845	Aliphatic CH stretching vibration
1709	Aromatic carbonyl/carboxylic C=O stretching
1613	Aromatic C=C ring stretching
1504	Aromatic C=C ring stretching
1370	Aliphatical CH_3 deformation
1226	Aromatic CO- stretching
1030	Aliphatic ether C-O and alcohol C-O stretching
Apricot stone	
3342	-OH stretching
2916	Aliphatic CH stretching vibration
1729	Aromatic carbonyl/carboxylic C=O stretching
1590	Aromatic C=C ring stretching
1499	Aromatic C=C ring stretching
1370	Aliphatical CH_3 deformation
1231	Aromatic CO- stretching
1030	Aliphatic ether C-O and alcohol C-O stretching
897	Aromatic stretching
Grapeseed	
2923	Aliphatic CH stretching vibration
2855	Aliphatic CH stretching vibration
1709	Aromatic carbonyl/carboxylic C=O stretching
1613	Aromatic C=C ring stretching
1317	Aliphatical CH_2 deformation
1036	Aliphatic ether C-O and alcohol C-O stretching

Factors influencing the process of abiotic oxidation

Biochar was considered to be inert to chemical and biological oxidation (Smith and Noack, 2000) to such an extent that the mineralization of biochar was considered to be negligible (Cheng and Lehmann, 2009). In recent times however, the inertness of biochar has been brought under the microscope with some scientists arguing that biochar properties can be altered over time due to natural oxidation (Cheng and Lehmann, 2009).

Reasons for the questionability over the biological and chemical inertness of biochar came about, because biochar plays an important role in soil fertility (Glaser et al., 2000), global scale carbon cycling (Hockaday, et al. 2006), sequestration (Kuhlbusch and Crutzen, 1996), and the humification processes in soils (Shindo et al. 1986). Spectroscopic evidence of humic substances formed during natural weathering and oxidative depolymerisation has proven this fact. Factors having a direct influence on the rate of oxidation include the following:

1. *Type of charred material*

Different biomass precursors can be used to derive biochar which include poultry manures, hardwood and softwood materials each with differences in their elemental and structural composition. Biochars derived from different precursors show differences in their surface chemistry (Fig. 2) and morphology (Fig. 3-4) as seen from the FT-IR scan absorbance peaks done by Steinbeiss et al. (2009) on glucose and yeast derived biochars. FT-IR analysis (Table 1) done by Özçimen and Ersoy-Meriçboyu (2010) also showed alterations in the aromatic structures of biochar samples derived from different precursor materials.

Furthermore, the amount of volatile matter from different precursors, is a determining factor in the rate at which devolatilization occurs. This process causes differences in pore structure and density (Pastor-Villegas et al. 2007, Özçimen and Ersoy-Meriçboyu, 2010) of biochars prepared from different precursors. In turn the pore structure determines the specific area exposed to atmospheric oxygen and the accessibility of the biochar to microbial populations. The type of charred material, therefore, can have a strong influence on the rate of oxidation of different biochars. Pastor-Villegas et al. (2007) concluded that charcoal characteristics depend on both the starting material and the carbonisation system used.

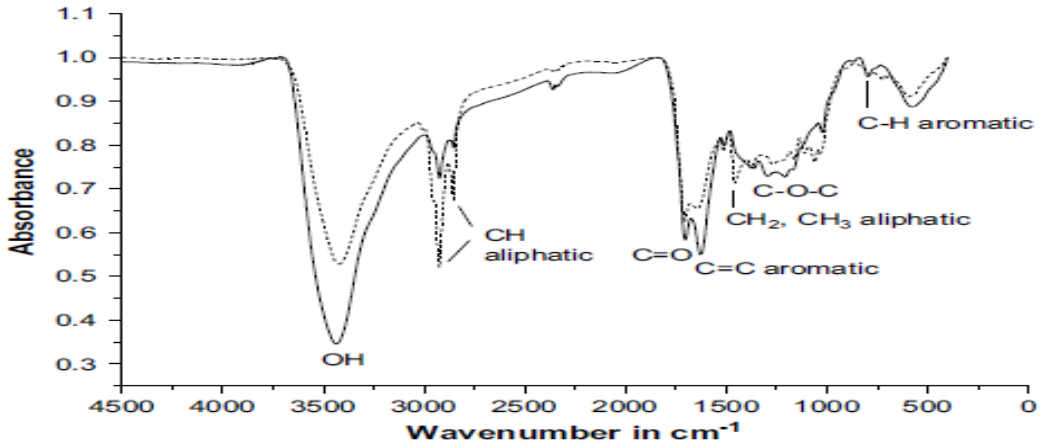

Figure 2: FT-IR Scan of biochar derived from glucose (solid line) and yeast (dotted line) with the SEM picture of each below (Steinbeiss et al. 2009).

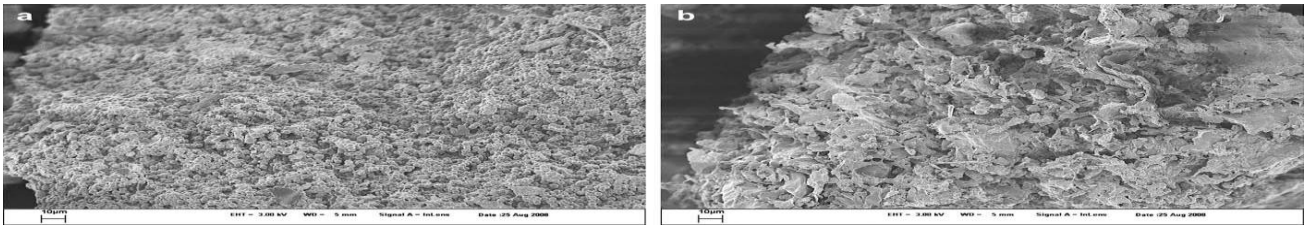

Figure 3: SEM micrographs showing different morphology of biochar derived from different precursors (a) glucose- and (b) yeast. Scale bar 10 µm (Steinbeiss et al. 2009).

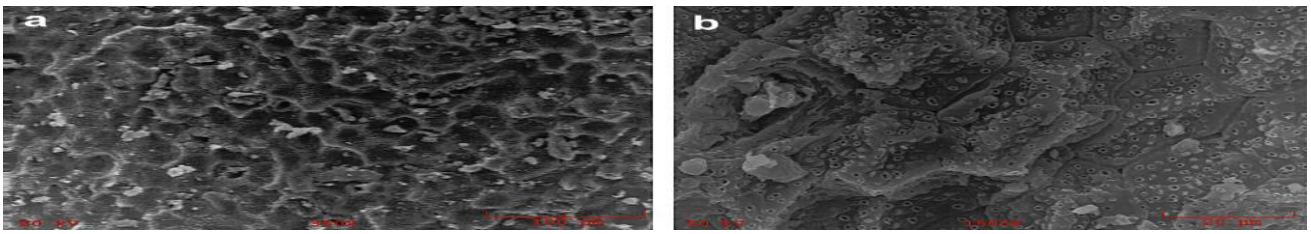

Figure 4: Surface morphology of (a) apricot stone and (b) its biochar - SEM micrographs. Scale bar 100 µm (a) and 20 µm (b) (Özçimen and Ersoy-Meriçboyu, 2010).

2. *Charring temperature*

Thermal conditioning of organic biomass leads to alterations in its internal structure, elemental composition and surface characteristics. Many studies conducted have shown that an increase in charring temperature leads to an increase in the aromaticity of the biochar, and thus its recalcitrance. ^{13}C NMR revealed a loss in signal intensity associated with cellulose and gains in signal intensity in the aryl and O-aryl regions due to the increase in aromaticity during the charring process of pine wood (Baldock and Smernik, 2002). The increase in charring temperature also led to a decrease in the amount of biochar mineralized over an incubation period of 120 days (Baldock and Smernik, 2002) due to the increase in recalcitrance of the biochar with the increase in aromaticity.

Furthermore, increases in the final charring temperatures led to a decrease in the H/C and O/C ratios as a result of dehydration and depolymerisation of the plant materials. The increase in the amount of fixed carbon gives a relative measure of the increase in the stable components of the chars relative to the more labile components as charring temperature increases (Keiluweit et al. 2010). Nguyen and Lehmann (2009) produced biochar from corn residues and oak wood and charred each of these plant materials at 350˚C and 600˚C. In both cases the increase in charring temperature led to an increase in the degree of aromaticity and thus increased the amount of fixed carbon (Corn residues: 77.6-85.2%; Oak wood: 61.8-68.4%). Carbon lost from red pine wood during a four month incubation trial done by Baldock and Smernik (2002) decreased from 20%, 13% to 2% from the uncharred material to the material charred at 150˚C and 350˚C, respectively. From these studies one can conclude that increases in the charring temperature, increases the aromaticity and stability of the bonds of biochar and decreases its vulnerability to microbial decomposition.

3. Mean annual temperature

Schneour (1966) found that the chemical degradation of biochar in soils is strongly correlated to factors such as the regional climatic conditions and soil properties. Cheng and Lehmann (2009) found that the ageing (oxidation) of biochar can occur over a wide range of temperatures stretching from -22 to 70˚C. Incubations done at 30˚C all led to an increase in oxygen concentrations, surface acidity and accordingly a decrease in pH and hydroquinone adsorption. This trend was the same for biochar incubated at 70˚C but the increases and decreases were more pronounced than for the incubations at 30˚C and from these results Cheng and Lehmann concluded that higher temperatures and longer incubation times enhanced biochar ageing.

Furthermore, Cheng et al. (2008) found a very strong positive correlation between the mean annual temperature of a region and the amount of oxidation occurring on biochar. Cheng et al. (2008) found that the amount of biochar oxidized increased by 87 mmole kg C^{-1} per unit Celsius increase in the mean annual temperature. This relationship was more pronounced at the surface of the biochar particle than for the entire particle.

4. Water regime

The water regimes occurring in terrestrial ecosystems and under cultivated land can have a marked effect on the dynamics of biochar within the soil system. Biochar found in marine sediments was shown to be 13900 years old (Masiello and Druffel, 1998), whilst biochar under well aerated tropical soils persisted for only a few decades to centuries (Bird et al.

1999). This may lead to a conclusion that the exclusion of oxygen from a system is responsible for this substantial difference in residence time.

However, the specific effect the water regime has on biochar oxidation is also strongly dependant on the biomass quality (biomass type and charring temperature) and possibly the alternating drying and wetting cycles (Nguyen and Lehmann, 2009). Corn biochar for instance was found to mineralize and oxidize much faster under unsaturated conditions, whilst oak biochar mineralized at a more rapid tempo under alternating saturated-unsaturated conditions with its oxidative status not differing between different water regimes (Nguyen and Lehmann, 2009).

5. Soil texture

Soil porosity is a function of soil texture and increases from sandy soils to clayey soils. Conversely, the field air capacity (defined as the volume of air within the soil at field capacity water content) decreases from sandy (± 25%) to a clayey (≤ 10%) soil (Daniel Hillel, 1980). Pore-distribution also differs between soils with different textures, with clayey soils consisting mostly of micro-pores and sandy soils of macro-pores. Soil air mostly occupies the larger pore fraction within soils and strongly influences the gas exchange (which takes place via diffusion and convection) and the maximal rate at which it can take place between the lithosphere and atmosphere and, therefore, indirectly influences microbial respiration.

Brodowski et al. (2006) found the highest biochar contents are found within the micro-aggregate fraction of the soil, with the macro-aggregates containing lower amounts of biochar. Micro-aggregates, therefore, play an integral role in reducing biochar decomposition by increasing the encapsulation of the organic fractions.

Biochar can initiate increased aggregation when they are broken down to humic acids. The addition of coal-derived humic acids to soils for instance led to an increase in macro-aggregate stability of between 20-130% (Glaser et al. 2002). However, micro-aggregates get turned over every 88 days, whilst biochar has a residence time of thousands of years. Therefore, aggregation may only play a small role in reducing the decomposition rate of the biochar (De Cryze et al. 2006) with its own inherent recalcitrance playing the major role.

6. Cultivation

Cultivation leads to the active disturbance of the soil system by physical actions, such as ploughing. These practices leads to the destruction of soil structure and aggregates and leads to a decrease in the amount of physical and chemical protection the soil system can provide to

stabilize soil organic matter against microbial attack. The quantity of biochar lost under managed ecosystems (agricultural soil) may differ a lot from unmanaged ecosystems.

Biochar under agricultural management may be more susceptible to erosion by wind and water (due to its low density), leaching (due to fragmentation of particles) and surface oxidation due to the disturbance of the soil and improved oxygen supply. Agricultural practices such as ploughing, leads to physical breakdown of biochar into smaller particles and decreases the degree of aggregation within the soil (Nguyen et al. 2009). Accordingly, Nguyen et al. (2009) found that biochar formed during the conversion of undisturbed land to agricultural land by the burning of the natural vegetation, led to the formation of biochar and irrespective of its origin, the initial biochar content per unit soil mass decreased rapidly by 30% over a period of 30 years as estimated by NMR. Afterwards the decrease in the biochar content reached a relatively steady state of decrease. These results corresponded with a decrease in carbon content at the surface of the biochar from 44.6% to 18.8% with the entire particle carbon content decreasing from 51.1% to 34.2% over the first 40 years. This decrease was followed by an increase in the oxygen content from 45.2% to 50.6% as natural oxidation occurred.

Nguyen et al. (2009) calculated a mean residence time of 8.3 years for biochar up to a soil depth of 0.1 m and attributed this rapid decrease to the decomposition of the easily degradable portion of biochar and to the mass movement with clay and silt to the deeper horizons. Furthermore, chemical oxidative reactions play a larger role at the topsoil because gas exchange takes place at a higher rate near the surface.

Biodegradation of biochar

Biodegradation of biochar refers to the decomposition of biochar by the biological component of soils, which includes macro-, meso- and micro-fauna. The decomposability of organics in soils depends on both the substance properties (porosity, pore size distribution etc.) and the accessibility to the soil microbial population (Ekschmitt et al. 2008; Lützow et al. 2006).

Ever since Fakoussa (1981) revealed that microorganisms can indeed metabolize coal, numerous studies were conducted and found that especially fungi and some bacteria have the ability to solubilise coal. However, estimating the degradation rate of the biochar will need very long periods to obtain measurable transformations (Kuzyakov et al. 2009) and because of this only a few studies describe biochar transformations.

Hockaday et al. (2005) investigated the natural degradation of charcoal particles over a period of 100 years by using ultrahigh resolution mass spectrometry with electrospray ionization and found condensed aromatic ring structures extensively substituted with oxygen containing functional groups. Hockaday et al. (2005) thereby concluded that oxidation and dissolution of charcoal black carbon occurs on a centennial timescale and that saprophytic fungi may be important to these biochar degradation processes because the charcoal particles at these sites were penetrated by filamentous microorganisms.

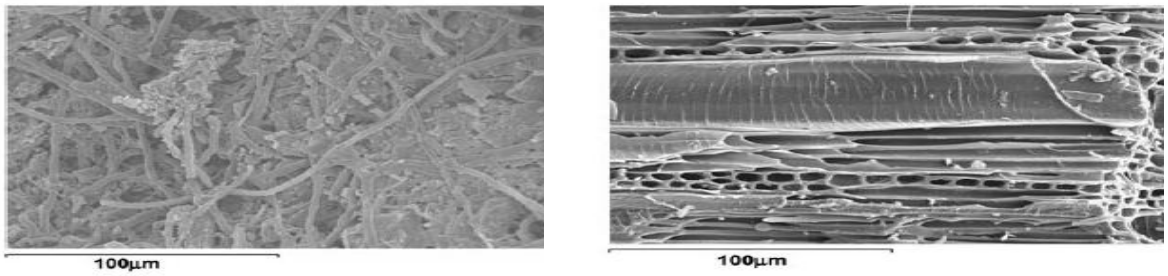

Figure 5: Optical light micrograph to illustrate the abundance of filamentous microorganisms from the edges of a 100-year old charcoal fragment (Hockaday et al. 2006).

Many microorganisms species have been reported to be able to solubilise low-rank coals, predominantly wood decaying basidiomycetes and micro-mycetes (Hofrichter and Fritsche, 1996). Cohen and Gabriele (1982) revealed that two fungal species had the ability to totally solubilise leonardite (highly oxidized lignite). Laborda et al. (1995) found that different fungal strains from the *Trichoderma* genera could solubilise both low rank and high rank coals. SEM images revealed that that the fungi produced a fibrilar extracellular polymer on the coal particle for adhesion, and to enable it to attack the particle (Fig. 5).

Hofrichter and Fritsche (1996) found that 38 wood and litter decaying basidiomycetes had a strong bleaching effect on coal derived humic-acids and 49 types having a weak effect. *Nematoloma frowardii* b19 was found to be the most effective in bleaching. Charcoal inoculated with the white-rot fungi species, *Pleurotus pulmonarius* and *Coriolus versicolor* was colonised on the surface and in the interior along structural weaknesses (Ascough et al. 2010).

These results led to many incubation studies, where biochar was used as the principle carbon source for the microbes to utilize. Most of the incubation studies have shown that microbes are able to use biochar as a carbon source. Hamer et al. (2004) reported a 0.8, 0.7 and 0.3% loss of carbon as CO_2 evolving from biochar, derived from rye, maize and oak wood during 60 day incubation period with microbial inoculants.

Zimmerman (2010) found a direct relationship between the logarithmically transformed experimental degradation rate (k in unit of years^{-1}) and time (in year units) data collected over a period of a year from laboratorial incubations done for both abiotic and biotic incubations. His results revealed that 3-26% of the biochar carbon will be remineralized after a period of 100 years and that the biochar carbon had half lives stretching from 10^2 to 10^7 years. This incredible inertness of the biochar makes it an effective tool to sequester carbon from the atmosphere and to store it in the soil.

Mechanisms used to degrade biochar

The mechanisms used by these microorganisms to degrade and solubilise coal are still a region of uncertainty. More than one mechanism have been proposed for the biodegradation of coal and include oxidative enzymes, hydrolytic enzymes, alkaline metabolites and natural chelators (Fakoussa and Hofrichter, 1999). The production of alkaline metabolites like ammonium and biogenic amines excelled the liquefaction of complex substrates, like coal, by solubilisation of the coal acidic groups through deprotonation (Quigley, 1987, 1988b, 1989b). The natural chelators produced by microorganisms can complex with metal ions like Fe^{3+} and Mn^{2+} in the structure of the coal, and remove them from the structure. Degradation of organic material from coal is often accompanied with the release of heavy metals into the environment (Littke et al. 1991) and microorganisms, therefore, play an important role in heavy metal mobilization (Lin, 1997). Yet, neither of these mechanisms can break carbon to carbon bonds and therefore cannot oxidise coal to carbon dioxide. For total reduction in molecular weight of coal, microorganisms produce non specific, stable extracellular enzymes to degrade the complex coal structure.

Ligninolytic fungi like white-rot and brown-rot fungi (wood rotting basidiomycetes) have the ability to biologically degrade macromolecules, like lignin, through the production of exo-enzymes with an extremely high oxidative potential (Leonowics et al. 1999). The ligninolytic enzyme system by which lignin is degraded, consists mainly of peroxidase (with the most common being manganese peroxidase (MnP) and lignin peroxidase), phenol oxidases (laccase), secondary enzymes like H_2O_2-oxidases and low molecular weight organic acids (Fakoussa and Hofrichter, 1999). These enzymes can perform radical reactions and oxidize macromolecules like lignin. Based on this fact, many scientists have hypothesized that these ligninolytic basidiomycetes would also be able to degrade, transform and depolymerise high molecular weight compounds like coal (Wengel et al. 2006; Ascough et al. 2010; Hofrichter and Fritsche, 1996; Fakoussa and Hofrichter, 1999).

Hofrichter et al. (1999) investigated the degradation of low-rank coals by white-rot fungi's, *Nematoloma frowardii* b19 and *Clitocybula duseni* and found extracellular manganese peroxidase to be the crucial enzyme in the depolymerisation of coal derived humic substances and native coal. These results overlap with a previous study done by Hofrichter and Fritsche (1996) using *N. frowardii* which caused extreme bleaching of the high molecular mass humic substances because of the formation of low molecular mass fulvic acids during the depolymerisation reaction (Hofrichter et al. 1999). This decolourisation effect was furthermore effectively enhanced by the addition of Mn^{2+} to the system.

The involvement of the different enzymes contributing to the degradation of coal is still a debateable topic, but it seems fair to say that ligninolytic basidiomycetes can solubilise coal to some degree with MnP playing an integral role in the depolymerisation of the coal substances. We hypothesize that the same mechanisms of coal degradation would be viable for biological degradation of biochar because manganese is a natural compound of wood present in high concentrations (10-100 mg/kg dry wood). Lignin degradation is strongly promoted by the presence of Mn^{2+} since it stimulates MnP production (Fig. 6) by white-rot fungi and functions as a MnP substrate (Have et al. 2001). Manganese not only promotes delignification, but also the depolymerisation of coal substances (Hofrichter et al. 1999). Hardie (2010) found that *Pinus* wood biochar (from Allbrick, Thembalethu) charred at 400˚C contained 10.83 mg Mn kg^{-1} biochar. This can, therefore, possibly promote degradation of the biochar by stimulating MnP production by white-rot fungi.

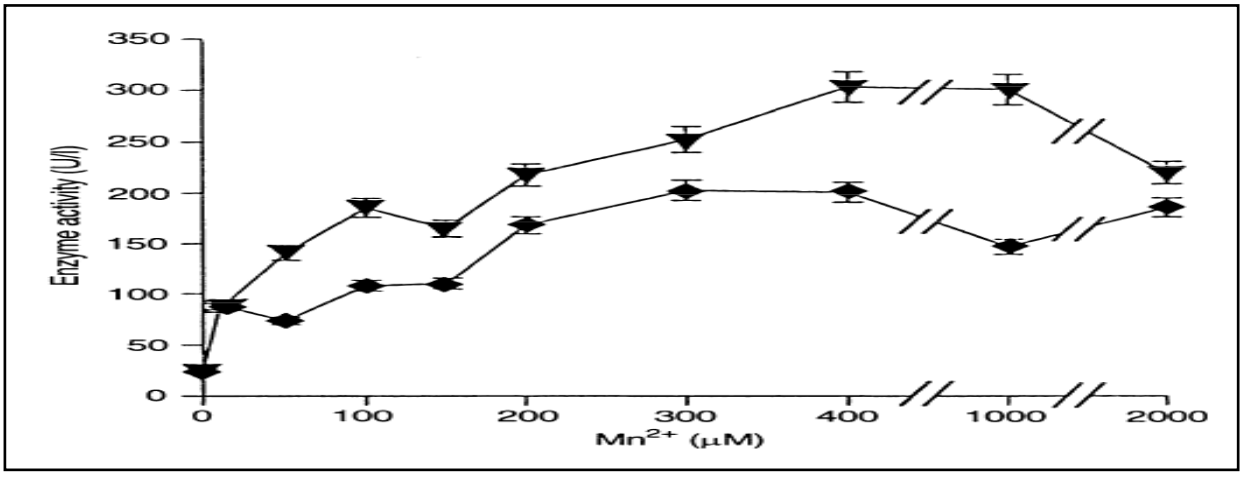

Figure 6: Influence of Mn (II) of the medium on the production of MnP by surface cultures of white rot fungus *Clitcybula dusenii* (Hofrichter et al. 1999).

Effects of biochar on microbial activity in the soil system

The composition of the soils micro-fauna and micro-flora depends on a few factors which include the type and quantities of soil organic matter (SOM) present (Alexander, 1977). Soils with a high turnover rate of organic matter are usually associated with a high level of microbial activity. Practices like the application of mulches, compost and manures have been shown to increase soil fertility (Glaser et al. 2001), because it can readily be mineralized and in areas like the tropics this mineralization rate is very rapid (Tiessen et al. 1994). Rapid mineralization causes a rapid depletion of SOM, with only a small portion of the SOM being stored in the soil. Eventually all of the SOM will be released back into the atmosphere as respiratory CO_2. Carbonized materials like pyrogenic carbon and charcoal, however, are more recalcitrant and are responsible for maintaining high levels of SOM and available nutrients in anthropogenic soils (Glaser et al. 2000, 2001a). Biochar‘s recalcitrant nature may, however, prove to be the limiting factor regarding the influence it has on chemical and biological processes which controls the carbon and nitrogen cycling within the soil system (Novak et al. 2009).

Most commercially produced biochars consist of over 60% carbon. However, this carbon is stored in a very stable aromatic backbone of biochar and might not serve as an easily accessible carbon source for soil microbes. Studies conducted by Kuzyakov et al. (2009) found that 2.6 and 1.5% of the biochar ^{14}C input derived from perennial ryegrass and incubated in the Ah of a Haplic Luvisol and a loess, respectively, were incorporated into the microorganisms after 624 days as seen in Figure 7b. DeLuca (2006) also revealed that biochar formed during wild fires stimulated the gross/net nitrification rates by adsorbing inhibiting compounds such as phenols.

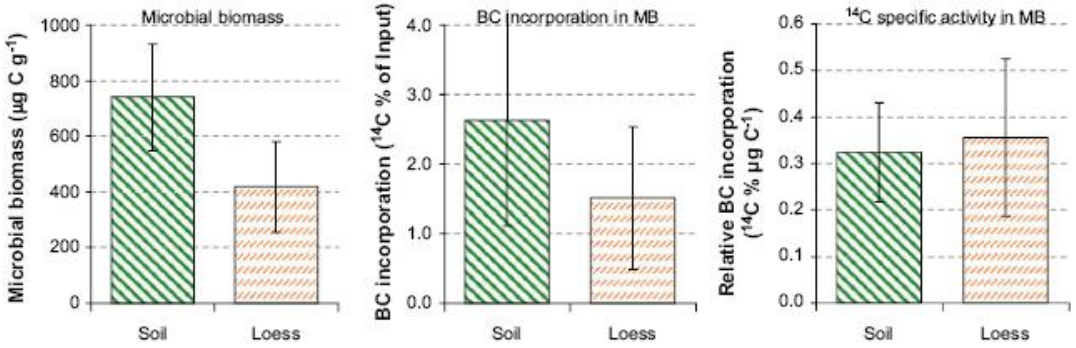

Figure 7: Incorporation of 14C from Black Carbon (BC) into microbial biomass (MB) after 624 days of incubation in soil and loess. Left (7a): MB content; middle (7b): 14C from BC into MB; right (7c): relative incorporation of 14C into MB. Error bars show standard errors (n ¼ 4) (Kuzyakov et al. 2009).

Biochar also has the ability to promote co-metabolic decomposition of organic matter by the microbial community as shown in studies where an increase in mineralisation of indigenous soil organic carbon and exogenous organic carbon was measured after biochar addition (Rogovska et al. 2008; Wardle et al. 2008). Kuzyakov et al. (2009) also found that the addition of glucose stimulated the decomposition of biochar up to six times and that co-metabolic activities of microbes can, therefore, work in both directions. This priming effect (Jenkinson, 1966) induced by labile carbon can either increase or decrease the mineralisation rate of stable carbon because of an increase in microbial activity caused by the easily degradable substrate.

Although it is clear that biochar can be utilized by microorganisms as a carbon source it can still cause a decrease in microbial activity as seen in incubation studies done by Baldock and Smernik (2002) where biochar served as the sole carbon source. Using biochar derived from ^{13}C labelled glucose and yeast, Steinbeiss et al. (2009) found that the glucose derived biochar were unable to support the microbial biomass and resulted in a significant reduction of the total microbial biomass during the incubation period, whilst yeast derived biochar did not cause any significant change. The effect on the microbial community also differed between the two types of biochar with glucose derived biochar utilized by gram negative bacteria whilst yeast derived biochar brought about a 16% increase in fungal biomass in both soils, but a decrease of 7-14% in gram negative and positive bacteria (Steinbeiss et al. 2009).

Apart from serving as a carbon source to microbes, biochar can increase soil microbial activity by increasing pH, cation exchange capacity, waterholding capacity and increasing nutrient availability, including phosphor and other base cations (Glaser et al. 2002; Lucas and Davis, 1961). The porous nature of biochar not only increases the water holding capacity of soils but also provides suitable micro habitats for microorganisms to colonise (Joseph et al. 2010). These micro pore habitats provide microorganisms with refuges (Saito, 1998), moisture, and greater protection against predators and climatic extremes and also allow for a wide variety of microbial communities to colonise them (Thies and Rilling, 2009).

Symbiotic associations between biochar and mycorrhizal fungi are very important in agricultural and natural ecosystems through its ability to promote plant growth and increase crop yields. Warnock et al. (2007) looked at the effect of biochar on mycorrhizal associations, abundance and activity by assessing the effect of biochar on the soil physico-chemical properties, indirect effect of biochar on other microorganisms, plant fungus signalling interference and detoxification of allelochemicals and the protection biochar provides to mycorrhizae against fungal grazers by serving as a refuge.

Biochar not only directly stimulate mycorrhizal activity, but also promotes soil microbes (phosphate solubilising bacteria and Mycorrhization Helper Bacteria), which facilitates hyphal growth and root colonisation by the ecto-mychorrhizal (ECM) and arbuscular fungi (AM) (Founoune et al. 2002; Duponnois and Plenchette, 2003; Hildebrandht et al. 2002, 2006). Signalling between the microbes and plant roots can be influenced by biochar due to its sorptive capacity and effect on soil pH. Angelini et al. (2003) found flavenoid compounds can either stimulate or inhibit certain groups of soil biota as a function of the soil pH. Biochar also has the ability to adsorb signalling compounds and can serve as a signalling reservoir or sink which remove both stimulating and inhibiting compounds from the soil (Warnock et al. 2007). This effect can, therefore, result in a decrease in the number of signalling compounds within the soil system and directly decrease or even increase mycorrhizal hyphae growth and spore germination (Warnock et al. 2007) depending on the sorptive characteristics of the specific biochar used. This can lead to decreased associations between plant roots and mycorrhizal fungi.

The physical characteristics of biochar (high micro porosity) can serve as a physical barrier between the hyphae and bacteria which colonizes it, and the soil predators (Saito, 1990; Ezawa et al. 2002). Pore size of the biochar is often large enough for many bacteria and fungi which allows successful colonisation of the biochar particles by arbuscular mycorrhizal fungi for instance (Saito, 1990; Ezawa et al. 2002). Biochar can, therefore, provide synergistic possibilities with arbuscular, ericoid and ectomycorrhizal symbiosis, but it is important to mention that under certain circumstances depending on the plant in use, the soil pH and nutrient status, biochar can bring about certain negative effects which can influence mycorrhizae-root associations (Warnock et al. 2007).

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RESEARCH QUESTIONS, GOAL AND OBJECTIVES

The literature study above has shown that the existing knowledge does not answer a few important questions:

1. If biochar is highly resistant to degradation in the environment and has a life span of thousands of years, why are there no concentrated terrestrial or marine deposits of charcoal produced through natural or man-made fires followed by erosion/accumulation of charred material?
2. Although detailed characterization of biochar from various feedstocks exist – very little is known of it‘s degradation reactions and their products, while CO_2 is only assumed as the sole product of degradation. That may be so if the oxidation process is complete. The question remains to what extent is charcoal oxidizable by various reagents and are there stable intermediate products of such oxidation being formed in the process?
3. Although, biochar may be resistant to oxidation by atmospheric oxygen, it is known that charcoal, as part of gunpowder, is highly (explosively) reactive with nitrates which are constantly produced in well-aerated soils. The question is: what is the influence of oxidative soil minerals and soil nitrates on charcoal degradation?
4. The observed degradation rates are largely extrapolated into timespans far exceeding observation periods. The question is: which environmental parameters control the degradation rates of biochar and to what extent?
5. All the prior studies of charcoal degradation are focussed on oxidation processes. However, charcoal is a product of incomplete reduction and further reduction to methane as end product is still possilbe. Formation of methane in coal deposits indicates a high probability of reduction pathway for charcoal degradation in the environemnt. The questions are whether anaerobic decay may be a more rapid pathway of charcoal degradation and what are the controlling factors of charcoal degradation in anaerobic conditions?
6. The interaction of charcoal with soil organisms and higher plants is insufficiently studied. There are still multiple questions regarding the interactions with specific groups of organisms and mechanisms of such interaction, some of which may be at least partially answered with special emphasis on plant roots and their symbionts (mycorrhyzal fungi and nitrogen-fixing bacter in legumes).

The above questions inspired by the literature study have allowed us to formulate the overall goal of this thesis:

Improve the understanding of charcoal chemical and biological degradation in aerobic and anaerobic conditions and its interaction with plant roots, their symbionts and soil microbial communities.

To achieve this goal the following objectives were set:

1. Conduct *in vitro* oxidation of biochar by hydrogen peroxide, observe the oxidation rate, changes in biochar‘s elemental composition and chemistry and determine the intermediate products formed as a result of incomplete oxidation.
2. Set up a physical simulation of biochar degradation in a sandy soil as influenced by the presence of wood-rotting microbial community, fertilizers and oxidative minerals, while monitoring liquid and gaseous degradation products.
3. Determine the rates and products of anaerobic degradation of biochar *in vitro* by a methanogenic consortium as influenced by the addition of nitrogen, phosphorus, pH, alkalinity and different charcoal loads.
4. Incubate biochar *in vivo* in contrasting soil conditions and observe the effects on the composition changes.
5. Establish pot trials to observe and quantify interaction of biochar with plant roots, mycorrhyzal fungi and symbiotic nitrogen-fixers and total microbial communities of soil amended with varying rates of biochar applications.

The specific tasks of individual experiments conducted to meet the above objectives are detailed in the respective ensuing chapters.

CHAPTER 1: *IN VITRO* OXIDATION OF BIOCHAR WITH HYDROGEN PEROXIDE

Introduction

It is well known that white rot basidiomycetes are good degraders of both wood polysaccharides (cellulose and hemi-celluloses) and lignin, whilst brown rot fungi are good degraders of cellulose and hemi-celluloses, leaving behind lignin (Enoki et al. 1988; Highley, 1987, Enoki et al. 1997). A few studies have reported the production of extracellular hydroxyl radicals (•OH) by white rot fungi (Backa, et al. 1993; Kremer and Wood, 1992) which is the strongest reactive oxygen species (ROS) occurring in aqueous systems (Enoki et al. 1997), able to oxidise almost any organic material. A few studies have also reported on the ability of basidiomycetes to degrade and liquefy coal (Fakoussa and Hofrichter, 1999; Hofrichter et al. 1999)

Four mechanisms have been proposed to explain the depolymerisation of coal by microbes and include the production of extracellular enzymes with an extremely high oxidative potential (Leonowics et al. 1999) such as lignin peroxidase (LiP) and manganese peroxidase (MnP) which are known to play a role in lignin degradation (Enoki et al. 1997). LiP has broad substrate specificity for aromatic compounds and requires H_2O_2 for its catalytic activity (Fakoussa and Hofrichter, 1999) to produce ROS such as the •OH radical through the well established Fenton reaction pathway (Hammel et al. 2002).

The biochar used in this study was produced from the slow pyrolysis of woody biomass (Pine wood). This type of feedstock is chemically composed by three main macro components which are hemicelluloses, cellulose and lignin. Due to the polyphenolic structure of lignin, this carbon source is considered to be more recalcitrant to biochemical degradation compared to other constituents in plant biomass. We propose that the pyrolysed lignin in the biochar produced from wood feedstocks also forms the backbone of the recalcitrant fraction in biochar. According to Keiluweit et al. (2010) the presence of lignin/cellulose derived transformation products occur as multiple peaks between 1600-700 cm^{-1} on a FT-IR spectra of biochar pyrolysed at 400 ˚C.

We hypothesize that the use of different concentrations of hydrogen peroxide (H_2O_2), to facilitate the progressive oxidation of biochar, can mimic the depolymerisation of biochar as catalysed by lignocelluloses-degrading enzymes through the Fenton reaction pathway as the

biochar do contain catalysts. Removal of the labile C fraction of charcoal by progressive oxidation with H_2O_2 will allow us to identify the changes occurring in surface chemistry step by step and hopefully identify the more recalcitrant fraction of biochar.

Furthermore, the intermediates released when biochar is oxidised by hydrogen peroxide will be investigated as it has been shown that the depolymerisation of the high molecular-mass fractions of lignite by basidiomycetes through the production of enzymes, (i.e. LiP and MnP) releases a complex mixture of lower-molecular-mass-fulvic-acid-like compounds (Hofrichter and Fritsche 1996).

Materials and methods

Biochar and reagent

The raw biochar for this project was produced by the small-scale commercial producer, Allbrick, Thembalethu in the Eastern Cape, South Africa. Pine sawmill waste was used as the feedstock and slow-pyrolysed at 450˚C. The charred material was then milled to an average particle diameter of 3 mm. The pH, surface acidity and basicity, cation exchange capacity (CEC*), FT-IR and elemental composition of the fresh and treated biochar was analyzed according to the methods described under the heading, analytical methods. All determinations were carried out in duplicate. The results of the biochar characterization are shown in Table 1.

Different concentrations of H_2O_2 (0.333, 0.166, 0.083 and 0.042 M) was used as oxidant as it can be catalyzed to various radicals (depending on the pH of the system) by transition metals such as ferric and ferrous iron, manganese and copper present in the matrix of the biochar. Xu et al. (2004) found the catalytic activity of metal ions to be in the order: $Fe^{2+} > Cu^{2+} > Mn^{2+} > Ag^{+}$. The biochar in this trial was assumed to be exposed to a •HO-producing system due to the pH range in use and the biochar being able to provide the catalysts. (Petri et al. 2011). However, other radicals such as superoxide ($•O_2^-$) could also have been produced.

Table 1.1: Biochar characteristics

Biochar	pH (H_2O)	% Volatile matter	% C	% N	C/N ratio	Ash%	$CEC_{7.0}$ ($cmolc.kg^{-1}$)
	9.72	19.89	82.71	0.53	156:1	3.04	34.30

The biochar used in this trial has a very high pH, rich in carbon and contained very low amounts of N. As a result, the biochar had a very wide C/N ratio (156:1).

Complete chemical oxidation using hydrogen peroxide

Ten grams of biochar (< 2 mm) were treated with 50ml of 0.333 M H_2O_2 and placed on a warm water bath, kept at 60˚C, to accelerate the reaction rate. After all the H_2O_2 reacted (gas production stopped for a day), the samples were dried at 105˚C for 24 hours and weighed to determine the mass lost as CO_2 during the oxidative process. The above treatment was then repeatedly applied to the residual mass until almost all the biochar were successfully oxidized.

Progressive oxidation of biochar using hydrogen peroxide

This trial consisted of two main steps which include the oxidation of fresh biochar and lixiviation of biochar, to investigate and characterize the intermediates released as biochar depolymerises and the changes occurring in the surface chemistry.

The trial consisted of 4 treatments done in triplicate. Ten grams of biochar, dried at 60˚C in 500 ml flasks, were oxidised using 100 ml of 0.333, 0.166, 0.083 or 0.042 M H_2O_2 supplied by Merck. The flasks were left on the laboratory bench, exposed to the atmosphere and light until the completion of the oxidative reaction. When the reaction reached its endpoint (end of gas production for a day), the solution leftover was decanted and filtered through a Whatmann 40 ashless filter paper and analysed by HPLC-UV/IR.

The treated biochar leftover after completion of the oxidative step was then dried at 60˚C for 24 hours. A subsample of 1 g treated biochar was then shaken in 100 ml distilled water at 250 rpm for 24 hours. The suspension was filtered using Whatmann 40 ashless filter paper and the lixiviated intermediates were determined.

Analytical methods

The surface characteristics of the fresh and incubated biochar samples were characterized with a Thermo Nexus^{TM} Nicolet FT-IR Spectrophotometer using the KBr-pellet method (0.5%). The spectra were obtained using Omnic version 7.2 software. The spectra were recorded between 4000-500 cm^{-1}, averaged over 64 scans.

The total C, N, H and ash contents of the biochar and soils were determined by the EuroVector Elemental Analyzer using the dry combustion method (Nelson and Sommers 1996). Oxygen was determined by subtraction

Acidity and alkalinity surfaces were determined after treatments and carried out according to the method described by Cheng and Lehmann (2009), which is based on the classic Boehm titration method used for the characterization of charcoal.

The cation exchange capacity (CEC*) of the biochar was determined according to the method for alkaline soils as described by Rhoades (1982). The pH of the hydrogen peroxide treated biochars was determined in distilled water using a 1:20 biochar to solution ratio (Cheng and Lehmann, 2009).

The HPLC-UV equipment used was a Thermo separations product (Spectra Systems HPLC system) with a UV detector adjusted to a wavelength of 215 nm and equipped with a 300 mm by 7.8 mm Aminex HPX-87H cation exchange column for the determination of organic acids. The standard solution consisted of the following organic acids: Formic acid (supplied by BDH laboratory suppliers with purity of minimum 98%); acetic acid (supplied by Riedel de Haën with purity 99.8 - 100.5%); malic, tartaric, citric and oxalic acid (supplied by Sigma-Aldrich). A temperature of 65˚C was used and a fixed eluent flow rate of 0.5 ml/min. The mobile phase composed of 0.005 M H_2SO_4. The samples were filtered through a 0.22 µm nylon filter before chromatographic separations were conducted.

Results

Complete chemical oxidation using hydrogen peroxide

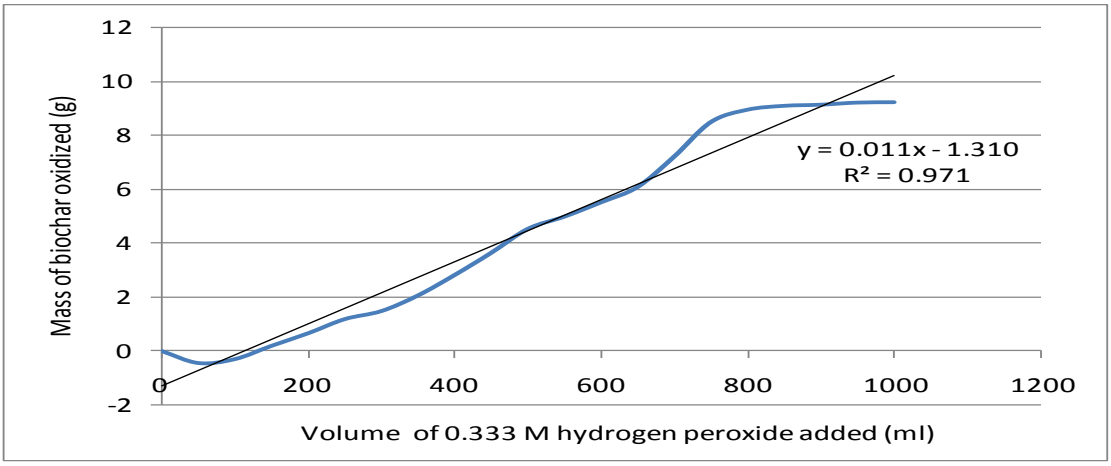

Figure 1.1: Mass of biochar lost during successive oxidation with 50 ml 0.333 M H_2O_2

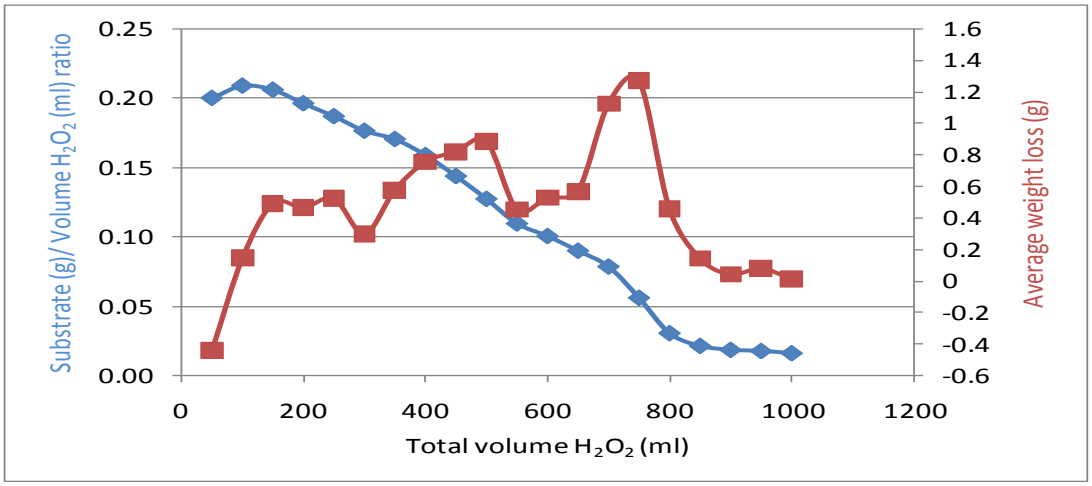

Figure 1.2: Relationship between residual substrate mass (g) to volume H_2O_2 (ml) ratio and the average weight loss during successive oxidation with 50 ml 0.333 M hydrogen peroxide.

The total oxidation of the biochar was completed in order to identify the fraction showing the greatest resistance to oxidative reactions. The oxidation of biochar using the oxidant H_2O_2 initially did not cause a mass loss in the biochar samples, but caused an unexpected increase in biochar mass (Fig. 1.1; Appendix 1.1). Only after the addition of a 150 ml H_2O_2 did the biochar weight decrease from the initial ten grams added. Furthermore, variations occurred in the weight loss during successive oxidation periods (Fig. 1.2) until the complete oxidation of 9.211 g biochar with a 1000 ml of 0.333 M H_2O_2. The weight loss following successive oxidation periods with 50 ml H_2O_2 steadily increased up to 750 ml H_2O_2 with exception of a few points (Fig. 1.2). In contrast the substrate/volume H_2O_2 decreased steadily up to 750 ml. A steep increase in weight loss was measured between 650-750 ml with a total of 2.402 g biochar oxidized during these treatment periods. However, after 750 ml H_2O_2 a decrease

occurred in the weight loss with successive oxidation period even though the substrate to solution ratio was the lowest compared to earlier in the trial. Only 0.277 g of biochar was lost from biochar with the addition of the last 200 ml 0.333 M H_2O_2 which is less than any of the previous weight loss with only 50 ml H_2O_2.

In summarizing the results; a 1000 ml of 0.333 M $H_2O_2$was needed to fully oxidize 9.211 g (92.1%) of biochar to CO_2. The oxidation of biochar with H_2O_2 followed a linear trend (Fig 1.1; R^2= 0.97) with very little variation between replicates (average St.Dev for all the treatments was 0.035 calculated from Appendix 1.1).

Progressive oxidation of biochar using different hydrogen peroxide concentrations

Elemental and chemical analyses

Table 1.2: Chemical and elemental analysis of fresh- and treated biochar

Treatment	pH_{H2O}	Surface basicity (mmol g^{-1})	Surface acidity (mmol g^{-1})	% C (wt %)	% H (wt %)	% N (wt %)	% O (wt %)	% Ash	Molar H/C	Molar O/C
Fresh Biochar	9.72	0.08	0.78	81.86	3.84	bd	11.80	2.51	0.567	0.108
0.042 M	8.65	0.67	11.47	82.90	4.44	bd	10.80	1.86	0.642	0.098
0.083 M	8.17	0.47	49.53	81.12	4.26	bd	12.24	2.59	0.630	0.113
0.166 M	7.43	0.23	49.50	81.15	4.20	bd	12.11	2.53	0.621	0.112
0.333 M	5.64	0.13	49.80	79.11	4.11	bd	13.94	2.84	0.623	0.132

*bd-below detection of 0.001 wt%

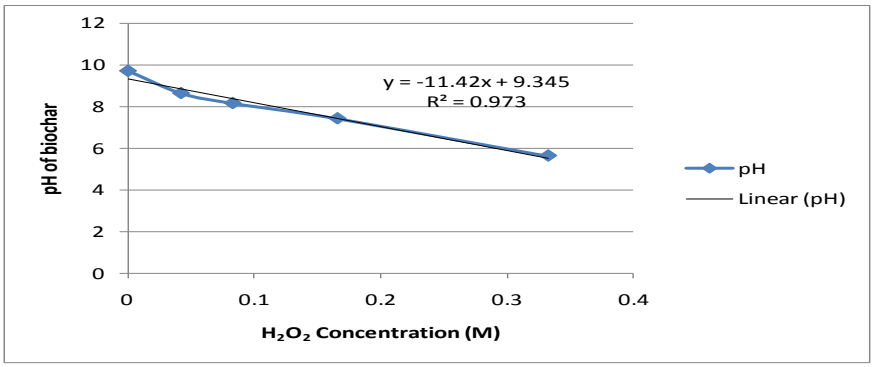

Figure 1.3: Decrease in pH of biochars with progressive oxidation using different [H_2O_2]

The catalytic oxidation of biochar with different concentrations of H_2O_2 of the biochar and brought about several changes in surface chemistry and elemental composition. The treatments caused a significant decrease ($P \leq 0.001$) in biochar pH from 9.72 for the untreated biochar to 5.64 for the biochar treated and leached with 0.333 M H_2O_2.

This translated into a significant increase in surface acidity. However, the biochar oxidized with 0.083, 0.166 and 0.333 M H_2O_2 had similar surface acidities. The surface acidity seems to have a maximum value of between 49.5-50 mmol g^{-1} and even if it is oxidized further, the increase in surface acidity was marginal as seen by the difference of only 0.27 mmol g^{-1} in surface acidity between the biochars oxidized with 0.083 M and the highest H_2O_2 concentration of 0.333 M. Surface basicity of the biochar that reacted with 0.042 M H2O2 increased to 0.67 mmol g^{-1} compared to the surface basicity of 0.08 mmol g^{-1} of the fresh biochar. However, progressive oxidation caused a decrease in surface basicity of 0.54 mmol g^{-1} between the 0.042 and 0.333 M H_2O_2 treated biochar.

Changes occurred in the elemental composition and included decreases in carbon content, increases in the oxygen content and decreases in the hydrogen content with progressive oxidation of biochar between 0.042 and 0.333 M H_2O_2 This resulted in an increase of 0.034 units in the molar O/C ratio from the 0.042 M to 0.333 M H_2O_2 treated biochars. Due to the carbon being mineralized and the decrease occurring in hydrogen content of the biochar, the H/C ratio remained reasonably constant between 0.042 and 0.333 M H_2O_2 treated biochar, but a slight decrease was detected with progressive oxidation. The nitrogen content was found to be below detection point of 0.001 wt% (Table 1.2).

Spectroscopic analysis

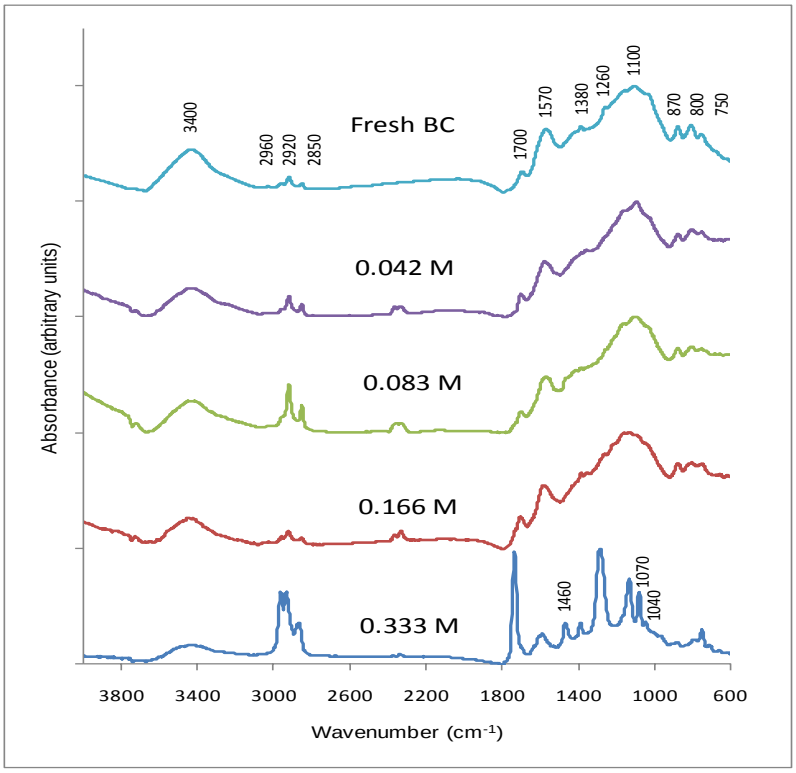

Figure 1.4: FT-IR spectras of fresh biochar and biochar treated with different concentrations of H_2O_2 (Band assignments given in Appendix 1.2)

The most prominent peaks for the fresh biochar occurred at the following wavenumbers (Fig. 1.4): A very broad peak at 3400 cm^{-1} associated with hydrogen bonded O-H stretching (Cheng et al. 2006; Cheng et al. 2008; Keiluweit et al. 2010), 2960-2850 cm^{-1} associated with aliphatic C-H_x stretching (Chen et al. 2005), 1700 cm^{-1} associated with C=O stretching (Chen et al. 2008; Koch et al. 1998; Pradhan and Sandle, 1999) which includes carboxylic C components and traces of ketones, aldehydes and esters, 1570 cm^{-1} associated with the aromatic components of biochar skeleton (Chen et al. 2008; Bustin and Guo, 1999), 1380 cm^{-1} associated with in plane bending of phenolic –OH related to ligneous syringyl units (Lehmann et al. 2005; López-Pasquali and Herrera,1997; Liu et al. 2008), 1280 cm^{-1} associated with phenolic C-O stretching derived from guaiacyl units of lignin (López-Pasquali and Herrera,1997), 1150-1100 cm^{-1} associated with symmetric and assymetric C-O-C ester groups/stretching derived from cellulose and hemicelluloses (Keiluweit et al. 2010) although the presence of silicates, which has intense adsorption in this region, cannot be excluded (Francioso et al. 2011). Wavenumbers below 1000 cm^{-1} delivered three peaks at 875, 800 and 750 cm^{-1} which is associated with out of plane C-H bending in aromatic structures containing two to three adjacent C-H bonds (Bornemann et al. 2008; Keiluweit et al. 2010). Minor peaks were also seen at wavelengths of 1380 cm^{-1}, associated with O-H bonds and phenols derived

from ligneous products, and 1260 cm^{-1}, associated with cellulose derived C-O stretching of pyranone rings and guaiacyl monomers, respectively (Keiluweit et al. 2010).

The oxidation of biochar with 0.042 M H_2O_2 only caused slight differences in spectral intensities of the O-H bonds at 3400 cm^{-1} and 1380 cm^{-1} and the aromatic components at 1570 cm^{-1} when compared to the fresh biochar. No noticeable changes occurred with regards to the carboxylic bonds and C-O-C stretching vibrations.

Biochar oxidation using 0.083 M H_2O_2 had very similar FT-IR spectra compared to the biochar oxidized with 0.042 M H_2O_2. However, a pronounced increase in the intensity of the aliphatic C-H peaks between 2950-2850 cm^{-1} were observed with respect to the fresh and 0.042 M H_2O_2 oxidized biochar. The oxidation of biochar with 0.166 M biochar did lead to an increase in the intensity of the peak at 1700 cm^{-1} indicating the formation of mainly carboxylic C (Pradhan and Sandle, 1999; Chen et al. 2008; Keiluweit et al. 2010). The pronounced increase in the intensity of the aliphatic C-H peaks shown when biochar was oxidized with 0.083 M H_2O_2 was not repeated in this treatment. The intensity and number of peaks still remained the same between 1600-1000 cm^{-1} and it seemed like the components associated with this region was the least affected by oxidation.

However, the oxidation of the biochar with 0.333 M H_2O_2 caused considerable shifts and changes in peak intensity and sharpness, indicating purification of the biochar. Three peaks increased considerably in their intensity and sharpness. The intensity of the aliphatic C-H vibrations, between wavelengths of 2960-2850 cm^{-1}, increased considerably compared to the fresh biochar. The degree of carboxylation was greater than in any of the previous treatments as shown by the increase in intensity and sharpness of the carboxylic vibrations at 1730 cm^{-1}. Furthermore, the peak at 1280 cm^{-1} which was identified as one of the minor peaks on the surface of the fresh biochar had the greatest intensity now. Various studies ascribe the peaks occurring at ±1260 and 1380 cm^{-1} to phenolic acid functional (C-O) and COOH groups and carboxylate respectively (Starsinic et al. 1983; Guo and Bustin, 1998; Cheng et al. 2008).

However, not all the peaks identified on the fresh biochar increased in intensity. The peaks at 1580 cm^{-1}, 1380 cm^{-1} and 1125 cm^{-1} associated with aromatic components, phenolic C-O stretching (as mentioned before) and C-O-C stretching vibrations in cellulose and hemi-celluloses and aliphatic –OH respectively (Pretsch et al. 2009; Keiluweit et al. 2010), decreased in their intensities compared to the fresh biochar. In contrast to the decrease in intensity, the sharpness of these peaks increased considerably due to the purification of the biochar, possibly as a result of the removal of aliphatics and VOCs during progressive

oxidation. The larger degree of oxidation also increased the number of peaks identified between wavelengths of 1600-1000 cm^{-1}. New peaks occurred at 1460 cm^{-1} and 1070 cm^{-1} associated with the C=C stretching of aromatic carbon, bound to an unsaturated group (indicative of lignin) and C-H deformation vibrations typical for substituted aromatics, respectively. Two peaks at 875 and 750 cm^{-1} indicating out of plane aromatic C-H stretching (Bustin and Guo, 1999) disappeared, with the peak at 745 cm^{-1} remaining.

Below are the FT-IR spectrum of the biochar treated with 0.333 M H_2O_2 (blue line) overlapped with the FT-IR spectrum of several substances (red line)(Omnic software libraries, Version 7.2 Thermo Scientific), which show strong relationships in certain wavenumber ranges. These include phenol, anthracene, acetophenone, *p*-benzoquinone, benzoic acid and benzaldehyde.

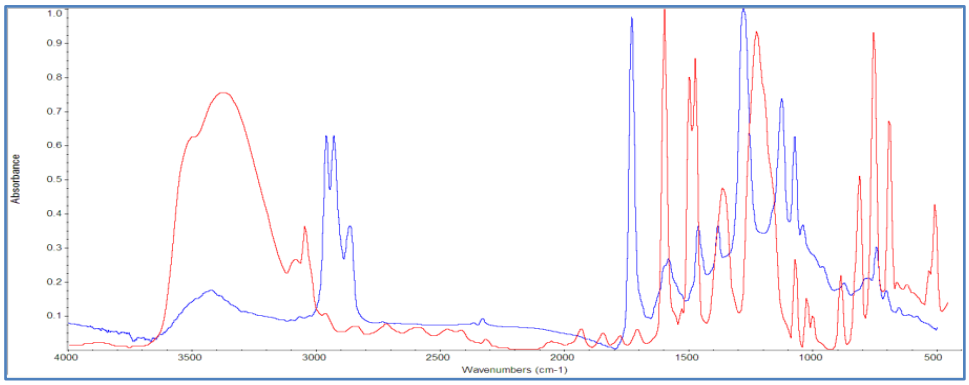

Figure 1.5.1: FT-IR spectrum of phenol (red line) and 0.333 M H_2O_2 oxidized biochar treatment (blue line)

The FT-IR spectrum of phenol shows strong overlap at wavenumbers 1580 cm^{-1}, 1460 cm^{-1}, 1380 cm^{-1} and 1280 cm^{-1}. A degree of overlap also occurs at 740 cm^{-1} and 700 cm^{-1} although the peaks of phenol are much more intense.

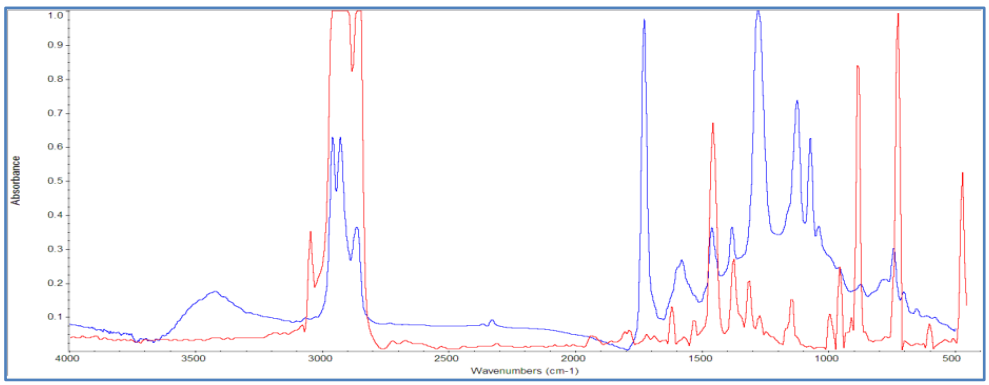

Figure 1.5.2: FT-IR spectrum of anthracene (red line) and 0.333 M H_2O_2 oxidized biochar treatment (blue line)

The FT-IR spectrum of anthracene shows strong overlap at wavenumbers 2960-2860 cm^{-1}, 1460 cm^{-1}, 1380 cm^{-1} and 740 cm^{-1}.

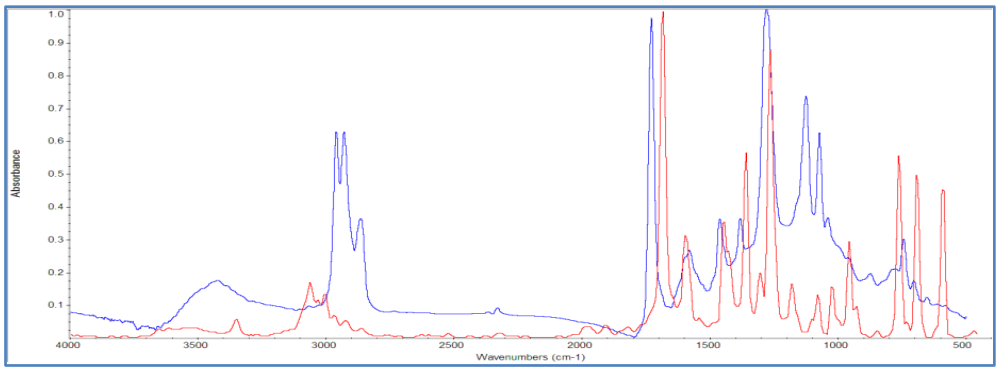

Figure 1.5.3: FT-IR spectrum of acetophenone (red line) and 0.333 M H_2O_2 oxidized biochar treatment (blue line)

The FT-IR spectrum of acetophenone shows strong overlap at wavenumbers 1730 cm^{-1}, 1580 cm^{-1}, 1460 cm^{-1}, 1380 cm^{-1} and 1280 cm^{-1}. Strong overlap also occurs at the lower wavenumbers of 740 cm^{-1} and 700 cm^{-1}.

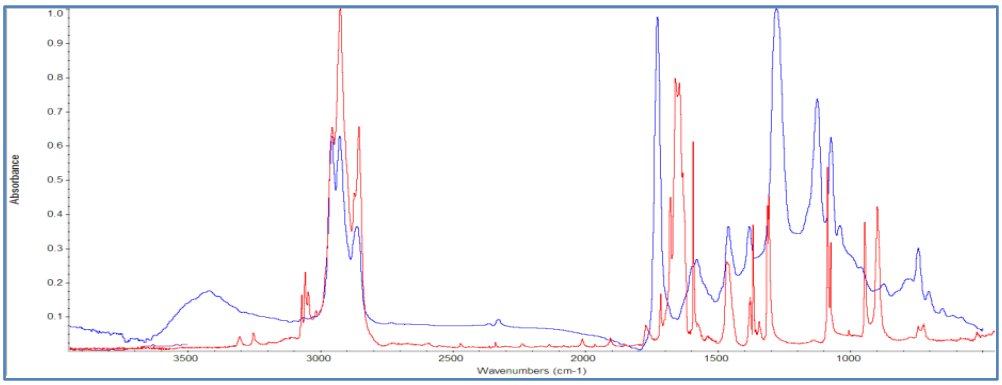

Figure1.5.4: FT-IR spectrum of p-benzoquinone (red line) and 0.333 M H_2O_2 oxidized biochar treatment (blue line)

The FT-IR spectrum of *p*-benzoquinone shows strong overlap at wavenumbers 2960-2860 cm^{-1}, 1460 cm^{-1} 1580 cm^{-1} and 1380 cm^{-1}.

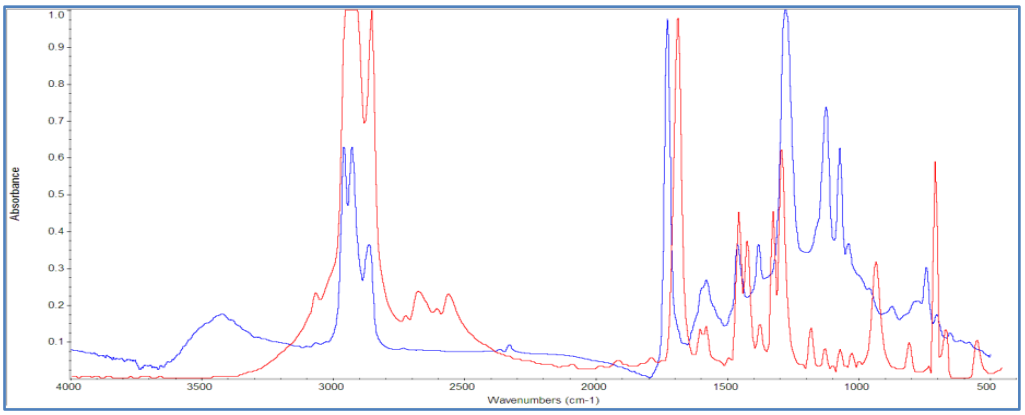

Figure 1.5.5: FT-IR spectrum of benzoic-acid (red line) and 0.333 M H_2O_2 oxidized biochar treatment (blue line)

The FT-IR spectrum of benzoic-acid shows strong or good overlap at wavenumbers 2960-2860 cm^{-1}, 1730 cm^{-1} 1580 cm^{-1}, 1280 cm^{-1} and with the small peak at 700 cm^{-1}.

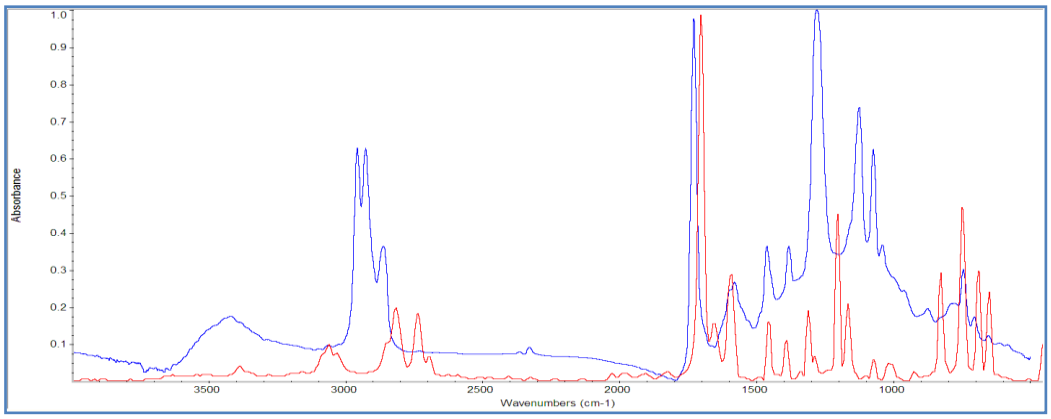

Figure 1.5.6: FT-IR spectrum of benzaldehyde (red line) and 0.333 M H_2O_2 oxidized biochar treatment (blue line)

The FT-IR spectrum of benzoic-acid shows strong or good overlap at wavenumbers 1730 cm^{-1}, 1580 cm^{-1} and with the peaks at the lower wavenumbers of 740 and 700 cm^{-1} respectively.

It is clear that anthracene, p-benzoquinone and benzoic acid show very strong overlap with the spectra of the oxidised biochar in the region associated with aliphatic C-H stretching (2950-2850 cm^{-1}) even though these substances contain no aliphatics and only C-H stretching associated with benzene rings (aromatics).

Acetophenone, benzaldehyde and benzoic acid have very strong absorbencies at wavenumbers in-between 1685-1700 cm^{-1} which strongly overlaps with the intense peak at 1730 cm^{-1} of the treated biochar, associated with C=O stretching.

Acetophenone and phenol almost fits the treated biochar like a fingerprint in the interval 1580-1280 cm^{-1}, with clear overlapping of the peaks identified at 1580, 1460, 1380 and 1280 cm^{-1}. Antracene also overlapped with the peaks at 1460 cm^{-1} and 1380 cm^{-1}.

All of the spectras (except p-benzoquinone) showed some degree of overlap at the lower wavenumbers of 700-750 cm^{-1}.

Intermediates released during oxidation and lixiviation

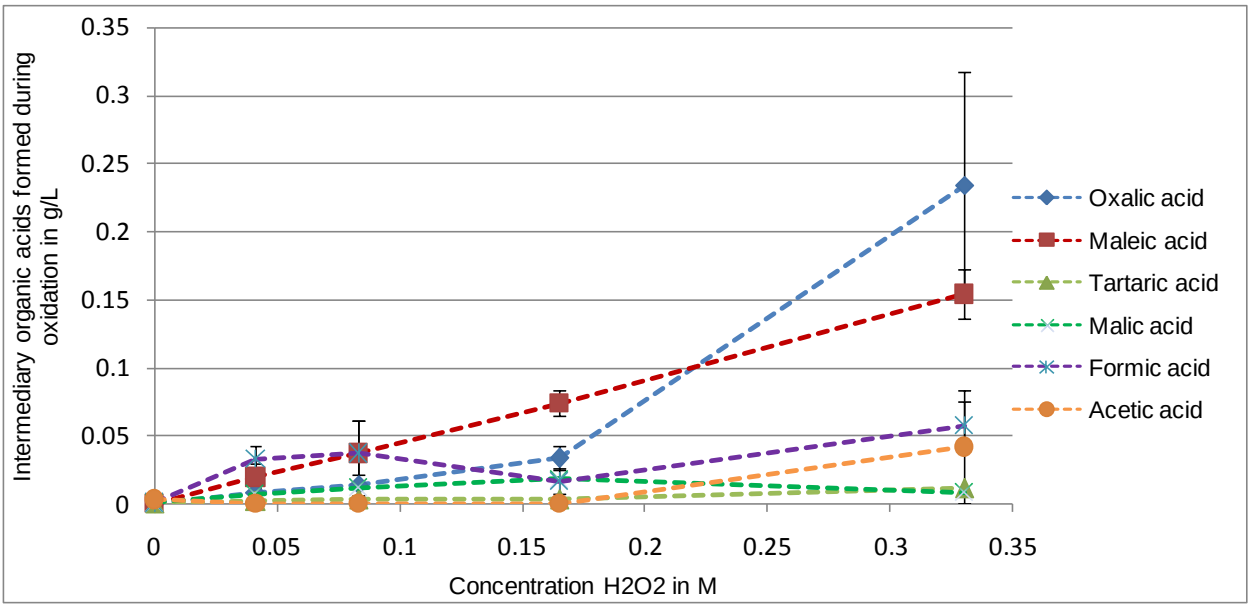

Figure 1.6: Short chain carboxylic acids released with progressive oxidation of the biochar using different concentrations of H_2O_2

The evolution of several short chain organic acids was followed during the progressive oxidation of biochar. The most predominant acids formed during oxidation with 0.042 and 0.083 M H_2O_2 was formic and maleic acids (Fig. 1.6). The formic acid concetration was the highest at 0.042 M H_2O_2 whilst maleic acid was the highest at 0.083 and significantly higher at 0.166 M H_2O_2 compared to any other organic acid. However, at 0.333 M a significant increase occurred in the production of oxalic acid, which was the dominant organic acid accumulating at 0.333 M H_2O_2. The maleic acid increased continuously with progressive oxidation of the biochars surface and followed a strong positive linear correlation (R^2= 0.99) with increasing oxidation of the biochars surface. Oxalic acid increased to 0.033 and 0.234 g. L^{-1} when the biochar was treated with 0.166 and 0.333 M H_2O_2 and seems to accumulate with progressive oxidation. With extensive oxidation using 0.333 M H_2O_2, all of the organic acids tested for was present, with maleic and oxalic acids being the dominant organic acids with concentrations of 0.15 and 0.23 g. L^{-1} respectively. Very low concentrations of acetic and tartaric acids formed with progressive oxidation up to 0.166 M H_2O_2. A noticeable increase in the concentrations of these acids only occurred when biochar was oxidized with 0.333 M H_2O_2. Especially the acetic acid increased in concentration to 0.042 g. L^{-1}. The only organic acid that decreased in concentration when oxidized with 0.333 M H_2O_2 was the malic acid.

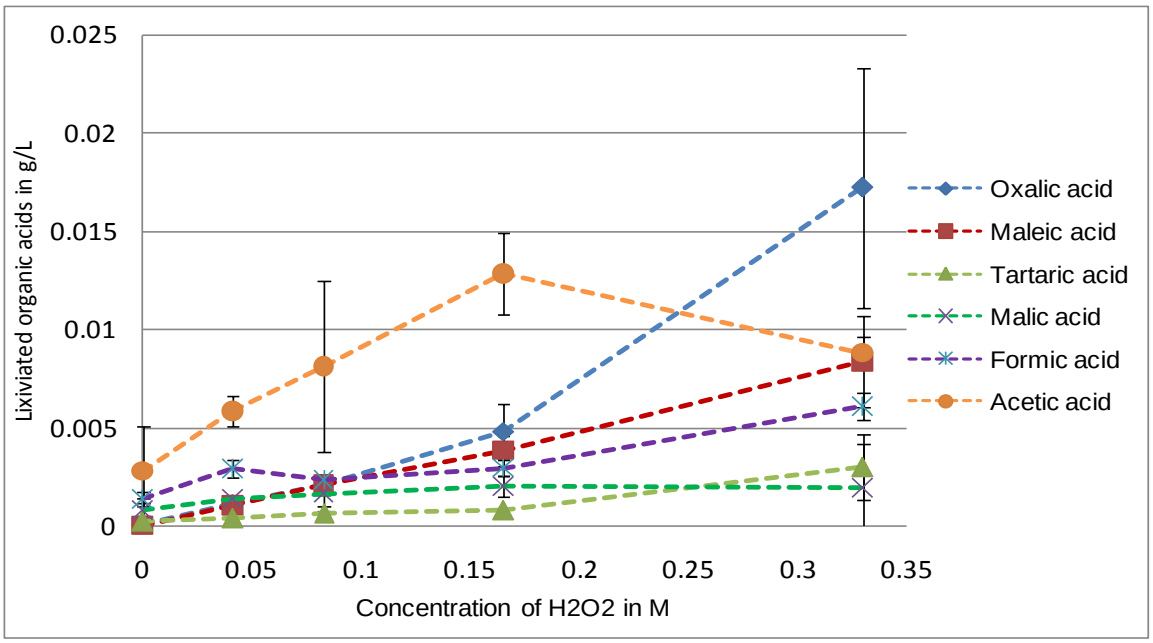

Figure 1.7: Short chain organic acids lixiviated from treated biochar.

All of the lixiviated organic acids tested for increased continuously increased (except acetic acid at 0.333 M H_2O_2) as the biochar was progressively oxidized (Fig. 1.7). Except for acetic acid, allmost the same pattern was repeated with the lixiviated acids compared to the oxidized intermediate organic acids (Fig. 1.6). Malic and tartaric acid again only occurred in trace amounts, followed by formic acid. Formic acid produced similar amount of lixiviated acid from the biochars treated with 0.042, 0.083 and 0.166 M H_2O_2, but an increase occurred in the amount of lixiviated formic acid collected from the 0.333 M H_2O_2 treated biochar.

The lixiviated acetic acid was the dominant organic acid lixiviated from biochar up to oxidation with 0.166 H_2O_2 where after a significant decrease occurred with extensive oxidation of biochar using 0.333 M H_2O_2. The lixiviated maleic acid concentration showed a strong positive linear correlation (R^2= 0.99) with increasing oxidant concentration. A significant increase occurred in the lixiviated oxalic from the biochar treated with 0.333 M H_2O_2 (Fig. 1.7). As a result the lixiviated oxalic acids had the highest concentration of 0.017 g. L^{-1} at the biochar treated with 0.333 M H_2O_2 followed by acetic and maleic acids with 0.009 and 0.008 g. L^{-1}.

Discussion

Complete chemical oxidation of biochar using hydrogen peroxide

Many studies have indicated that certain chemical fractions within biochar can last for millennia in the environment (Schmidt et al. 2002; Gavin et al. 2003), whilst incubation studies have indicated that a certain fraction can be degraded to some extent (Hamer et al. 2004; Zimmermann, 2010; Kuzyakov et al. 2009). Foereid et al. (2011) suggested that biochar consists of different chemical fractions that turn over at different rates.

It is clear from the data that the average amount of biochar oxidized with successive treatment with 0.333 M H_2O_2 steadily increased over time due to the decrease in biochar/H_2O_2 ratio. However, after the complete oxidation of about 90% of the biochar with 800 ml H_2O_2 the average amount of biochar oxidized decreased considerably with only 0.277 g of biochar being oxidized between 800-1000 ml H_2O_2. From this one can conclude that the last 10 % of the biochar left over showed clear resistance to oxidation and requires more of the oxidant to be fully oxidized to CO_2 and water.

Progressive oxidation of biochar using hydrogen peroxide

1. Changes in elemental and chemical characteristics

Progressive oxidation causes an increase in o-containing functional groups and oxygen content of biochar by producing oxidised weakly condensed aromatic structures on the surface (Knicker et al. 2007; Cheng et al. 2006). Progressive oxidation also results in a decrease in carbon content due to the mineralization of the carbon to various carboxylic acids and eventually CO_2. Aromatic carbons do have a redox potential and act mainly as reducing agents with oxygen being the most common electron acceptor (Joseph et al. 2010). In this trial, hydroxyl radicals were the prominent oxidant. Hydroxyl radicals react with organics via three mechanisms: hydrogen abstraction, addition to multiple bonds and direct electron transfer (Bosmann et al. 1998; Petri et al. 2011). Many studies have shown that progressive oxidation of biochar causes an increase in the atomic ratio of O/C and H/C as a result of oxygen addition and carbon mineralization to CO_2 (Cheng et al. 2006; Cheng et al. 2008; Cheng and Lehmann 2009). In this trial the H-content of the biochar decreased with progressive oxidation due to hydrogen abstraction which leads to the formation of radicals which dimerize in high yields (Coffmann et al. 1958).

The nature of the O-containing functional group can be acidic or basic and co-exist on the surface of biochar (Joseph et al. 2010). With progressive oxidation, the surface basicity

decrease and acidity increase (Cheng et al. 2008; Cheng and Lehmann 2009) due to the predominant formation of acidic functional groups such as carboxyl, lactonic, phenolic, carbonyl, o-quinone-like structures and ether type oxygen (Boehm 2001). From Figure 1.4, an increase in carboxylic-C (1690-1730 cm^{-1}) stretching bands occurred with progressive oxidation. The sharp intense peak seen in the most oxidized biochar at 1280 cm^{-1} can be ascribed to phenolic acid functional (C-O) and COOH groups (Starsinic et al. 1983; Guo and Bustin 1998). As a result a significant increase occurred in surface acidity. However, surface acidity seemed to reach a maximum after treatment with 0.083 M H_2O_2, where after only small increases occurred between biochar oxidized with 0.083 and 0.333 M H_2O_2 (Table 1.2). It would seem as if the surface of the biochar were completely saturated with acidic O-containing functional groups. This can however not be explained by the observed FT-IR spectras.

The initial increase in surface basicity of the biochar compared to the fresh biochar (Table 1.2) can be ascribed to a possible increase in the O-containing functionalities correlated with surface basicity. However, a decrease can be seen during progressive oxidation of biochar with 0.042 to 0.333 M H_2O_2. This is in accordance with previous studies (Cheng et al. 2008; Cheng and Lehmann, 2009). The reason for the decrease in surface basicity can be ascribed to the disappearance of basic o-containing functionalities such as pyrone and chromene (Montes-Morán et al. 2004) with progressive oxidation. According to Leon y Leon et al. (1992) the decrease in surface basicity is due to the incorporation of oxygen, which causes the dislocation of π electrons in the periphery of the basal planes. These delocalised π electrons in the basal planes has the ability to adsorb protons onto these planes through electrostatic interaction and because these electrons are abundant enough, it may represent an important percentage of basic sites (Montes-Morán et al. 2004).

Due to the increase seen in the peaks associated with carboxylic and phenolic groups (Fig. 1.4) during progressive oxidation of biochar, a significant drop occurred in the pH of the biochar. Even though surface acidity did not change significantly from the biochar oxidized with 0.083 to 0.333 M H_2O_2, the pH continued to decrease. Factors other than surface acidity, therefore, contributed to the drop in pH. The short chain organic acids that were lixiviated from the treated biochar (Fig. 1.8) and the decrease in surface basicity with progressive oxidation also contributed to the lower pH due to the smaller buffer capacity of the biochar. However, the most important factor in the decrease in pH seen is the dissolution of basic cations such as Na, K, Mg and Ca from the matrix of the biochar as it is degraded. The biochar was filtered after the oxidation with H_2O_2 thereby removing alkaline metals that

could compensate for acidification of the biochar surface by oxidation. (Shinogi et al. 2003; Major et al. 2009b; Joseph et al. 2010).

Changes in surface composition

*For the purposes of the spectroscopic analysis conducted, attention will mainly be devoted to the biochar oxidized with the highest concentration H_2O_2

Hydroxyl radicals can be produced via the ―Fenton process" (Petri et al. 2011) or Fenton like reactions. Hydroxyl radicals have been shown to play a role in lignin degradation as it can react with non-phenolic lignin model compounds by demethoxylation and the introduction of phenolic group to the non-phenolic model compound (Henrikson et al. 2000; Hildén et al. 2000; Have and Teunissen, 2001). Hardwood lignin consists of mainly syringyl and guaiacyl units (Obst, 1982). The FT-IR spectra clearly show a considerable increase in the sharpness and intensity of the peak at 1280 cm^{-1} which according to López-Pasquali and Herrera (1997) corresponds to the phenolic C-O groups associated with guaiacyl monomers in lignin. Demethoxylation of guaiacyl monomers by the hydroxyl radical and the introduction of phenolic groups (hydroxylation) could have led to the considerable increase seen in the peak intensity at 1280 cm^{-1} due to the formation of phenolic acid functional (C-O) and COOH groups (Cheng et al. 2008). Oxidation of benzene via hydroxylation causes the formation of phenol which contributes to the increase in surface acidity, as a result of the formation of phenolic components on the surface. Furthermore, the FT-IR graph (Fig. 1.5.1) shows that phenol has strong absorbance intensities at 1225 cm^{-1}, which is close to the intense peak seen at 1280 cm^{-1}.

An increase in the carbonyl/carboxylic stretching at 1690-1700 cm^{-1} from the fresh biochar to the biochar oxidized with 0.333 M H_2O_2 was evident (Fig. 1.4), due to the progressive carboxylation of the biochar's surface (Francioso et al. 2011; Brodowski et al. 2005a; Cheng et al. 2006). Hydroxyl radicals have been shown to be able to carboxylate various O-containing functional groups such as phenol, ketones, aldehydes, and esters to carboxylate (Choi et al. 1988; Zazo et al. 2005; Scheck and Frimmel, 1995). The FT-IR graph of benzoic acid (Fig. 1.5.5) also showed very strong absorbance at this wavenumber.

Surprisingly, and in contrast with the results of Cheng et al. (2006) and Nguyen et al. (2009) who found decreases in the aliphatic C-H groups (2850-2950 cm^{-1}) with progressive oxidation of biochar, a considerable increase occurred in the peak intensity between wavenumbers 2850-2950 cm^{-1} in our trial. From the overlapping of the treated biochar's FT-IR with the spectrums of acetophenone, p-benzoquinone and benzoic acid (Fig. 1.5.3-1.5.5),

it was clear that these pure chemical substances also had very strong absorbencies in this region even though these chemical substances do not contain aliphatics. Furthermore, with progressive oxidation it was expected that phenol and carboxylic groups would increase on the surface as a result of hydroxylation and carboxylation. This would result in an increase in benzoic acid and p-benzoquinone (phenol oxidation discussed next) on the surface of the biochar which would explain the increase in intensity of the peaks at 2950-2850 cm^{-1}.

In contrast to the increases seen in the previous peaks, considerable decreases occurred in the intensity of several peaks in-between 1600-1000 cm^{-1}. The aromatic C=C stretching in the range of 1560-1580 cm^{-1} decreased. Latriano et al. (1985) found that benzene ring fission can occur in a chemical hydroxyl generating system. Hydroxylation of benzene by the hydroxyl radical leads to the formation of phenol followed by the addition of another hydroxyl group to the C=C bonds of aromatics to from di-hydroxybenzene. Hydrogen abstraction then results in the formation of ortho-benzoquinone and the breaking of the double bond between the carbons of the benzene ring. Carboxylation of the muconic acid followed, leading to the formation of various short chain carboxylic acids (Scheck and Frimmel, 1995; Zazo et al. 2005) on the surface and also the release of these acids into suspension (discussed in following heading). This resulted in the considerable increases in the peaks seen at 1680-1700 cm^{-1} and 1280 cm^{-1}, which is associated with carboxylic and phenolic acid functional C-O groups as mentioned before.

The sub-range of 1850–500 cm^{-1} is known to encompass lignin related information (Hergert 1971) when FT-IR is done on wood material. The peaks at 1460, 1380, 1120 and 1070 cm^{-1} are all associated with functional groups related to lignin, cellulose and hemicelluloses as mentioned previously. In essence they are the transformation products deriving from the pyrolyses of lignin, and cellulose. In other words, the backbone of the wood‘s structure used to produce the pine derived biochar. However, processes which include demethoxylation of syringyl and guaiacyl associated units, hydroxylation, cleavage and eventual carboxylation of aromatics and esters led to the decreases and shifts that took place in these peaks with progressive oxidation of the biochar (Petri et al. 2011; Choi et al. 1988; Hammel et al. 2002). Still these peaks were the predominant peaks left after extensive oxidation with 0.333 M H_2O_2.

Interpretation of the intermediary carboxylic acids formed

The pyrolization of biochar produces mainly toluene, benzene and polycyclic aromatic hydrocarbons (PAHs) (Kaal et al. 2008). From previous studies conducted on biochar

oxidation, it seems as though depolymerisation and surface oxidation causes the initial alterations in the chemical and elemental characteristics of biochar (Kaal et al. 2008). The oxidation of aromatic hydrocarbons can lead to the formation of various carboxylic acids (Kawamura and Ikushima, 1993).

Benzene rings have been shown to be susceptible to attack from hydroxyl radicals through hydroxylation of benzene to phenol and catechol (benzene-diols) followed by hydrogen abstraction to form *o*-benzoquinones (Petri et al. 2011; Karakhanov et al. 2010; Scheck and Frimmel, 1995). These intermediates are very important as it serves as reducing reagents (reducing Fe^{3+} to Fe^{2+}), regenerating the catalyst via the Fenton reaction (Lu, 2000; Lu et al. 2002; Petri et al. 2011). It is important to know that the primary position of attack by the hydroxyl radical would be on those atoms with the largest electron density as the hydroxyl radical is an electrophile (Carrier et al. 2006). The benzene ring of the intermediates is then cleaved through hydrolysis to produce muconic acid from catechol and *o*-benzoquinones (Jones, 1999) and maleic acid from *p*-benzoquinones (Zazo et al. 2005; Sanchez et al. 2007). Successive cleavage of muconic acids leads to the formation of various carboxylic acids (Scheck and Frimmel, 1995). According to Zazo et al. (2005) the main organic acids produced with the oxidation of phenol by Fenton‘s reagent in aqueous solution resulted in the formation of mainly maleic, acetic, oxalic and formic acid and lower amounts of muconic, fumaric and malonic acids. These short chain carboxylic acids have a lower susceptibility to attack from the hydroxyl radical in the system, which allows for these short chain acids to accumulate in suspension (Koyama et al. 1994).

In this trial the oxidation of volatile organic carbon (VOCs) aliphatics and aromatics by hydroxyl radical attack led to the production of 6 short chain carboxylic acids namely maleic, malic, tartaric, oxalic, acetic and formic acids. Many simplified reaction pathways for the oxidative processes that take place to give rise to the various carboxylic acids from the initial oxidation of phenol have been derived (Scheck and Frimmel, 1995; Sanchez et al. 2007; Zazo et al. 2005). Among all the organic acids, muconic acid has the highest oxidation rate as it has the largest number of double bonds (Zazo et al. 2005) which facilitate attack by the electrophilic hydroxyl radical. Muconic acid can undergo successive oxidative steps to form maleic-, fumaric-, oxalic-, formic acid which is eventually oxidized to CO_2. Maleic and fumaric acid can be hydroxylated at the double bond between C_2-C_3, followed by hydrogen abstraction to produce malic acid (Scheck and Frimmel, 1995). Malic acid can further be oxidized to oxalacetic acid which is then decarboxylised and eventually oxidized to malonic acid (Scheck and Frimmel, 1995; Walling and El-Taliawi, 1973). Milas et al. (1937) has

shown that hydrogen peroxide under the influence of UV-light can hydroxylate the double bond in maleic acid to form tartaric acid. Hydroxyl attack on the double bonds of maleic acid can also lead to the formation of malonic, oxalic, formic and acetic acid (Zazo et al. 2005). However, the acetic acid has been shown to mainly derive from the oxidation of malonic acid, which appeared only in trace amounts in the trials conducted by Zazo et al. (2005). Finally, the formic acid formed with oxidation of the other organic acids is then completely oxidized to CO_2 (Scheck and Frimmel, 1995). Figure 1.8 (pp. 48) is a simplified scheme for the complete oxidation of phenol.

As biochar is described as a highly heterogeneous material, it is difficult to identify whether the chain reaction, as shown in Figure 1.8, is followed or if oxidation commenced from other starting points and not from the oxidation of benzene to phenol to short chain organic acids. At low concentration of H_2O_2 (0.042-0.083 M), it can be postulated that the aliphatics and volatile organic carbons adsorbed on the surface, served as the initial substrates for oxidative processes and resulted in the formic acid concentration being the highest at 0.042 M H_2O_2 as it is easier to oxidize chain C-C bonds (Scheck and Frimmel, 1995) and completely oxidize the VOCs that solubilised into the oxidative solution to formic acid. Furthermore, the removal of aliphatics and VOCs was also clear from the FT-IR spectra of the 0.333 M H_2O_2 oxidized biochar, as removal of VOCs and aliphatics demasked the biochar in the region 1600-1000 cm^{-1} with less overlap between absorbance bands resulting in the formation of clear, sharp peaks. The formation of formic acid can also be ascribed to the complete oxidation of the maleic acid (Fig. 1.8).

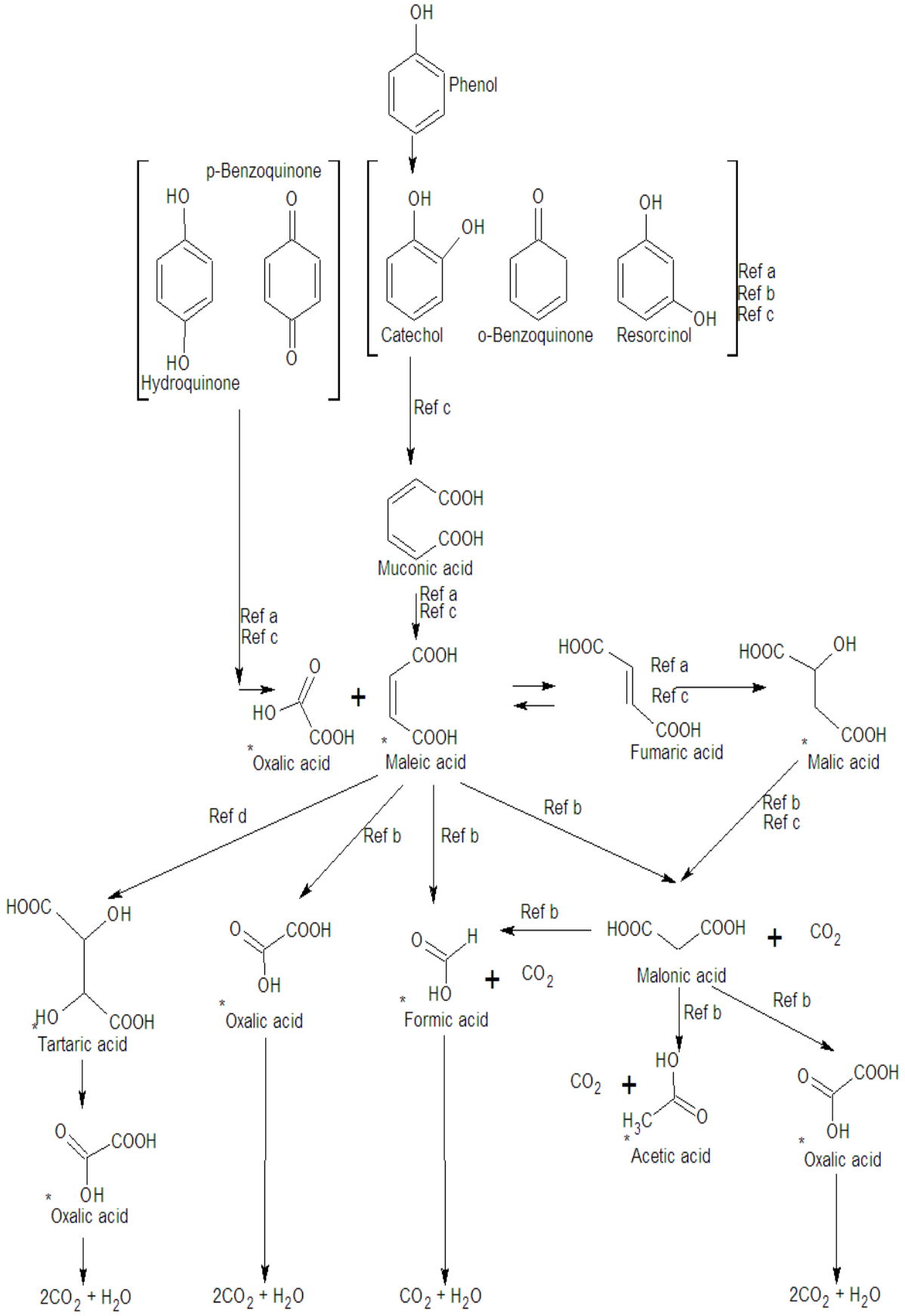

Figure 1.8: Simplified reaction pathway of phenol oxidation using the articles of (a) Sanchez et al. (2007); (b) Zazo et al. (2005); (c) Scheck and Frimmel (1995) and (d) Milas et al. 1937. The organic acids marked with * have been measured in this trial.

The oxidation of benzene (or aromatic compounds on biochars surface) resulted in the formation of maleic acid, which showed a positive linear increase in concentration with increasing concentration of the oxidant. As biochar is a highly aromatic substance, consisting mostly of benzene rings linked together, oxidation of these rings would be the most predominant, giving rise to muconic and maleic acids. Very low amounts of malic and oxalic acids were found, indicating that low amounts of muconic and maleic acids were completely oxidized at low concentrations of H_2O_2. This is further confirmed by the complete absence of acetic acid during these periods and only traces of tartaric acid were found.

The increase in H_2O_2 concentration to 0.166 M resulted in a linear increase in maleic acid. As a result more maleic acid could be oxidized to oxalic and formic acid. The oxidation of formic acid to CO_2 occurred at very similar rates during the trial as formic acid concentrations remained reasonably constant with progressive oxidation. Maleic and oxalic acid was the most dominant organic acids (Fig. 1.6) at high concentrations of H_2O_2. Oxalic acid can be formed from the cleavage of the intermediates of phenol oxidation (Scheck and Frimmel, 1995) and various longer chained organic acids which include tartaric, maleic and malonic acid (Fig. 1.8). Also the increases in direct cleavage of the intermediates by radicals resulted in an increase in maleic acid formation, which then ranslated into the increase seen in oxalic acid. The slight increase in malic acid concentration can also be ascribed to an increase in maleic acid oxidation. Acetic acid was absent again and only traces of tartaric acid were measured showing that these routes are of less importance at lower concentrations of H_2O_2.

The oxidation of biochar with the highest concentration of H_2O_2 (0.333 M) caused the formation of all the acids tested for in this trial. A significant increase in maleic acid concentration occurred and the formation of acetic and tartaric acid also increased considerably (Fig. 1.6). This is a clear indication that higher amounts of maleic acids were available for oxidation according to these routes. As a result of this, a significant increase in the formation of oxalic acid was measured. Zazo et al. (2005) observed almost the same trend during the oxidation of high amounts of phenol with high concentration of H_2O_2 and 1 mg L^{-1} Fe^{2+} and found that the production of oxalic acids was initially low, but steadily increased as oxidation proceeded, to stabilize at a higher concentration where it remained. According to Zazo et al. (2005) all of the intermediates will finally be oxidized to formic acid which eventually oxidizes to CO_2 and water under high Fe^{2+} and H_2O_2 concentrations except for oxalic acid which showed to be quite refractory to oxidation and remained in solution. Oxalic acid will eventually be oxidized directly to two molecules of CO_2 and water. The reason for

the stability of oxalic acid against oxidation could be due to the formation of Fe^{2+} and Fe^{3+} complexes (Alegría et al. 2003).

Several variables have to be considered when assessing the concentrations of organic acids formed during the oxidation of biochar. From Figure 1.8 it was clear that maleic acid continuously increased with an increase in the oxidant's concentration. This is because the biochar (substrate) is never fully oxidized and therefore never the limiting factor in maleic acid production, but rather the H_2O_2 concentration and the availability of a catalyst. Due to the large quantity of aromatic substrate (biochar) available to react with H_2O_2 the maleic acid concentration continuously increased with increased oxidation of biochar. The oxidation of biochar occurred at a faster rate compared to the oxidation of maleic acid. According to Zazo et al. (2005) the aromatic intermediates show faster oxidation rates than organic acids. Therefore, one can expect an increase in maleic acid concentration with progressive oxidation, followed by increases in the concentrations of the other organic acids derived from maleic acid oxidation as seen in Figure 1.8.

Other variables includes the decrease in pH that occur with progressive oxidation and the dissolution of transition metals such as Fe^{2+}, Cu^{2+} and Mn^{2+} which serve as catalyst, from the biochar's structure as it gets degraded. According to various studies (Potter and Roth, 1993; Jones, 1999) the optimal pH for the Fenton reagent is between 3-3.5. The decrease seen in pH could have resulted in more efficient oxidation of biochar and its intermediates. Also, with progressive oxidation more of the catalysts are released from the structure and more intermediates are produced from phenol oxidation which serve as a reducing reagent for the reduction of Fe^{3+} to Fe^{2+}, thus regenerating the catalyst via the Fenton reaction (Lu, 2000; Lu et al. 2002; Petri et al. 2011). The increase in catalyst and H_2O_2 concentration could have accelerated the oxidative process.

Conclusions

It is clear that hydrogen peroxide can fully oxidize biochar to CO_2 and water as biochar contains transitional elements which can facilitate the generation of hydroxyl radicals which can oxidize aromatic substances. The biochar oxidized at a steadily increasing rate (Fig. 1.2) due to a smaller biochar to oxidant ratio with each successive oxidation period using 50 ml H_2O_2. A considerable decrease occurred after 90% of the biochar were successfully oxidised even though the biochar to oxidant ratio was at its smallest. This is a clear indication that about ± 7% of the biochar (±3% ash) was highly recalcitrant to oxidation by hydroxyl radicals.

The oxidation of the biochar with H_2O_2 resulted in an increase in the oxygen content and in a decrease in the hydrogen and carbon content possibly due to hydrogen abstraction and mineralization of carbon through the oxidation of aliphatics, VOCs and cleavage of benzene rings. The increase in oxygen content can mainly be attributed to hydroxylation and carboxylation reactions which caused an increase in C=O stretching vibrations associated with carboxylic groups and phenolic groups. This is evident from the intense absorbance at wavenumber 1730 cm^{-1} and 1280 cm^{-1} of the FT-IR spectra from the 0.333 M H_2O_2 treated biochar. The purification of biochar via the selective removal of less recalcitrant components on the surface (possibly VOCs and aliphatics) leaves behind the components associated with the recalcitrant fraction of biochar. An increase the number of peaks in the region between 1600-1000 cm^{-1} occurred, which is associated with lignin related components and C-O-C stretching vibration in cellulose and hemicelluloses related components. One can conclude that the main components of the wood structure (lignin) were also the most recalcitrant during oxidation of biochar and that pyrolysis only further enhanced the recalcitrance of these components.

Increases in the phenolic and carboxylic groups on the surface led to an increase in surface acidity with progressive oxidation. The surface acidity reached saturation point, whilst the surface basicity decreased continuously with progressive oxidation. Even though the surface acidity reached saturation point, the pH still decreased. This is due to the decrease in surface basicity, the increased amounts of lixiviated short chain organic acids derived from the more oxidized biochar, but mainly due to the release and removal of basic cations from the biochar.

As biochar is a highly heterogeneous material, it is difficult to identify the primary products that are responsible for the ratios between and the amounts of organic acids formed. Furthermore, it also makes it difficult to identify whether chain reactions, shown in Figure 1.8, took place. However, it is clear that the continuous increases seen in maleic acid production with increasing H_2O_2 concentration resulted in the increases seen in the other organic acids, especially during the oxidation of biochar with 0.333 M H_2O_2. Maleic acid, therefore, serves as the primary product from which the other organic acids are derived. The increase in maleic acid is also a clear indicator of increased oxidation of aromatics (phenol, catechol, *p*-benzoquinone) as it is mainly derived from ring cleavage of these intermediates.

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CHAPTER 2: *IN VITRO* AEROBIC DEGRADATION OF BIOCHAR IN SAND COLUMNS

Introduction

The inherent stability of biochar against oxidation led to the assumption that biochar is chemically and biologically inert to such an extent, that its mineralization rate was thought to be negligible (Goldberg, 1985). The majority of carbon in biochar is in a highly stable state and has a mean residence time of a 1000 years at a mean annual temperature of 10˚C (Roberts et al. 2010). These findings are supported by the Terre Preta de Indio (Amazonian Dark Earths) phenomenon, created by farmers 500-2500 years ago.

However, the findings of Fakoussa (1981) led to multiple studies being conducted over longer periods of time which proved that biochar could be solubilised and oxidized by microbes (Leonowics et al. 1999; Hofrichter, 1999; Baldock and Smernik, 2002). In 1982 Cohen and Gabriele revealed that two fungal species had the ability to totally solubilise leonardite (highly oxidized lignite). Laborda et al. (1995) found that different fungal strains from the *Trichoderma* genera could solubilise both low rank and high rank coals. Hofrichter and Fritsche (1996) found that 38 wood and litter decaying basidiomycetes had a strong bleaching effect on coal derived humic-acids with 49 having a weak effect. *Nematoloma frowardii* b19 was found to be the most effective in bleaching. These studies proved that basidiomycetes had the ability to effectively colonise and solubilise coal and charcoal through the production of exo-enzymes and therefore have been used in a number of studies. Furthermore, Zimmerman (2010) found that abiotic oxidation of biochar can also contribute significantly to the degradation rate of biochar. However, Schneour (1996) found that the rate of abiotic oxidation is strongly mediated by regional climatic conditions and soil properties.

In this chapter, the degradation rate of pine wood derived biochar, inoculated with a wood rotting consortium of organisms, was determined. The objective was to determine whether biochar can serve as the sole carbon source to these organisms and whether fertilization and the addition of glucose can stimulate or even have a priming effect on the degradation rate of the biochar. Furthermore, to investigate and assess the contribution of abiotic oxidation by atmospheric oxygen and the highly oxidative soil mineral, birnessite (δ-MnO_2).

Materials and Methods

The trial setup

The incubation study was conducted in eighteen closed columns installed at the laboratory of the Soil Science Department, Stellenbosch University. The columns were made using PVC-pipe and PVC-endcaps with dimensions 250mm × 110mm. Each column were filled with a 10 % (w/w) biochar-silica sand mixture (180 g biochar mixed with 1620 g silica sand from Consol glass (AFS 35 sand). The sand was added to increase the permeability and accessibility of water and oxygen to the char. Each column was fitted with a perforated tube, which stretched through the 10% (w/w) biochar-sand mixture to the bottom of the column. The perforated tube was capped with a rubber endcap through which gas extraction from the soil-airspace were made. Airspace gas extractions were made through a small hole in the top PVC-endcap which was sealed with silicone in-between incubation periods. The field capacity of the 10% (w/w) biochar-silica sand mixture in each column was determined as ± 35%. Each column was therefore leached with 600 ml distilled or fertilized water after every incubation period. Columns were fitted with a Whatman 54 filter through which leachates was collected. Leachates were filtrated again through a Whatmann 2 filter with a pore diameter of 8 µm. The trial consisted of the following treatments done in triplicate:

- Tr1: (Control) Sterilised Biochar leached with sterilised water (abiotic)
- Tr 2: Sterilised Biochar + 0.1% birnessite leached with sterilised water (abiotic)
- Tr 3: (Control) Biochar + Inoculant leached with distilled water
- Tr 4: Biochar + Inoculant leached with fertilised distilled water
- Tr 5: Biochar + Inoculant + 0.1% glucose leached with fertilised distilled water
- Tr 6: Biochar + Inoculant + 0.1% birnessite (w/w) leached with fertilised distilled water

Fertilization

The pine wood biochar used in this experiment contained in excess of 60% carbon. The conservative value of 60% was used to calculate the amounts of fertilizer and glucose application. The addition of 180 g of biochar meant that each of the columns contained 108 g of carbon. The total amount of N and P added to the column by the biochar were considered to be negligible. Treatments 4, 5 and 6 were fertilized with NH_4NO_3 and KH_2PO_4 to attain C/N and C/P ratios of about 200:1 and a 1000:1, respectively. Each fertilized column was leached with distilled water containing 2.5 g. L^{-1} NH_4NO_3 and 0.78 g. L^{-1} KH_2PO_4.

Additionally, 1 ml trace-element solution (Cornelissen et al. 2003) was added every three months.

Glucose and birnessite additions were only made at the start of the incubation trial by leaching 270 mg and 180 mg of each into the columns, respectively. This amount of glucose was added to attain a glucose-C to biochar-C ratio of 1:1000. The amount of birnessite was added to attain a 1:1000 w/w ratio. The birnessite used in this trial was synthetically prepared according to the method of McKenzie (1971).

The Inoculant

The inoculant was prepared from rotting pine wood log material collected at the Botanical gardens of Stellenbosch University. The rotting wood material was broken into smaller pieces and distilled water was added to prepare mulch, which was then sieved through a 250 µm sieve. The inoculants had a pH of 4.71 and an EC of 205 µS/cm. The columns were then inoculated with 150 ml of the supernatant.

Figure 2.1: Rotting pine wood log

Isolation and identification of the fungi and yeasts

The organisms in the supernatant were identified by preparing a dilution series and plating the series on agar media containing benomyl and dichloran as antifungal ingredients and streptomycin (BDS) to prevent any bacterial growth (Worall, 1991). This media is used for the selective isolation of hymenomycetes. The colonies were allowed to grow for a week after which the morphologically different colonies were counted (Appendix 2.1). A representative sample of the various colonies present was obtained using a Harrison‘s disc (Harrison, 1938). The colonies were transferred several times onto the BDS agar plates to purify the colonies and obtain pure cultures for DNA isolation and sequencing.

After the completion of the incubation study the composition of the microbial community was again assessed. A top section (0-3 cm) and a middle section (8-10 cm depth) of the biochar-sand mixture was removed and shaken in a 100 ml sterilized water for 15 minutes. The supernatant was filtrated through a 250 µm sieve before the organisms was seperated and purified using the same method used for the inoculant.

All of the pure cultures were identified by isolating the DNA using the CTAB extraction protocol as described by Saghai Maroof et al. (1984). Polymerase chain reaction (PCR) amplification then followed. The ITS primers, ITS 1 and ITS 4 was used. PCR was performed in a Verity (Applied Biosystems) with the following cycling conditions: 95°C for 5 minutes followed by 40 cycles of 95°C at 30 seconds, 50°C at 60 seconds and 72°C for 90 seconds and a final extension of 72°C for 10 minutes. Post-PCR purification was done using the NucleoFast Purification System (Separations).

The resulting fragments were then sequenced using ITS4 and BigDye Terminator V1.3 (Applied Biosystems) followed by electrophoresis on the 3730xl DNA Analyser (Applied Biosystems). Sequences were analysed and trimmed using Sequencing Analysis V5.3.1 (Applied Biosystems) and Sequence Scanner V1 (Applied Biosystems). BLAST analyses were performed at http://blast.ncbi.nlm.nih.gov/Blast.cgi using the blastn algorithm.

Analytical methods

Gas extractions were made from the column- and soil-airspace using a syringe which was immediately capped with a rubber stopper before GC analysis. The gas composition (CO_2, CH_4 and N_2) within the head- and soil-airspace were then determined by injecting 0.4 ml of the biogas sample into a gas chromatograph (Varian 3300) (Sigge, 2005). The GC was equipped with a thermal conductivity detector and an i.d. column (2.0 m × 3.0 mm) filled with Hayesep Q (Supelco, Bellefonte, PA), 80/100 mesh. The GC made use of helium carrier gas at a flow rate of 30 ml. min^{-1} and the oven temperature was set at 55 ˚C.

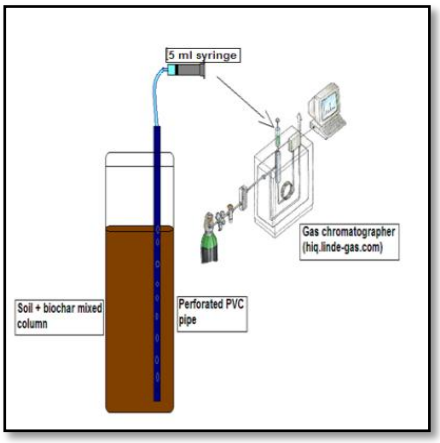

Figure 2.2: Column setup

Leachates were analyzed for electrical conductivity (only in the unfertilized columns) and pH, using an EDT conductivity meter and a Metrohm pH meter respectively. Total organic carbon (TOC) below 8µm was determined colometrically via the rapid oxidation spectrophotometric method of Heanes (1984) using an Ultrospec III spectrophotometer. A 5 ml sub-sample of the filtered extract was digested with a mixture of 1 ml 0.17 M $K_2Cr_2O_4$ and 5 ml concentrated H_2SO_4 (98%). Digestion was aided by microwaving each sample at 500 $J.ml^{-1}$. Samples were then allowed to cool and the volume adjusted to 30ml using distilled water. Absorption readings were taken at a wavelength of 590 nm.

Statistical analyses

Statistical analysis was conducted using SAS 9.1 for Windows. Significant differences between the total amount of carbon lost from the treatments were distinguished at the $P< 0.05$ level using Tukey‘s Studentized Range test. A single factor ANOVA was used to distinguish between the treatments means of the different treatments.

Results

Microbial composition of inoculum pre-incubation and post incubation

Table 2.1 below shows the species of fungi and yeast isolated from the inoculants pre-incubation and from the columns post-incubation. Illustrations of the fungi and yeast isolated are shown in Appendix 2.1.

Table 2.1: Composition of the microbial consortia

Pre-incubation	**Post-incubation**
Fungi	**Fungi**
• *Umbelopsis ramanniana* • *Mortierella turficola* • *1 Unidentified specie*	• *Fusarium oxysporum* • *Fusarium equiseti* • *2 Unidentified species*
Yeasts	**Yeasts**
• *Candida valdiviana* • *Rhodoturula sp.* • *Pichia guilliermondii* • *Trichosporon porosum* • *4 Unidentified species*	• *Trichosporon porosum* • *Pichia guilliermondii* • *2 Unidentified species*

From the table above it is clear that shifts occurred in the microbial consortia composition, especially regarding the fungi‘s with the disappearance of *Mortierella* species and *Fusarium* species becoming the predominant fungi isolated after incubation (Appendix 2.2 and 2.3).

Umbelopsis isabellina was previously known as *Mortierella isabellina. Mortierella* sp. belongs to the Zygomycota phylum. Watanabe et al. (1998) screened for lignocelluloses-decomposing fungi by isolating various fungi from soil and decayed wood and found *Mortierella isabellina* and *lignicola* always to be dominant as is the case with the decomposing pine wood material used for the preparation of the inoculums in this trial with *Umbelopsis* and *mortierella* species being the dominant fungies in the rotting wood microbial consortium.

Fusarium oxysporum belongs to the phylum ascomycota and is a ubiquitous soil inhabiting fungus. *Fusarium oxysporum* causes widespread plant diseases in agriculture and is considered to be very adaptable, able to degrade a wide variety of substrates such as lignin (Rodriguez et al. 1996; Sutherland et al. 1983) and complex carbohydrates. It is also considered to be one of the most potent cellulolytic degrading fungi‘s in nature as it can release a battery of extracellular enzymes to degrade cellulose and pectin (Fernández et al.

1993). Sampredo et al. (2007) found Mn-peroxidase and Mn-independent peroxidase activities in *F.oxysporum* solid state cultures which were utilized to decrease the phytotoxicity in dry olive mill residue by transforming phytotoxic aromatic components.

Previous attempts to isolate yeasts from rotten wood was done by Gringsberg in 1970 followed by Ramírez and González (1984a, b) which led to the isolation and description of 18 currently accepted novel species which consisted of 12 species of *Candida, 1 Pichia* species, *Schizoblastosporion* species. and *4 Rhodotorula spp.* (This is very similar to the yeast genera isolated from the rotting pine wood stump in this trial).

Pichia guilliermondii is a genus of *Pichia* belonging to the phylum Ascomycota and is very common in nature, with strains from this species commonly isolated from tree exudates, soil, plant and in the atmosphere. *Pichia guilliermondii* has an anamorphic form refered to as *Candida guilliermondii*. *Candida valdiviana* is a genus of yeast and belongs to the phylum Ascomycota. *C. valdiviana* was first discovered after it was isolated from rotting wood material by Grinbergs and Yarrow (1970).

*Rhodoturula sp.*falls under the phylum, basidiomycota and consist of three active species namely *Rhodotorula glutinis*, *R minuta*, and *R mucilaginosa*. *Rhodotorula* is very common yeast in the environment and has often been isolated from rotting wood. Gonzalez et al. (1989) found high numbers of the genera *Rhodotorula* during the medium stage of wood decay. Randhawa et al. (2000) also sporadically isolated *Rhodotorula mucilaginosa* and *R. glutinis* in decaying wood in tree trunk hollows. Furthermore, certain *Rhodotorula* species are able to utilize several aromatic compounds deriving from lignin breakdown (Cain et al. 1968)

Trichosporon porosum is basidiomycetous yeasts, commonly isolated from soil and has also been isolated from various rotting wood materials. Middelhoven (2006) isolated *Trichosporon porosum* from six rotten wood samples, collected from the Wageningen forest. Middelhoven et al. (1992, 1999, 2000) found that various benzene compounds are assimilated by *Trichosporon* spesies as listed in Middelhoven et al. (2004) and that these compounds can be used to distinguish between various strains of *Trichosporon.*

Respiratory CO_2 loss from columns

Table 2.2: Average CO2 efflux (µg CO2-C g^{-1} d^{-1}) produced during each incubation period

Days	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6
0	0.000	0.000	0.000	0.000	0.000	0.000
30	0.000	0.000	0.000	0.813	15.680	0.000
60	0.000	0.518	1.220	1.496	7.054	1.319
90	0.000	0.035	0.000	3.759	3.470	0.985
135	0.000	0.000	0.000	2.744	2.276	0.000
180	0.000	0.000	0.000	2.380	1.569	0.750
225	0.000	0.000	0.000	1.593	0.081	0.000

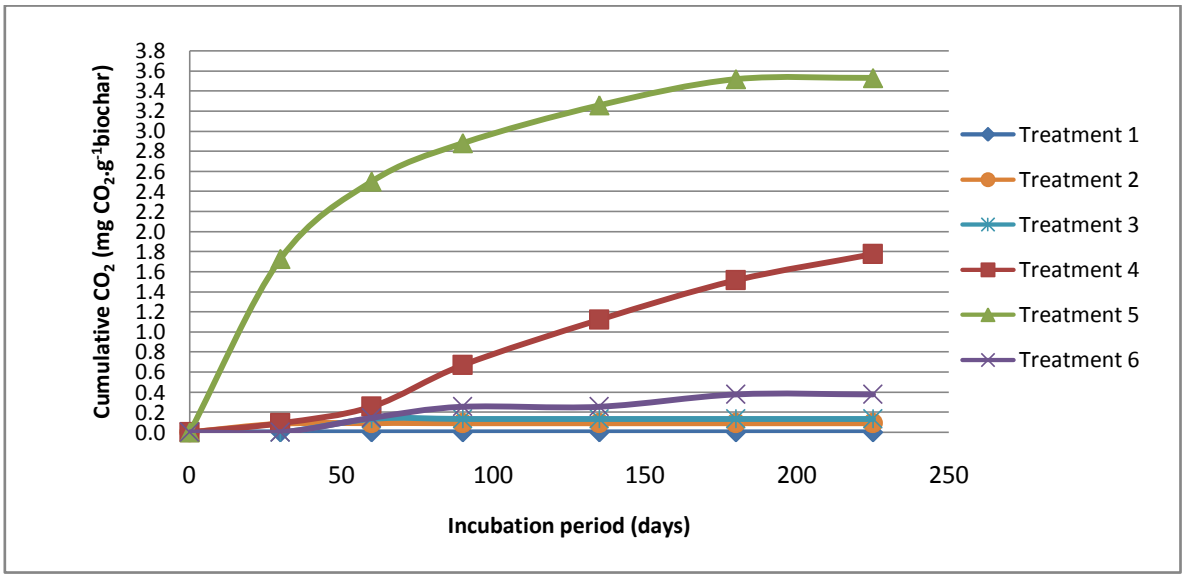

Figure 2.3: Trend of respiratory CO_2 produced over the incubation period

Treatment 1 (abiotic oxidation) did not produce any CO_2 during the entire incubation period, whilst treatment 2 produced CO_2 during the first 60 days from only one column where after no further CO_2 production occurred. Treatment 3 showed respiratory activity only during the second incubation period (Table 2.2). The respiratory activity stopped with no further production of CO_2 deriving from any of the replicates thereafter. The CO_2 effluxes were the greatest in treatment 4 and 5 (Fig. 2.3) compared to any of the other treatments. The addition of fertilizer and an easily available carbon source in the form of glucose, led to the production of CO_2 throughout the incubation period. However, at the end of the incubation period of 225 days the rate of respiration was more pronounced in treatment 4. Treatment 4 produced respiratory CO_2 from two of the three columns for most of the incubation trial. The cumulative CO_2 produced from these columns followed a linear trend (R^2= 0.983) and produced CO_2 consistently throughout the incubation period of 225 days. The initial CO_2 efflux from these columns was 0.813 µg CO_2-C g^{-1} d^{-1} after 30 days. The rate increased to 3.759 µg CO_2-C g^{-1} d^{-1} after 90 days of incubation, where after the rate steadily decreased to

1.593 µg CO_2-C g^{-1} d^{-1} at the end of the trial (Table 2.2). The average amount of carbon lost as CO_2 from treatment 4 were 87.175 mg over the 225 days. Treatment 5 followed a logarithmic trend (R^2= 0.984) with a high respiratory rate during the first 60 days of incubation. Thereafter, the rate steadily decreased over time and followed a similar linear trend (R^2= 0.878) compared to treatment 4. The CO_2 efflux was initially very high at 15.680 µg CO_2-C g^{-1} d^{-1} during the first incubation period of 30 days. However, the rate of respiration decreased rapidly to 7.054 µg CO_2-C g^{-1} d^{-1} during the next incubation period of 30 days. Thereafter the rate steadily decreased for the rest of the incubation period to 1.569 µg CO_2-C g^{-1} d^{-1} after 180 days and then to an almost stagnating slow rate of 0.081 µg CO_2-C g^{-1} d^{-1} after 225 days.

Liquefaction of biochar

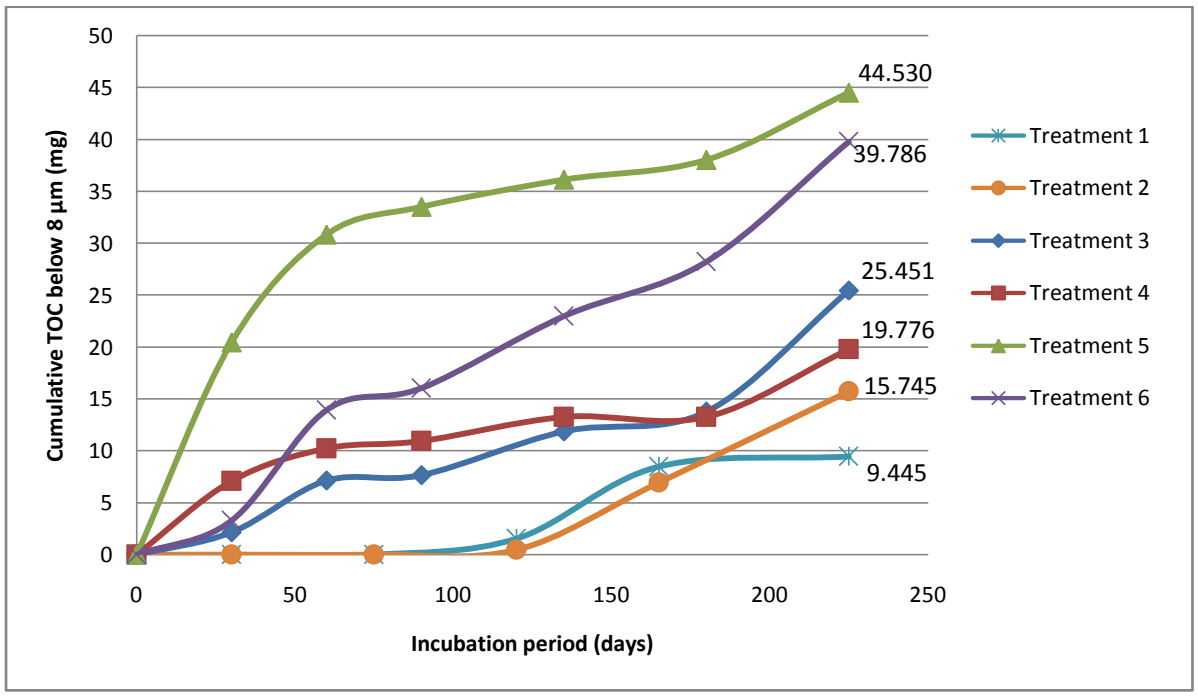

Figure 2.4: Total organic carbon (TOC) leached from columns during the incubation period

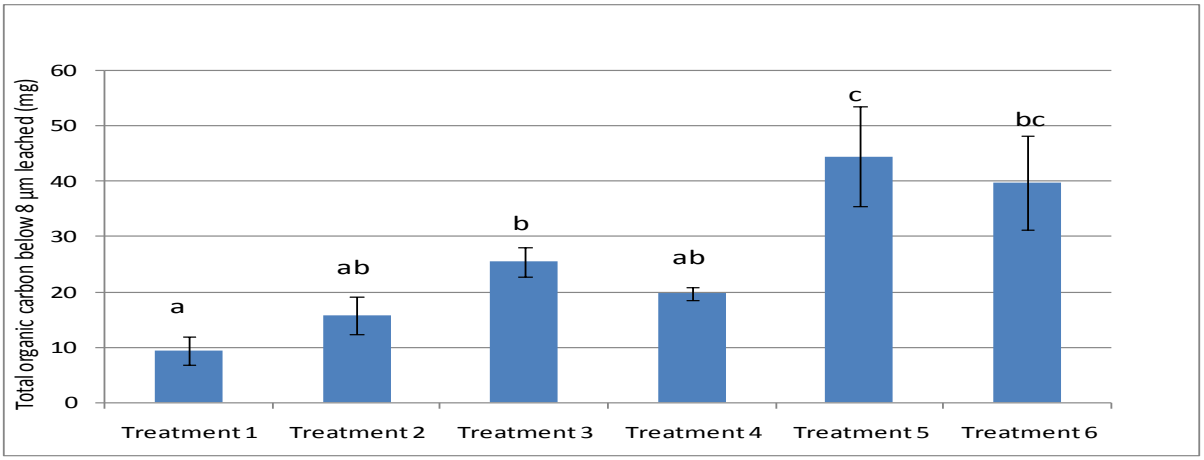

Figure 2.5: Total organic carbon (TOC) leached from the treatment columns after 225 days

Treatment 1 produced the least amount of leached organics (≤ 8 µm) when compared to any of the other treatments. Treatment 1 and 2 first started producing solubilised organic carbon between 90-120 days in one of the columns. With the next sampling event, all of the columns produced solubilised organics continuously for the rest of the incubation period up to 225 days. Treatment 1 was followed by treatment 2 and 4 (Fig. 2.4) which produced 66.7 and 109.4% more leached organics. Although these treatments led to an increase in the amount of leached organics, the increases were not significant (P> 0.05) compared to treatment 1. However, treatment 3 produced 25.452 mg of leached organic carbon (Table 2.3) during the incubation period and differed significantly from treatment 1, but not from treatment 2 or 4. A comparison between treatment 1 and 3 (Table 2.3) showed, that even though the inoculums did introduce additional organic carbon to the columns of treatment 3-6, it did not have a significant effect (P> 0.05) on the total carbon lost from these columns through leaching and respiration (Table 2.3). Treatment 2 also showed an increase of 67% in the amount of leached organic compared to its control (treatment 1) due to the addition of the highly oxidative soil mineral, birnessite.

Treatment 5 produced significantly more solubilised organics over the incubation period compared to the control (treatment 3 in this case) or any of the other treatments. Initially treatment 5 produced 20.475 mg solubilised organics after the first 30 days of incubation. However, the amount of solubilised organics decreased to 10.367 mg and 2.690 mg after 60 and 90 days respectively. In contrast, treatment 6 only produced 3.291 mg solubilised organics during the first leaching event. The amount of leached organics steadily increased thereafter to 11.588 mg at the last leaching event, which meant that the rate of solubilisation within these columns increased over time. Treatment 4 produced 29.3% less leached organics compared to treatment 3.

Total carbon lost

Table 2.3: Fraction of carbon lost during leaching events and as microbial respiration

	Respiratory CO_2 (mg)	Carbon lost as CO_2 (mg)	TOC (≤8 µm) leached (mg)	Total carbon lost from biochar in columns (mg)
Treatment 1	0.00	0.00	9.45	9.45 a
Treatment 2	16.43	4.49	15.75	20.23 a
Treatment 3	24.14	6.59	25.45	32.04 ac
Treatment 4	319.35	87.18	19.78	106.95 b
Treatment 5	634.87	173.31	44.53	109.84 b
Treatment 6	203.55	18.52	39.79	58.31 c

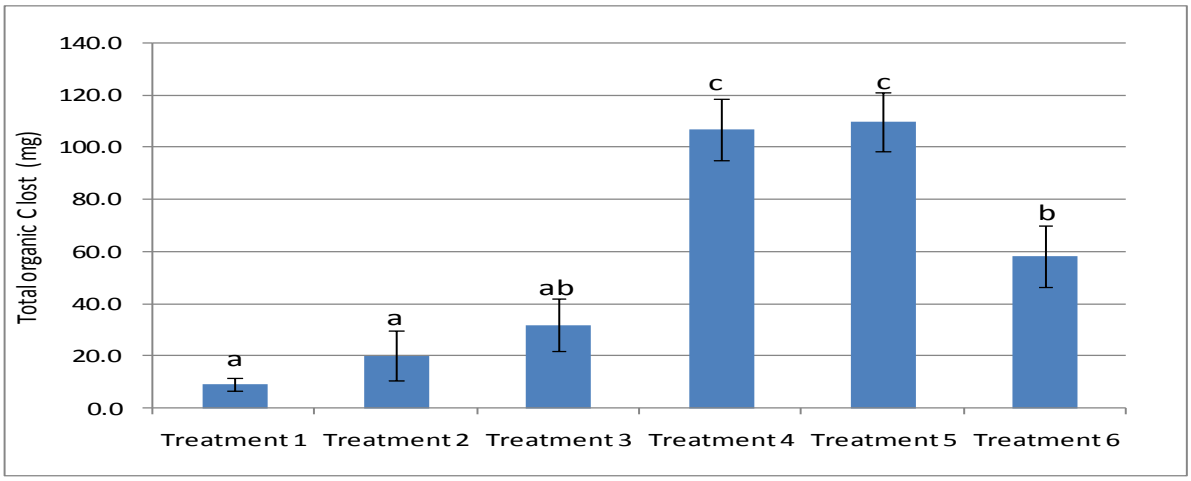

Figure 2.6: Total amount of carbon (mg) lost from the columns through leaching microbial respiration and abiotic oxidation.

The total amount of carbon lost as CO_2 and solubilised organics (TOC ≤8 µm) during leaching events showed a steady increase from treatment 1-3 (Fig. 2.6). These increases were, however, not significant ($P > 0.05$) and treatment 1-3 did not differ significantly from each other (Table 2.3). The results shown in Figure 2.6 clearly shows a significant increase ($P < 0.05$) in the total amount of carbon lost from treatments 4 and 5 compared to the rest of the treatments. Treatment 4 and 5 lost comparable amounts of carbon through respiration, natural oxidation and leaching. However, the rate of carbon loss from treatment 5 decreased at the end of the incubation period due to the decrease in microbial respiration (Table 2.2) within these columns. Treatment 4 lost carbon at a higher rate due to the higher rate of microbial respiration (1.593 µg CO_2-C g^{-1} d^{-1}) after 225 days of incubation. Treatment 6 caused a significant increase in carbon loss compared to treatment 1 and 2. Even though treatment 6 caused an increase of 26.267 mg (± 82%) in carbon loss compared to treatment 3, the treatments did not differ significantly ($P > 0.05$).

Column chemistry: pH and EC

Table 2.4: Column chemistry at the end of the trial

	Initial pH	End pH	EC (µS/cm)
Treatment 1	8.70	7.83	31.47
Treatment 2	8.70	7.66	32.70
Treatment 3	8.70	7.77	37.33
Treatment 4	8.70	3.54	n.a
Treatment 5	8.70	5.89	n.a
Treatment 6	8.70	4.20	n.a

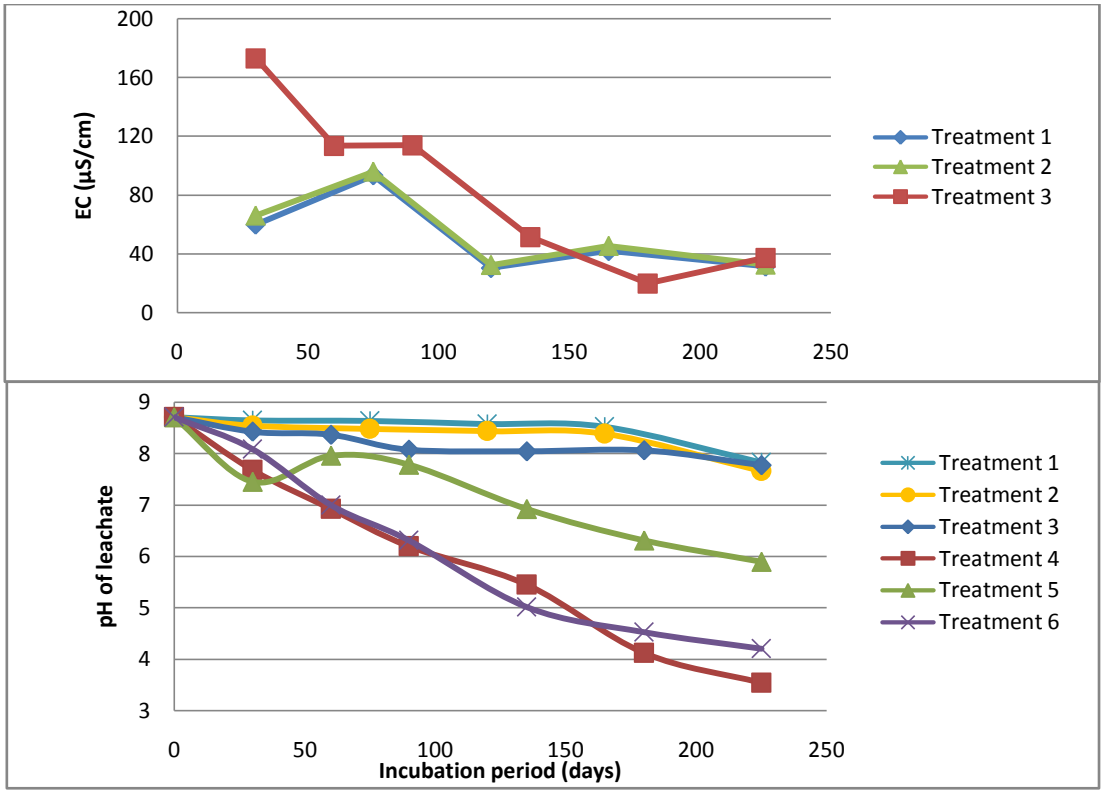

Figure 2.7: (a) Electrical conductivity (EC) and (b) pH of column leachate during the 225 day incubation period.

The EC (from non-fertilised columns) and pH of all treatments decreased from the initial 8.70 during the incubation period. The EC of treatment 1 and 2 were very similar as shown in figure 2.7a. Overall, the EC collected from these treatments decreased by 28.5 and 33.4 µS/cm from the initial EC measured with the first leaching event to the last at day 225. The initial EC of treatment 3 was much higher compared to the other two treatments due to the addition of the inoculum. The EC collected from treatment 3 also decreased from an initial 173.2 µS/cm to 20 µS/cm after 180 days before increasing to 37.3 µS/cm. Overall, the leaching events with distilled water caused a decrease in the EC of the biochar-sand columns (Fig. 2.7a).

The pH of the leachates also decreased in all treatment with varying magnitudes. The control showed a similar magnitude of decrease compared to treatments 2 and 3 and decreased by 0.87 pH units compared to the 1.04 and 0.93 pH units decrease from treatment 2 and 3, respectively. The fertilized treatments showed a significant amount of acidification leading to the large drop in pH over the incubation period. Acidification were the greatest in treatment 4 and 6 causing a decrease of 5.16 and 4.50 pH units respectively from the initial pH= 8.70. Even though treatment 5 received the same amount of fertilizer, the pH decreased to a lesser degree and was higher at the end of the incubation period compared to treatment 4 and 6 (Table 2.4).

Discussion

Respiratory CO_2 loss from columns

From the data collected it was clear that abiotic oxidation (Treatment 1) did not contribute to any carbon loss as CO_2 over the 225 day incubation period. This finding contradicts the findings of Zimmerman (2010) which showed that abiotic oxidation could contribute significantly to biochar mineralization via carbon lost as CO_2 from biochar incubations. This could be due to the fertilizer Zimmerman used in his abiotic treatments ($(NH_4)_2SO_4$ and KH_2PO_4) which possibly could have contributed to the oxidation of biochar.

The addition of the highly oxidative soil mineral, birnessite did cause the production of small amounts of CO_2 during the first 60 days of incubation. However, the measured CO_2 came from only one column and from different columns with each sampling period. Between the period 60-225 days no CO_2 evolution occurred and one can conclude that the addition of birnessite did not contribute to carbon loss from the biochar via oxidation to CO_2.

The additions of a microbial inoculate to the 10% (w/w) biochar-silica sand mix delivered similar results compared to treatment 2. Carbon dioxide evolution was only measured during the second incubation period (60 days) in two of the three columns. Thereafter no CO_2 evolution occurred in any of the columns for the rest of the trial. The CO_2 that did evolve after 60 days possibly came from the organic carbon introduced with the inoculum. The fact that no respiration occurred for the rest of the trial indicated that the microbes were unable to adjust and attain the essential nutrients needed for their survival from the new carbon source provided.

However, when fertilizer was added together with the inoculums, CO_2 evolution was more prominent and occurred until completion of the trial. Initially, the respiration occurred only from column 2 and after 60 days CO_2 evolved from column 3 as well and continued to evolve from these two columns for the rest of the trial. The initial lag phase seen in CO_2 evolution can be ascribed to the microbial consortia establishing and adjusting to the new conditions and carbon source provided and also the initial higher pH of the columns compared to the pH of the inoculums (pH= 4.71). After 60 days the rate of respiration increased considerably and continued to increase at a steady rate (Fig. 2.3). Compared to the non-fertilized treatments, it is clear that the addition of fertilizer is essential for the microbes to effectively utilize biochar as a carbon source as biochar alone cannot provide the essential nutrients needed for continued microbial activity.

The presence of a labile organic compound such as glucose has been shown to increase the rate at which biochar is mineralized via co-metabolic priming effect (Jenkinson, 1966; Kuzyakov et al. 2009). Glucose in this trial did not induce a priming effect on biochar mineralization as treatment 5 only lost 2.887 mg C more compared to treatment 4. The initial high rate of respiration was not due to biochar mineralization but due to glucose utilization and respiration by the microorganisms. Glucose caused an immediate activation of the microbial community. The rapid decrease in the rate of respiration is due to the depletion of glucose as an easily available carbon source. All the glucose was possibly utilized by day 90 as the respiration rate after 90 days was comparable to the respiration rate of treatment 4 which received no glucose. However, in contrast to treatment 4, the depletion of glucose from the columns in treatment 5 caused a significant decrease in microbial activity at the end of the trial with the respiratory rate almost coming to a complete standstill (0.081 µg CO_2-C g^{-1} d^{-1}) and only one column producing CO_2 compared to all the columns at the beginning of the trial. This is possibly due to the ‗Allee effect' (Begon et al. 2006). The addition of a 108 mg glucose-C could have possibly resulted in the competitive suppression of certain groups of the microbial community (responsible for the degradation of more recalcitrant substrates) and selected microbes that utilize more easily available organic compounds to become the dominant active microbes such as r-strategist bacteria (Nocentini et al. 2010). Instead of inducing a priming effect, the large amount of glucose added (compared to studies done by Hamer and Marschner, 2004; Nocentini et al. 2010) could have caused the extinction of the filamentous fungi (or any microbes) able to decompose recalcitrant substrates.

As seen in the Appendix 2.1-2.3, the fungi fraction of the consortium was affected the most by the change of carbon source. The *Mortierella* and *Umbelopsis* species were unable to utilise biochar as a sole carbon source and disappeared from the consortia, whilst biochar selected for *Fusarium* species in both treatments 4 and 5. The yeast also underwent considerable shifts in the composition of the microbial consortia, with the disappearance of *Rhodotorula* and *Candida* species after the incubation period. *Pichia guilliermondii* disappeared from the consortia in treatment 5, whilst *Trichosporon porosum* occurred before and after incubation in both treatments. Overall, the amount of colony forming units collected from treatment 5 were considerably less than those collected from the columns of treatment 4 and also decreased considerably in both treatments compared to what was added by the original inoculums (Appendix 2.1 and 2.2).

The addition of birnessite alone (treatment 2) have been shown not to induce the oxidation of biochar-C to CO_2. Even though a little amount of CO_2 did evolve from the columns in treatment 6, the results were erratic with CO_2 evolution occurring from different columns and only in three of the six sampling events.

Liquefaction of biochar

The inoculums introduced to treatments 3-6 did cause an increase in the amount of leached organics collected from these columns, but this did not have a significant effect on the total amount of carbon lost, when compared to treatment 1, which received no inoculant. The collection of leached organics from treatment 1 showed that biochar does contain a certain fraction of carbon that is able to solubilise in water over time. The biochar used in this trial had a volatile matter fraction of 19.89% due to adsorption of volatile gasses on the surface of biochar during the production process. Studies have shown that biochar contains different chemical fractions that are turned over at different rates (Hamer et al. 2004; Nguyen and Lehmann 2009; Cheng et al 2008). The volatile matter fraction is considered to be easily available to microbes and more easily oxidized and degraded. The solubilised organics possibly came from the volatile matter fraction that solubilised over time.

The addition of birnessite caused an increase in the amount of solubilised and leached organics in both treatment 2 and 6. Many poorly crystalline metal oxides such as Fe- and especially Mn-oxides contain a very reactive surface chemistry and has been proven to play an integral role in oxidative transformation of natural organic compounds such as polyphenols and low molecular weight organic acids within soils (Hardie et al. 2009; Chorover and Amistadi, 2001; Chang Chien et al. 2011). Birnessite promotes the oxidative potential of soil systems and can, therefore, play an integral role in humification processes within soils (Shindo and Huang, 1982). The increase (though not significant) in organic compounds leached from treatments 2 and 6 compared to their respective controls (treatment 1 and 3) emphasizes that birnessite can enhance the oxidative degradation of biochar and do play a role in redox reactions between oxic minerals and natural organic matter (or in this case highly reduced black carbon) (Chorover and Amistadi, 2001). Birnessite possibly promoted the solubilisation of the volatile matter fraction of the biochar.

The amount of leached organics in treatment 4 was less than any of the other treatments that received inoculums. This can be attributed to a higher microbial activity as seen in the rate of respiration (Fig. 2.3). More of the organics introduced by the inoculum and biochar were removed from the columns as respiratory CO_2 (Table 2.3). Treatment 3 produced about

28.7% more leached organic carbon compared to treatment 4 due to the smaller fraction that was utilized by microbes. From this one can also conclude that the fertilizer added did not increase the amount of solubilised organics leached from the columns and the increase in treatment 6 compared to treatment 3 can be attributed only to oxidative transformations of organics by birnessite into dissolved organic carbon.

The largest amount of leached organics was collected from treatment 5, due to the addition of glucose, added to activate the microbial population. A lot of the added glucose was leached during the first and second leaching events after which a rapid decline in the amount of leached organics was observed. Only 13.688 mg leached organics was collected in the following 165 days of incubation compared to 30.842 mg in the first 60 days (Fig. 2.4). This trend also coincides with the rate of respiration and indicated that most of the glucose had been removed from the columns either as CO_2 or leached organics. After 60 days of incubation, a total of about 153 mg of carbon on average were removed from the columns of treatment 5. This is 45 mg more than the amount of glucose-C that had been added with the start of the incubation.

Total carbon lost

The amount of carbon lost from treatment 2 and 3 increased compared to the control. Even though the addition of birnessite and a microbial inoculum did increase the amount of carbon lost from these treatments, the effect was not significant (Fig. 2.6). Birnessite did not facilitate the oxidation of biochar to CO_2, but did cause an increase in the amount of solubilised organic compounds leached from the columns. In treatment 3 the microbial population was unable to adapt to the new carbon source provided and the biochar were unable to stimulate the activation of any part of the microbial community. Therefore, most of the carbon lost from these columns came from the organic carbon introduced by the inoculum of which most was leached. In contrast, the addition of fertilizer (Treatment 4) and fertilizer with glucose (Treatment 5) did lead to a significant increase in the total amount of carbon lost due to the activity of the microbial consortium. The largest portion of carbon was lost as respiratory CO_2 (Table 2.3) in these two treatments. The addition of fertilizer and glucose did stimulate the activation of certain microbes within the microbial community. However, the data shows a significant decrease in microbial activity of treatment 5 at the end of the incubation period of 225 days. In contrast, treatment 4 produced significantly more respiratory CO_2 during the last incubation period of 45 days and continuously had a higher respiratory rate from day 90 until the end of the incubation period. It clearly shows that the addition of glucose in treatment 5 induced a strong selection process on the microbial

community. The microbes decomposing more recalcitrant organics (Nocentini et al. 2010) were possibly outcompeted, suppressed or eliminiated from the microbial community. The addition of fertilizer is therefore a pre-requisite for biochar mineralization by microbes and the addition of a labile co-metabolite is not required to stimulate biochar utilization by microbes (Zimmerman, 2010).

Column chemistry: pH and EC

All of the treatments showed decreases in pH over the 225 days of incubation. These decreases were more pronounced in the columns that received fertilizer additions throughout the trial. A decrease of 0.87 and 1.04 pH units was measured over the incubation period of 225 days for treatment 1 and 2 respectively and can be attributed to abiotic oxidation which leads to the formation of acidic functional groups on the surface. Furthermore, the inoculum added to treatment 3 had a pH of 4.71. Even with the addition of the inoculum to treatment 3, it did not cause a significant decrease in the end pH compared to treatment 1.

The dissolution and leaching of soluble salts and organic compounds is among the first reactions that would occur with continuous leaching of the columns (Joseph et al. 2010). The loss in soluble salts such as carbonates and sodium during leaching also contribute to the decrease in pH (Yao et al. 2010). This coincides with the gradual decrease in EC in all the treatments (Fig. 2.7a). Even though the addition of birnessite decreased the pH slightly more when compared to the control, the effect was minimal and can possibly be ascribed to the enhanced oxidative mineralization of biochar by birnessite. Fertiliser was the prominent agent in acidification in treatment 4-6. This is due to the nitrification of ammonium to nitrate, releasing protons and leading to the acidification of the incubated material.

Conclusions

The abiotic oxidation of biochar by atmospheric oxygen showed no production of CO_2 and the only losses of carbon came from the leaching of the water soluble organics from the biochar. However, the addition of the highly oxidative soil mineral, birnessite led to an increase in the amount of leached solubilised organics from the columns and clearly showed that oxidative soil minerals can facilitate oxidative degradation of biochar and increase the solubilisation of biochar. The addition of biochar alone did not stimulate any microbial activity or proliferation, whilst the addition of fertilizer, to provide the microbial community with the essential elements (especially nitrogen and phosphorus), stimulated the microbial consortium. The addition of fertilizer is therefore a pre-requisite if biochar is to be utilized by microbes. Fertilizer additions in this trial also caused a significant decrease in pH compared

to the control and possibly stimulated the microbes by decreasing the pH to a similar pH of the wood from which the inoculum was prepared. Furthermore, the addition of glucose did not lead to any priming effect, possibly due to the strong selection process it had on the microbial population. In trying to attain a 0.1% glucose-C to biochar-C ratio, too much glucose was added to each column and possibly led to the microbes, able to decompose more recalcitrant organics, being suppressed or outcompeted for nutrients and carbon. Therefore, when biochar degradation models are developed one should consider the oxidative potential (oxidative soil minerals), nutrient status and the composition and activity of the microbial population of the soil system.

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CHAPTER 3: ANAEROBIC DIGESTION OF BIOCHAR BY A METHANOGENIC CONSORTIUM

Introduction

The interest and commercial usage of biogas as a natural resource has increased dramatically over the past few years with the consumption of natural gas expected to increase by 127.4 × 10^9 m^3 from the year 2004 to 2030 in the United States alone (Harris et al. 2008). Methanogens are compulsory anaerobic microorganisms capable of converting substrates such as H_2CO_2 and acetate to methane as the end product (Green et al. 2008). The ability of microbial consortia to metabolize coal is a well known fact since the studies conducted by Potter (1908). A few microbial communities have been shown to degrade coal and produce methane as an end product (Green et al. 2008; Mclnerney and Bryant, 1981; Harris et al. 2008; Fakoussa and Hofrichter, 1999).

However, to convert complex and highly recalcitrant substrates such as coal to methane, fermentative and acetogenic bacteria are needed in addition to the methanogens (Mclnerney and Bryant, 1981). This consortium of microbes can be introduced into coalbeds together with extra nutrients to enhance the production of methane from coal. This concept is known as Microbially Enhanced Coalbed Methane (Scott, 1999). Studies done by Harris et al. (2008) on two coals from the Powder River Basin in the USA found that methanogenic microbes produced 140.5-374.6 ml CH_4/kg after being amended with H_2CO_2. Green et al. (2008) found that the methanogen consortium, using the Wyodak coal as the sole carbon and energy source, produced methane from acetate and methanol. Methane production after 624 hours were found to be 118% higher at 30˚C and 38˚C and cultures kept at pH 6.4 produced 200% more methane than those kept at pH equal to 7.4 and 7.6. Green et al. (2008) also found that methane production increased with a decrease in coal particle size with small particles (106-180 μm) producing 19% and 34% more methane when compared to the medium (250-600 μm) and larger (850-1700 μm) particle fractions, respectively. From these studies it is clear that the correct methanogenic consortium can stimulate in-situ bioconversion of coal to methane and that this stimulation can be enhanced by promoting the proper environmental conditions (Green et al. 2008).

The proposed extensive use of biochar as a soil amendment in agricultural practices and subsequent possible leaching of such charcoal into water resources and reservoirs justifies an assessment on the degradability and amounts and composition of the gas emissions released when biochar is degraded under anaerobic conditions. For example, rice paddy soils are characterised by anoxic conditions, anaerobic carbon turnover and significant amounts of methane production during anaerobic digestion of organics (Knoblauch et al. 2010).

The potential of biochar to serve as a carbon substrate and energy source for a methanogenic consortia to produce methane is still very uncertain and has not attracted a lot of attention thus far. Being less recalcitrant than coal, biochar might be a viable substrate to produce methane at an even higher rate than coal.

Materials and Methods

Trial setup

The trial had a factorial experimental design with all treatments done in triplicate. The trial was conducted in 500 mL Erlenmeyer flasks seeded with 50 grams of anaerobic granules, from a grain/wine distillery upflow anaerobic sludge bed (UASB) reactor. Each flask was fed with a biochar suspension to investigate the effect of pH and increasing COD inputs (different concentrations of biochar), on the biodegradability of biochar. Flasks were closed with a rubber stopper and further sealed with silicone before each incubation period of 7 days between sampling events and placed on a horizontal shaker at 130 rpm in an incubation room kept at 37˚C. With each sampling event the biochar suspension was seperated from the anearobic granules and completely removed from each flask by sieving the suspension through a 72 µm sieve before and then replaced with new fresh pH adjusted biochar feed.

The trial consisted of three main treatments assessed over the three 28 day intervals with different COD inputs:

- Treatment 1: pH= 5.5 ; COD inputs of ±900, ±1850 and ±2250 mg. L^{-1}
- Treatment 2: pH= 7.0 ; COD inputs of ±900, ±1850 and ±2250 mg. L^{-1}
- Treatment 3: pH= 8.0 ; COD inputs of ±900, ±1850 and ±2250 mg. L^{-1}

The pH of the flasks was adjusted using 0.1 M potassium hydroxide and 0.1 M hydrochloric acid. The COD input were increased every 28 days from ±900 to ±1850 to ±2250 mg COD. L^{-1} during period one, two and three respectively. Sampling was done four times within each incubation period.

Substrate and fertilisation

Biochar is a highly carbonaceous material presenting a complex mixture of aromatic structures which consist mostly of benzene like rings linked together in a complex heterogeneous structure. As a result the material is highly resistant to oxidation. The raw biochar for this experiment was characterized in Chapter 1 (Table 1.1; pp. 29). The COD of the biochar was determined as 1850 mg COD.L^{-1} when 1 g of biochar (< 63 µm) was shaken in 1 L distilled water.

The substrate feed was fertilized using potassium nitrate (KNO_3) and di-potassium hydrogen orthophosphate (K_2HPO_4) to maintain a COD/N/P ratio of 1000:10:1. Additionally, 0.1 mL of trace element solution was added weekly to each of the flasks (Nel et al. 1985). To adjust for the loss in alkalinity, 0.5 g. L^{-1} sodium carbonate (Na_2CO_3) was added after 49 days to each treatment.

Analytical methods

The substrate feed and the effluent parameters monitored with every feed included the following: COD reduction (APHA, 1998), pH, alkalinity (as $CaCO_3$), total biogas produced and its composition.

The biogas composition within the headspace was determined by injecting 0.4 ml biogas sample into a gas chromatograph (Varian 3300) (Sigge, 2005). The GC was equipped with a thermal conductivity detector and 2.0 m × 3.0 mm an i.d. column filled with Hayesep Q (Supelco, Bellefonte, PA), 80/100 mesh. The GC made use of helium carrier gas at a flow rate of 30 ml. min^{-1}. The oven temperature was set at 55 ˚C. The volume airspace of each of the flasks was determined at the end of the trial by filling up the airspace with water and weighing the amount added for determination of the quantitive amount of CO_2 and CH_4 produced.

Statistical analysis

Statystical analysis was conducted using Statistica data analysis software, version 10 (www.statsoft.com). Repeated measures ANOVA was done on the means of the treatments over the 3 time periods on each response from each experimental unit with factors: pH and period. The correlation structure over time was assumed to be that of compound symmetry. If the repeated measures ANOVA showed significant interaction between pH and period, the interactions was interpreted with a Bonferroni multiple comparisons procedure. In the case where the interaction were not significant, the main effect of pH and period was interpreted

directly using Bonferroni, Newman-Keuls or LSD multiple comparisons procedures. If the levels of the main effects differed significantly, a multiple comparisons procedure was done with the Bonferroni, Newman-Keuls or LSD comparisons tests.

Results

COD reduction trends

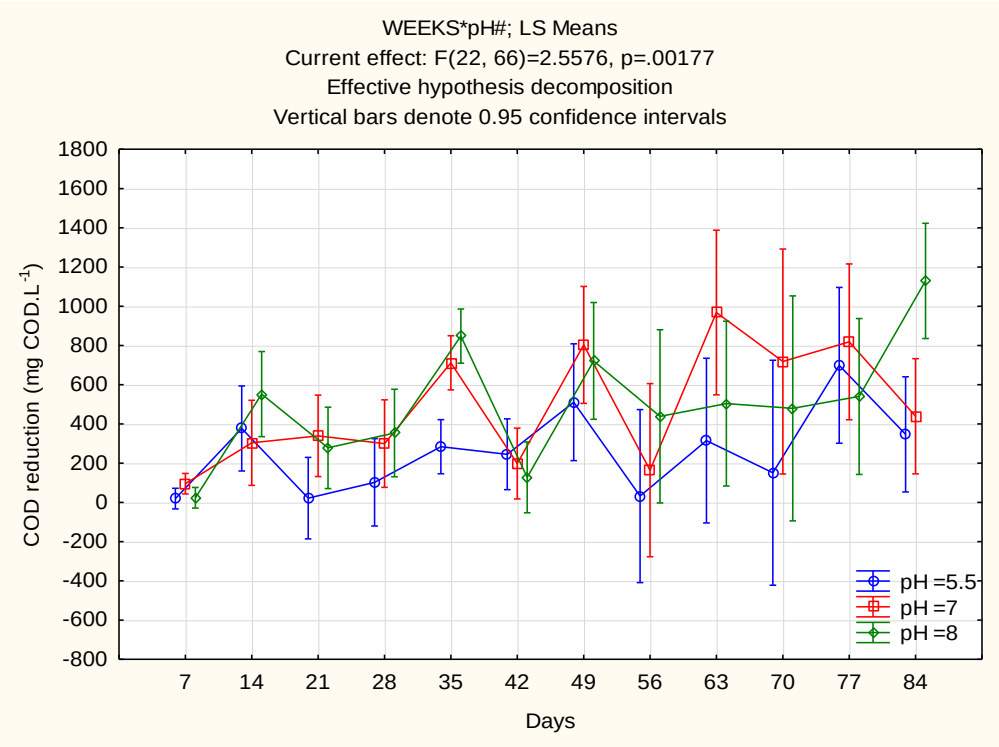

Figure 3.1: COD reduction of each treatment for the duration of the trial

Initially, the COD reduction in all the treatments was very low after the first incubation period of 7 days. However, the COD reduction of treatments 1 (pH= 5.5) and 3 (pH= 8), improved significantly (Appendix 3.1) with the following sampling period at 14 days. The COD reduction of treatment 2 (pH= 7) also improved considerably from an initial COD reduction of 95.2 to ±300 COD mg. L^{-1} and remained at these levels for the rest of the first period before COD input was increased after 28 days. Treatment 1 and 3 followed very similar trends and only produced stable COD reductions after 14 days (Fig. 3.1). The reason for the significant increases seen after the first sampling event is due to the time it took for the methanogenic consortia to acclimatize to the new carbon source. The COD reduction was the lowest in treatment 1, whilst COD reductions of treatment 2 and 3 were very similar (Fig. 3.1).

The COD input was increased from ±900 to ±1850 mg COD. L^{-1} with commencement of period 2. Consequently, a significant increase in COD removal was seen in treatments 2 and

3 (Appendicx 3.1), compared to the COD removed after 28 days. However, COD reduction from treatment 2 and 3 where very erratic during period 2 and never stabilized. COD reduction from treatment 1 increased considerably and was less erratic, but remained least efficient in reducing COD compared to treatment 2 and 3.

The COD input was increased by 20 % to 2250 mg COD. L^{-1} for period 3 (days 56 to 84) and accordingly caused an increase in COD reductions from treatment 1 and 2. Treatment 3 was very stable during this period and constantly produced COD reductions between 480-540 mg COD.L^{-1} up to 77 days after which a steep increase in COD reduction occurred at 84 days (Fig. 3.1).

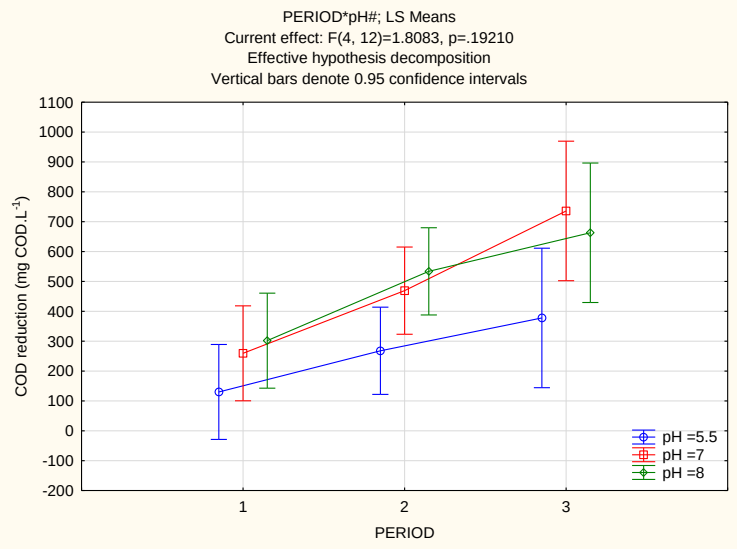

Figure 3.2: Average COD reduction of each treatment over the three periods

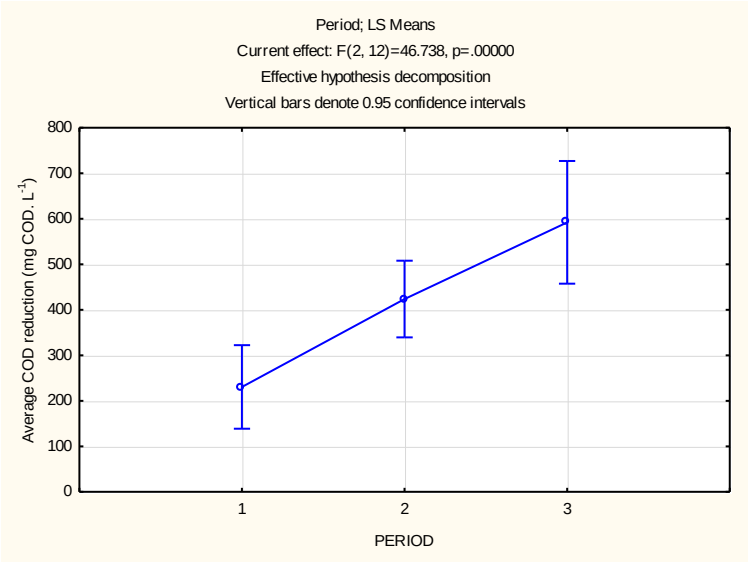

Figure 3.3: Average COD reduction of each treatment over all periods

Overall, when comparing the COD reduction means of each period, it was clear that no interaction occurred between pH and the period (p= 0.192) (Fig. 3.2; Appendix 3.2).

Therefore no significant increase in COD reduction occurred in any specific treatment over the 3 periods. The pH of the treatments also did not cause significant differences in COD reduction between treatments during specific periods (p= 0.067). However, a Newman-Keuls test (Appendix 3.4) between the average COD reduction from each treatment during period 2, did however show a significant difference between treatment 1 and 3. The COD reduction did however increase significantly over the periods (p< 0.001) (Fig. 3.3; Appendix 3.2). The significant differences between period of different treatments and within treatments are shown in Appendix 3.3.

Overall, the increases in pH and COD input did increase the amount of COD reduction considerably over periods 1 to 3 (Fig. 3.2). Treatments 2 and 3 produced very similar amounts of COD reductions in all periods, with the greatest difference being 73 mg COD. L^{-1} in period 3, were treatment 2 had the highest COD reduction of 735.9 mg COD. L^{-1}. Treatment 1 however caused the smallest amount of reduction in COD during all periods. The greatest COD reductions occurred during the last period between 56-84 days (Fig. 3.3) for all treatments.

COD removal efficiencies between treatments and periods

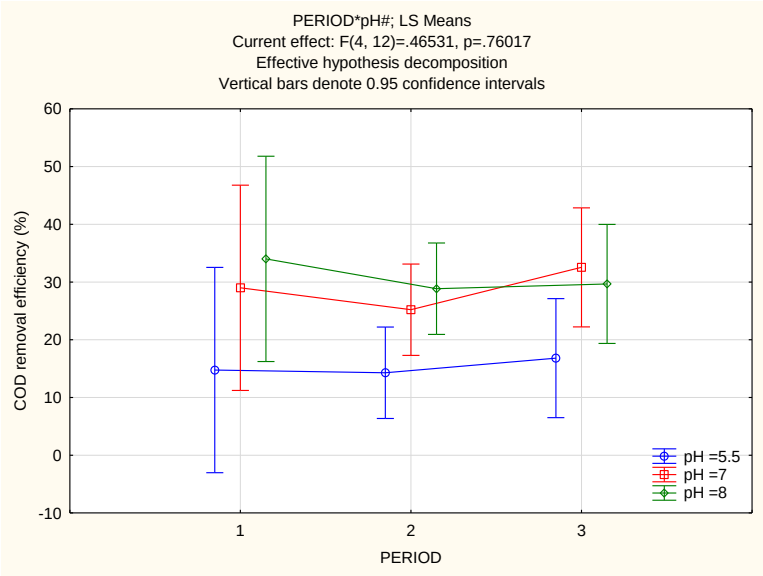

Figure 3.4: COD removal efficiencies of each treatment over the periods

No interaction occurred between the pH and period with no significant difference existing between COD removal efficiencies of treatments during any period (p= 0.76). The amount of COD removed by the methanogenic consortia, improved with an increase in pH from 5.5 to 7 and 8 (Fig 3.4), although it did not contribute significantly to COD removal efficiency (p= 0.084). Furthermore, the increase in COD input over the periods also did not lead to significant increases in COD removal efficiency (p= 0.42) as seen in Figure 3.4, which shows

considerable differences in COD removal efficieny between treatments (different pHs) but over periods only slight differences occurred (Appendix 3.5).

Overall, the average COD removal efficiencies from each treatment differed slightly between periods (p= 0.76). Treatment 1 had COD removal efficiencies of 14.8 to 14.3 to 16.8% between periods 1 to 3, respectively. The COD removal efficiency of treatments 2 and 3 between periods 1 to 3 were 29%, 25.3%, 32.5% and 34%, 28.8%, 34.6%, respectively. Treatment 3 therefore had the highest COD removal efficiency in period 1 (34%) and 2 (28.8%) compared to treatment 2, which conversely was the most efficient in COD removal during period 3 with 32.5%.

pH and Alkalinity

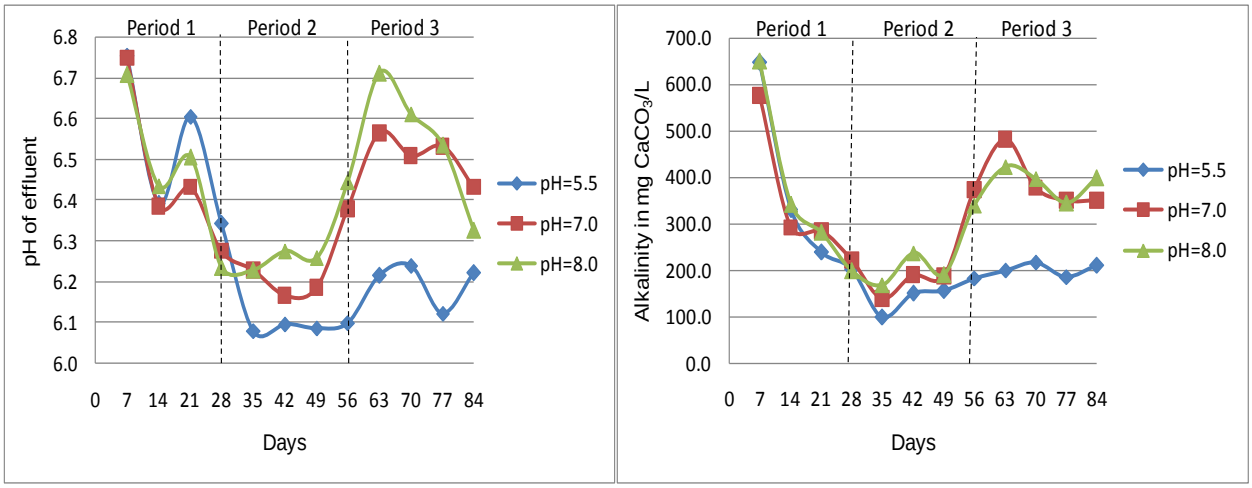

Figure 3.5: Changes in (a) pH and (b) alkalinity of the treatments for the duration of the trial.

The pH and alkalinity of all 3 treatments followed very similar trends throughout the whole trial. In Figure 3.5a it is clear that the pH of all the treatments were very erratic and decreased over period 1, stabilised during period 2 and increased from period 2 to 3 due to the addition of Na_2CO_3 after 49 days. The alkalinity (Fig. 3.5b) decreased up to day 35 in all treatments after which it stabilised. The addition of Na_2CO_3 on day 49 caused an immediate increase in alkalinity. In period 3, the alkalinity stabilised at higher levels in all treatments. However, the pH were less stable and decreased once again in treatments 2 and 3 from the maximum pH values of 6.6 and 6.7 it reached on day 63. Treatment 1 however maintained more stable pH levels during period 3.

Furthermore, the pH of treatment 1 always increased from the initial pH of 5.5, at which each incubation period started. The magnitude of the pH increase, however, varied over time. The pH of treatment 1 increased by 1.25 pH units to a pH of 6.75 after 7 days. The increases declined to 0.6 to stabilise at 6.1 after 35 days, were it remained up to day 56. The addition of

0.5 g.L^{-1} Na_2CO_3 on 49 caused an increase in pH which stabilised (except at day 77) at ± 6.2 in period 3. Alkalinity also decreased up to day 35 after which a steady increase in alkalinity followed for the rest of the trial (Fig. 3.5b).

Both treatments 2 and 3 followed very similar trends. In contrast to treatment 1, the pH of treatment 2 and 3 dropped to 6.2 from the initial starting pHs of 7 and 8, respectively. Alkalinity in both treatments also decreased to 170 mg $CaCO_3$ at day 35 after which a more stable period followed. The pH of treatment 2 and 3 increased considerably upon the addition of 0.5 g. L^{-1} Na_2CO_3 on day 49, to reach a maximum value of 6.6 and 6.7 after 63 days, respectively. Although alkalinity in the form of Na_2CO_3 were applied during period 3 to each of the treatments, both the pH and alkalinity decreased from day 63 to 84 in both treatments as shown in Figure 3.5a-b.

Gas composition

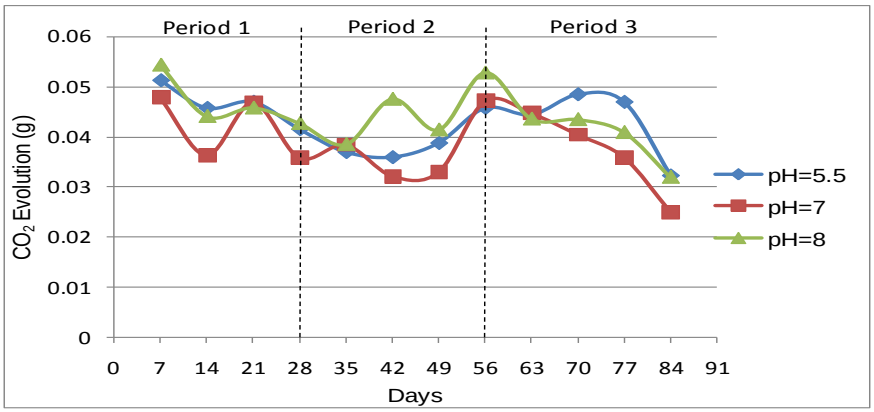

Figure 3.6: Average CO_2 evolution during successive periods

The CO_2 evolution during period 1 decreased from the initial amounts of CO_2 that evolved after 7 days (Fig. 3.6). This trend was less evident during period 2 in all treatments which coincide with the more stable pH and alkalinity (Fig 3.5a-b) during this period. The addition of Na_2CO_3 caused an increase in CO_2 evolution in the last week of period 2 (day 49-56), whereafter a continuous decrease in CO_2 evolution occurred in treatment 2 and 3. This trend corresponds with the trend seen in pH and alkalinity. In contrast, treatment 1 constantly produced higher levels of CO_2 in period 3, compared to the other treatments and to what it produced in period 2. However, a sudden decline in CO_2 evolution occurred in all the treatments between days 77-84 (Fig. 3.6).

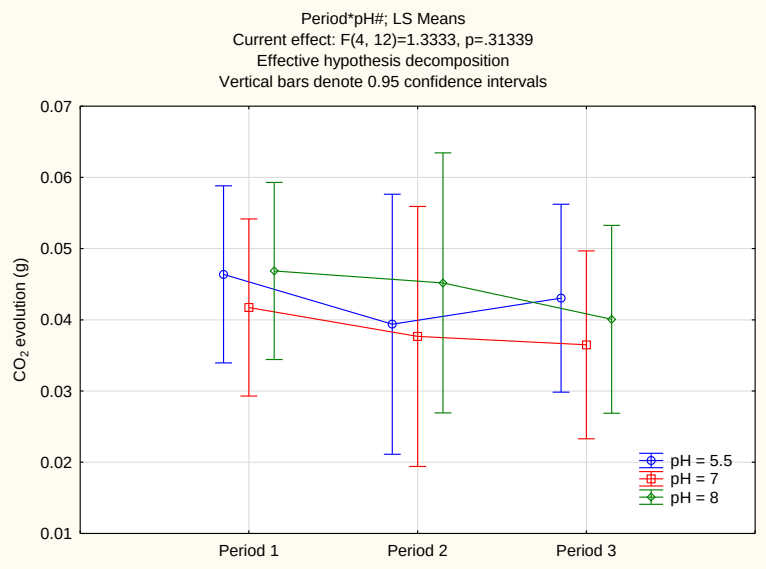

Figure 3.7: Average CO_2 evolution of each treatment over periods 1 to 3

Even though the CO_2 evolution did decrease over the periods (Fig. 3.7), there was no significant difference in CO_2 evolution in any specific treatments over period 1 to 3 (p= 0.313). No significant difference in CO_2 evolution existed between the pHs for the duration of the trial (p= 0.795). However, significant differences in the average CO_2 evolution did occur between the periods (p= 0.014) (Appendix 3.6). According to the Bonferroni test (Appendix 3.7) a significant decrease occurred between period 1 and 2. The increase in COD input, therefore, was the main contributor to the decrease seen in CO_2 evolution during the trial.

Table 3.1: Average amount of CO_2 and CH_4 (g) produced from each treatment

Days	pH=5.5		pH=7		pH=8	
	CO_2	CH_4	CO_2	CH_4	CO_2	CH_4
7	0.051	0.000	0.048	0.057	0.055	0.033
14	0.046	0.043	0.036	0.000	0.044	0.035
21	0.047	0.023	0.047	0.064	0.046	0.019
28	0.042	0.000	0.036	0.018	0.043	0.036
35	0.037	0.000	0.038	0.000	0.039	0.000
42	0.036	0.000	0.032	0.000	0.048	0.000
49	0.039	0.000	0.033	0.000	0.042	0.000
56	0.046	0.000	0.047	0.052	0.053	0.000
63	0.044	0.000	0.045	0.054	0.044	0.000
70	0.048	0.000	0.040	0.019	0.044	0.000
77	0.047	0.000	0.036	0.000	0.041	0.000
84	0.032	0.000	0.025	0.000	0.032	0.000
Total	**0.515**	**0.066**	**0.464**	**0.264**	**0.528**	**0.123**

Overall, the most carbon lost as respiratory CO_2 came from treatment 3; with 528 mg CO_2 followed by treatment 1 and 2, with 515 mg and 464 mg CO_2, respectively (Table 3.1). Methane evolution showed unstable behaviour, especially in treatments 2 and 3. Treatment 1 only produced methane in some of the flasks up to day 21, after which methane production stopped for the remainder of the trial. Treatment 2 was more efficient in methane production compared to any of the other treatments. Methane production occurred in period 1 but decreased to only one replicate and no replicates producing methane after 28 and 35 days. Treatment 3 produced methane only during period 1, but even during this period methane production only occurred in certain replicates.

The addition of 0.5 g. L^{-1} Na_2CO_3 from day 49 onwards, did not stimulate methane production in treatment 1 and 3, but did restore the normal methane production in all of the replicates of treatment 2 between day 49 to 63, after which a decrease was seen with only a single replicate producing methane after 70 days and none thereafter. The most carbon lost through methane evolution derived from treatment 2 with 264 mg followed by treatment 3 and 1 with 123 and 66 mg, respectively.

Discussion

Evaluation of COD reduction and removal efficiency

The anaerobic digestion of biowaste occurs in three main consecutive stages: the hydrolysis stage, followed by the acid forming stages of acidogenesis and acetogenesis and lastly the methanogenesis stage, as shown in Figure 3.8 (Bitton, 2005).

The anaerobic digestion process consists of an acid forming and methane forming phase and the microorganisms responsible for these reactions differ widely in terms of their physiology, nutritional needs and optimum environmental conditions (Pohland and Gosh, 1971). A delicate balance between these two groups is needed as it is the primary cause of reactor instability (Demirel and Yenigün, 2002).

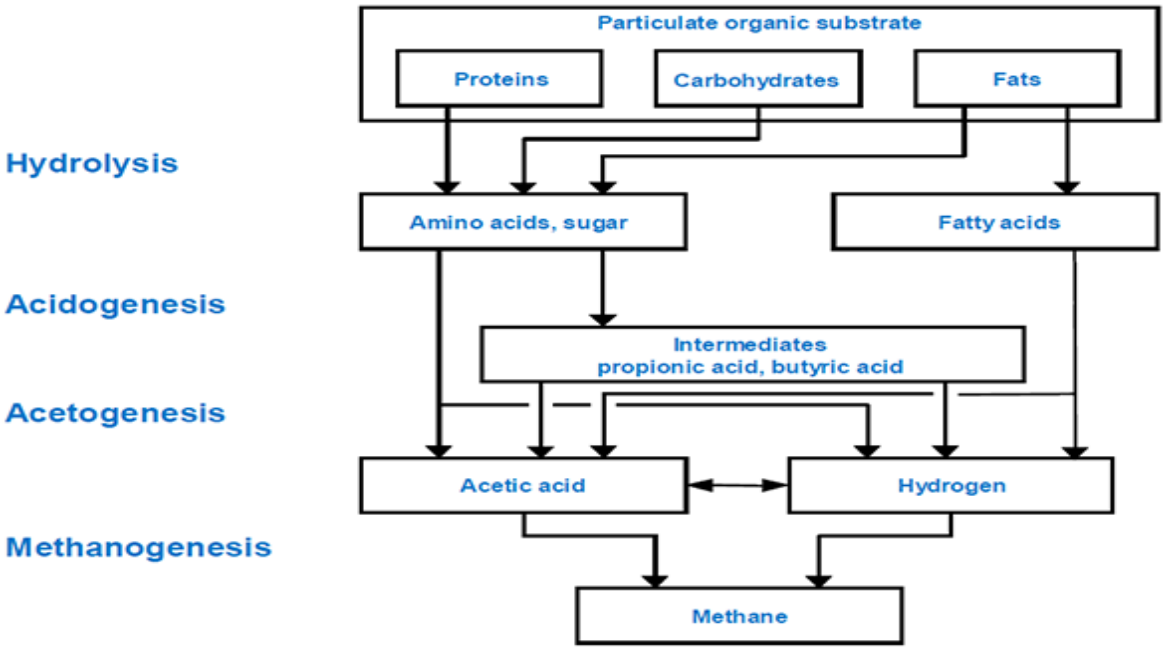

Figure 3.8: Four main degradative steps in anaerobic digestion of biowaste (http://www.wtert.eu)

The quantitative amount of the COD input that was removed by the methanogenic consortium increased considerably with increasing COD input between periods 1 to 3 in all treatments. This increase can be attributed to an increase in the available specific surface area of the biochar to facilitate the depolymerisation of the particulate phase (Oboirien et al. 2008) as a result of the increase in the organic carbon load. However, the efficiency with which the COD load was removed stayed more or less constant throughout the trial at an average of 15.3, 28.9 and 30.8% for treatments 1 to 3, respectively. These COD removal efficiencies are very low compared to studies on distillery grains wastewater (Gao et al. 2007) and olive mill wastewater (Marques, 2001), which had COD removal efficiencies of more than 80%. The results were more comparable to the study conducted by Harade et al. (1996) on the anaerobic digestion on recalcitrant sugar cane vinasse which revealed a COD removal efficiency of 39-67%.

Studies have shown that the addition of recalcitrant biowaste such as woody waste, containing a high lignin content, slows down the hydrolysis phase, as lignins are not readily degraded by anaerobic microorganisms and results in low COD removal efficiencies and methane production (United Tech, 2003; Harade et al. 1996; Bitton, 2005). Biochar has been described as a material very resistant to chemical and biological oxidation due to its aromatic nature (Goldberg, 1985; Roberts et al. 2010) and therefore would not be easily depolymerised during hydrolysis. The hydrolytic depolymerisation in anaerobic digestion of biochar would therefore be the rate limiting step in determining the efficiency of the system to remove the COD (Harris et al. 2008).

Furthermore, it is suggested that biochar contains different chemical fractions that are turned over at different rates (Hamer et al. 2004; Nguyen and Lehmann 2009; Cheng et al 2008). Biochar is a highly heterogeneous substance and consist of highly recalcitrant and more readily degradable compounds which turn over at different rates (Knoblauch et al. 2010; Nguyen and Lehmann, 2009; Knicker et al. 2008). The biochar used in this trial contained a volatile organic carbon fraction of 19.89% which may consist mainly of transformation products such as anhydro sugars, pyrans and furans according to Keiluweit et al. (2010). This fraction is more labile and can easily be utilized by microbes. Most of the COD removed probably occurred from this fraction.

Other factors that contributed to the poor COD removal efficiencies are the hydraulic retention time (HRT), pH and alkalinity of the influent. Many studies have shown that the degradation of organics and COD removal efficiency is primarily dependant on the HRT for complex substrates (Dinopoulou et al. 1988; Elefsiniotus and Oldham, 1994; Banerjee et al. 1998). Very complex material needs longer HRT to reach satisfactory COD removals. The pH of the suspension dropped considerably from day 7 to day 35 (Fig. 3.5a). The same trend was seen in alkalinity (Fig. 3.5b) and can be attributed to the production of organic acids and acetate by acido- and acetogenic bacteria. These groups of bacteria were not severely affected by the pH differences used in this trial and the changes that occurred over each incubation period. The drops in pH however, inhibited the methanogens which resulted into the accumulation of organic or acetic acids and inhibition of the anaerobic process (Bitton, 2005). It also removed the buffer capacity provided by the methanogens in terms of removing acetic acid from the system to prevent considerable decreases in pH and alkalinity.

Even though the COD removal efficiencies were only 30% (treatments 2 and 3), which according to normal standards of anaerobic digestion is rather low, considering the recalcitrant nature of the substrate supplied, it is quite a significant amount. This amounted to 581, 728 and 651 mg of biochar-C lost through the production of CO_2 and CH_4 in treatments 1 to 3 over an 84 day trial were the methane forming stage was suppressed most of the time. Compared to the aerobic digestion trial in the previous chapter where the fertilized incubation treatment (treatment 4) produced 319 mg respiratory CO_2 during its incubation period of 225 days (Table 3.1), it is clear that anaerobic digestion is rather efficient in biochar degradation.

Biogas evaluation

The biogas composition evolving from stable anaerobic digesters usually consists of 60-65% methane and 30-35% carbon dioxide (Gerardi, 2003). The biogas from this trial mainly consisted of CO_2 and in most sampling periods the complete absence of methane, especially in treatments 1 and 3. Carbon dioxide evolution occurred throughout the trial and was not inhibited by fluctuations in pH or alkalinity in any of the treatments. The acid forming stages were not affected by the increase in COD influent or the initial pH of the treatment as it did not translate into significant differences in CO_2 evolution between treatments or periods. This is because acid forming bacteria can maintain an acceptable enzymatic activity at pH values above 5 (Gerardi, 2003). However, a slight decrease in CO_2 evolution did occur from day 7 to 49, before the addition of Na_2CO_3 to increase alkalinity. This coincides with the slight decrease seen in COD removal efficiency over these periods (Fig. 3.4).

The addition of Na_2CO_3 to increase the buffer capacity of the digesters against acidification promoted CO_2 evolution in all treatments after day 49. However, it also caused an increase in the rate of acidogenesis and COD removal efficiency during period 3, especially in treatment 2 and 3, which resulted in a second decrease in pH over period 3. The decrease in pH and loss of alkalinity contributed to the decrease seen in treatments 2 and 3 in CO_2 evolution over period 3, whilst the more stable pH and alkalinity of treatment 1 resulted in more constant levels of CO_2 production during period 3.

Methane production in all treatments was severely affected by the decrease in pH and loss of alkalinity as seen over the duration of the trial. Methane production was totally inhibited for most of the trial in treatments 1 and 3 (Table 3.1) due to the initial pH values of 5.5 and 8 at which the granules were incubated. Treatment 2 was the most successful in methane production during the trial as it was incubated at pH= 7. Methane production stopped as soon as the buffer capacity of the system was overcome by the rate of acido- and acetogenesis, resulting in the decrease in pH and loss of alkalinity. Methanogens function optimally in the pH range between 6.7-7.4 and their enzymatic activity is inhibited by high acidity levels and pH values below 6.2. Furthermore, methanogenesis is inhibited by a variety of benzene ring compounds which includes benzene, phenol, phenolic compounds and toluene (Gerardi, 2003; Bitton, 2005) of which benzene and toluene are common volatiles on the surface of biochar most biochars (Spokas et al. 2011).

Conclusion

The balance between acid forming and methane forming microorganisms failed to be maintained in this trial due to poor pH control, loss of alkalinity and the recalcitrant nature of the biowaste used. This eventually resulted in the inhibition of the methanogens and accordingly methane production. Also, the possibility of inhibition caused by benzene ring compounds such as toluene and benzene cannot be excluded.

The initial low pH of treatment 1 (pH= 5.5) and high pH of treatment 3 (pH= 8), with exception of certain replicates during period 1, caused complete inhibition of methanogens. Even though methane formation in treatment 2 with an initial pH= 7 was also very erratic, it did occur in all replicates when methane production did occur and the biogas consisted of 26-27% CO_2 and very high methane concentrations of >70%. This is a ratio of between 2.6-2.8. In contrast to the methanogens, the acid forming bacteria were not inhibited by the factors mentioned above, and contributed to the decrease in pH and alkalinity loss. Furthermore, CO_2 evolution occurred in all replicates of each treatment throughout the trial, but decreased with each period as the COD inputs was increased. The pH and alkalinity of all treatments stabilised between 6.1-6.3 and 100-200 mg. L^{-1}, respectively, in period 2 when the methanogenesis was inhibited and before the addition of alkalinity.

Overall, the COD removal efficiency of the digesters was low compared to anaerobic digestion of organic waste, due to the recalcitrant nature of biochar against hydrolytic depolymerisation. Furthermore, the anaerobic digestion of biochar has the ability to produce high amounts of methane as seen in treatment 2. Methods to improve digester performance will include better control over pH (6.8-7.2) and significant increases in the alkalinity, which will result in a stable digester with possibly higher COD removal efficiencies and biogas containing between 70-80% methane. However, to reach these levels long HRT would be required.

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CHAPTER 4: BIOCHAR OXIDATION *IN VIVO*

Introduction

Biochar is promoted as a tool to sequester carbon in a very stable form which is inert to chemical and biological oxidation (Goldberg, 1985; Swift, 2001; Knicker, 2007). However, in 1981, Fakoussa revealed that microorganisms can indeed metabolize coal and this was confirmed by numerous studies thereafter, which showed lignite to be susceptible to a wide range of fungi and bacteria (Cohen and Gabriele, 1982; Ward, 1985; Strandberg and Lewis, 1987). More recently, Hofrichter and Fritsche (1996) tested 486 fungal strains from different ecophysiological and taxonomic groups and found that 77 wood- and litter decaying basidiomycetes to have a bleaching effect on coal derived humic acids.

Besides biotic oxidation of biochar, many other abiotic factors can play a significant role in biochar oxidation. Zimmermann (2010) showed that abiotic oxidation can contribute significantly to biochar oxidation. Abiotic factors such as cultivation (Nguyen et al. 2009), temperature (Cheng and Lehmann, 2009) and soil water regimes (Nguyen and Lehmann, 2009) can have a considerable effect on the rate of biochar oxidation.

A few studies have observed and described the changes that occur in the elemental composition, surface chemistry and adsorption properties when biochar gets oxidized via biotic or abiotic factors (Cheng and Lehmann, 2009; Cheng et al. 2008; Nguyen and Lehmann, 2009; Nguyen et al. 2009). However, there is still a strong need to correlate soil chemistry, - biological activity and -physical properties to the degree of oxidation occurring in the biochar and to look at the intermediates that is released into the environment upon biochar degradation (Chapter 1).

In this chapter in field trials were conducted. Biochar was incubated for 10 months under three different environmental conditions, namely fynbos, improved irrigated pasture and wetlands. The objective of this trial was to investigate the transformations biochar undergoes under each of the environmental conditions. Each of the mentioned environments differs in terms of their vegetation, soil water regimes, micro-climate, and soil physical and chemical properties.

Materials and methods:

The incubation sites

Figure 4.1: The (a) pasture, (b) fynbos and (c) wetland incubation sites (Google earth)

The biochar samples were incubated under irrigated pasture conditions on Welgevallen experimental farm, Stellenbosch, South Africa (33° 56" 40' S; 18° 51" 57' E), fynbos conditions in the J.S. Marais park, Stellenbosch, South Africa (33° 56" 00' S; 18° 52" 31' E) and under wetland conditions in Jonkershoek, South Africa (33° 57" 37' S; 18° 55" 37' E)

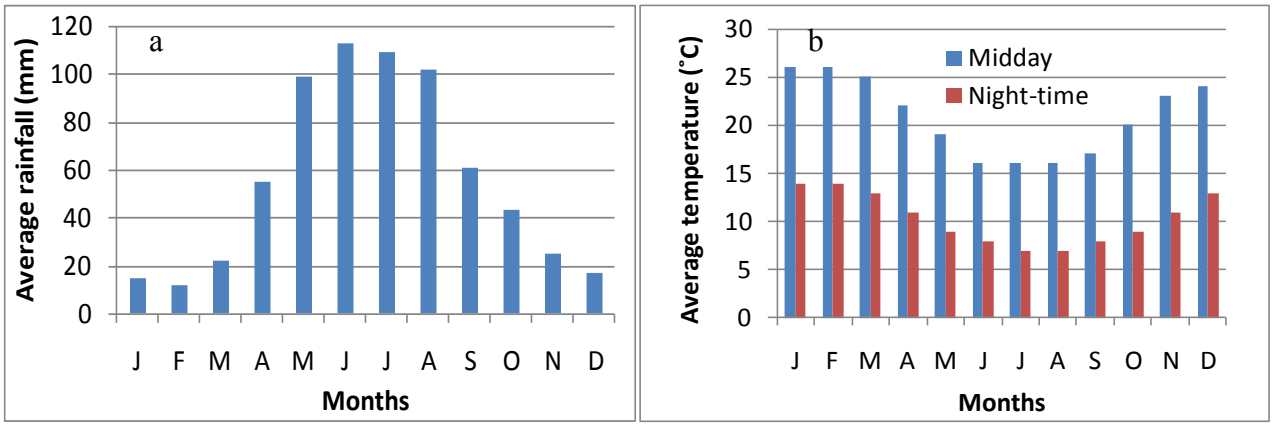

Figure 4.2: The (a) average rainfall (mm) and (b) midday and night-time temperatures for Stellenbosch (http://www.saexplorer.co.za)

Stellenbosch has a Mediterranean climate with a annual rainfall of ± 673 mm of which most is received during the winter months. The average temperature varies between seasons as shown in Figure 4.2b.

The biochar

The biochar used for this has been described previously (Chapter 1; pp. 29). For the purposes of this trial the biochar was characterized again. The pH, elemental composition, surface acidity and basicity, dissolved organic carbon (DOC) and FT-IR spectra of the fresh biochar was analysed according to the methods under analytical methods. Results are shown in Table 4.3 and Figure 4.3.

In field incubation setup

Ten grams of biochar (sieved fraction between 1-2 mm) was placed in perforated plastic containers which was overlaid with perforated material to allow easy access to microbes and oxidative soil minerals transported in the soil water during rain events. Six of these samples were prepared and placed in the field under different environmental conditions and vegetation, namely wetland, fynbos and grass pasture conditions. Two samples were placed in the topsoil (5cm depth) at each location and were incubated for 10 months (Aug, 2010-June, 2011) under these field conditions. Soil samples were collected from each location and the textural class of each soil was also determined (Table 4.1). The soil was also analysed for pH in distilled water (1:20 biochar solution ratio), EC, Eh, total microbial biomass C (C_{TMB}), % C and % N (Table 4.2).

Analytical methods

Three random soil samples were taken from the top 5 cm soil at each of the incubation sites after the incubation period elapsed, for the determination of the soil microbial biomass using microwave sterilisation and extraction with 0.5 M K_2SO_4 as described by Islam and Weil (1998). The microbial biomass carbon was then determined via the rapid oxidation spectrophotometric method of Heanes (1984) using an Ultrospec III spectrophotometer.

Dissolved organic carbon (DOC) extracted from the fresh and incubated biochar was determined colometrically via the rapid oxidation spectrophotometric method of Heanes (1984) using an Ultrospec III spectrophotometer. Five grams of the incubated biochar, dried at 60˚C for 24 hours, were shaken in 25 ml distilled water for 30 minutes. The suspension was then filtered using Whatmann 40 filter papers followed by filtratrion through a 0.45 µm Whatmann filter. A 5 ml sub-sample of the filtered extracts was digested with a mixture of 1 ml 0.17 M $K_2Cr_2O_4$ and 5 ml concentrated H_2SO_4. Digestion was aided by microwaving each sample at 500 $J.ml^{-1}$. Samples were then allowed to cool and the volume adjusted to 30ml using distilled water before absorption readings were taken at a wavelength of 590 nm.

The surface characteristics of the fresh and incubated biochar samples were characterized with a Thermo Nexus^{TM} Nicolet FT-IR Spectrophotometer using the KBr-pellet method (0.5%). The spectra were obtained using Omnic version 7.2 software. The spectra were recorded between 4000-500 cm^{-1}, averaged over 64 scans.

The total C, N, H and ash content of the biochar and soils was determined by the EuroVector Elemental Analyzer using the dry combustion method (Nelson and Sommers 1996). Oxygen was determined by subtraction.

Surface acidity and basicity was determined, before and after the incubation period elapsed, according to the method described by Cheng and Lehmann (2009), which is based on the classic Boehm titration method used for the characterization of charcoal.

The pH of the fresh and incubated biochars was determined in distilled water, using a 1:20 biochar to solution ratio (Cheng and Lehmann, 2009).

The textural class of the soils from the pasture, fynbos and wetland conditions were determined according to the method by Gee and Bauder (1986).

Statistical analysis

As the trial was conducted in duplicate not a lot of statistical analysis were conducted, but trends were described. A single factor ANOVA was used to distinguish between the treatment means of the different incubation sites.

Results

Soil and biochar analysis

Table 4.1: Particle size distribution

Sieve pore size (µm)	Fynbos soil %	Wetlands soil %	Pasture soil %
>500	34.87	9.65	10.93
>250	38.23	15.75	22.00
>106	13.70	15.35	32.63
>53	4.15	8.73	12.45
<53	0.12	0.43	0.50
Total	**91.07**	**49.90**	**78.50**
Clay Fraction	1.96	18.95	9.95
Fine silt	2.00	17.00	4.00
Coarse silt	4.97	14.16	7.56
Total	8.93	50.10	21.50
Sum	**100**	**100**	**100**
Textural Class	**Sand**	**Loam**	**Sandy Loam**

The textural class of soils from the fynbos, wetlands and pasture incubation site were determined as sandy, loamy and sandy loam. Soil texture can play a significant role in the rate of oxidation occurring on the surface of incorporated biochar.

Table 4.2: Soil chemistry and microbial analysis

	pH (H2O)	EC (µS/cm)	Eh (mV)	%C (% wt)	%N (% wt)	C/N Ratio	C_{TMB} (mg C. g^{-1})
Fynbos	4.56	69.5	243.5	2.41	0.11	25.6	0.32
Wetland	5.13	247.0	237.0	3.38	0.26	15.2	0.35
Pasture	5.95	264.5	164.5	3.50	0.36	11.3	1.61

Soil factors that can play a significant role in biochar oxidation were analysed as shown in Table 4.2. The fynbos had the lowest pH and EC of the incubation sites, followed by the wetlands and pasture soil, respectively. The fynbos soil had the highest Eh followed by the wetland and pasture soils respectively. Soil texture and microbial activity plays the determining role in Eh. As expected, the pasture soil had the highest carbon and nitrogen levels and the fynbos soils the lowest. The fynbos soils also had the widest C/N ratio of 26:1 compared to the wetlands and pasture which had C/N ratios of 15:1 and 11:1, respectively (Table 4.2).

The fynbos had the lowest microbial biomass of 0.32 mg C. g^{-1} compared to the wetland and pasture soils, which had a total microbial biomass of 0.35 and 1.61 mg C. g^{-1}, respectively. The average microbial biomass of the pasture was considerably higher than the fynbos and wetland soils, but the difference was not significant ($P > 0.07$) due to the variation in the data.

Table 4.3: Chemical and elemental analysis of biochar after 10 month incubation

	pH (H2O)	Surface acidity (mmol g^{-1})	Surface basicity (mmol g^{-1})	DOC (mg g^{-1})	Elemental composition (% wt)			Molar O/C	Molar H/C
					%C	%H	%O		
Fresh Biochar	8.25	0.43	0.00	0.37	81.86	3.84	10.46	0.10	0.56
Fynbos biochar	7.23	0.35	0.51	0.78	80.85	3.98	14.17	0.13	0.59.
Wetland biochar	7.25	0.51	0.00	0.72	78.24	3.85	16.12	0.16	0.59
Pasture biochar	6.20	1.07	0.00	0.91	75.63	4.17	16.60	0.17	0.66

*% N was below detection point of 0.001 %w/t

The low pH of the soils from the incubation sites caused a decrease in the pH of the biochar, compared to that of the fresh biochar before incubation. Even though the fynbos soils were the best aerated soil with the lowest pH and highest leaching intensity, it did not lead to the largest decrease in pH. The fynbos and wetland incubated biochar resulted in a pH decrease of 1 pH unit over the ten months of incubation. The pasture incubated biochars showed a pH decrease of 2 pH units compared to the fresh biochar (unincubated), even though the pasture soil had the highest pH and lowest Eh.

Accordingly, the increase in surface acidity was also the highest in the pasture incubated biochar with an increase of 0.64 mmol g^{-1}, followed by the wetlands incubated biochar with 0.17 mmol g^{-1}. No basicity was detected on the non-incubated biochar or on the wetlands and pasture incubated biochar. However, the biochar incubated under fynbos vegetation revealed an increase in surface basicity and decreased surface acidity compared to the fresh biochar (Table 4.3).

Dissolved organic carbon fraction also increased considerably in all incubated samples compared to the fresh biochar. The DOC from the pasture incubated biochar increased by 0.54 mg g^{-1} followed by fynbos and wetlands incubated biochar with increases of 0.41 and 0.35 mg g^{-1} biochar, compared to the fresh biochar.

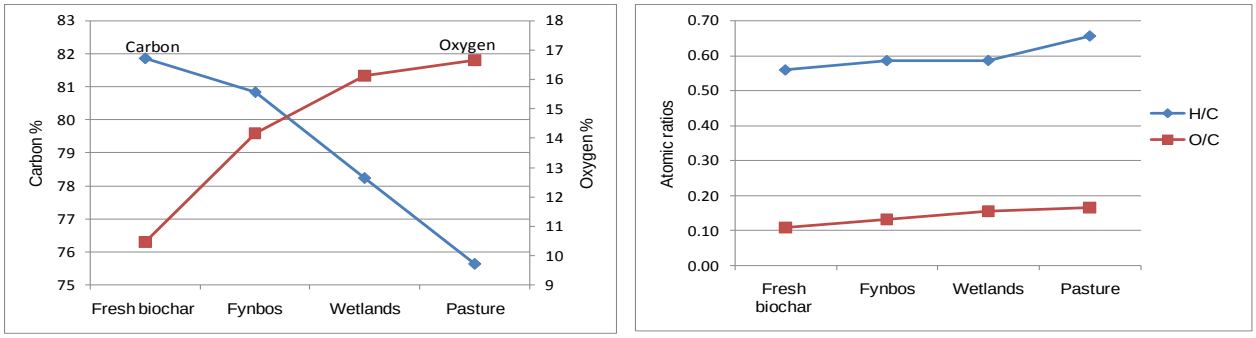

Figure 4.3: (a) Carbon and oxygen content of fresh biochar compared to the incubated biochars and (b) their respective molar H/C and O/C ratios

Furthermore, prominent changes occurred in the elemental composition of the incubated biochars compared to the fresh biochar. These include decreases in carbon content and increase in the hydrogen and oxygen content of the biochars over the incubation period. This is illustrated in Figure 4.3a and b, which clearly shows the greatest decrease in carbon and increase in oxygen content occurring in the pasture incubated biochars, whilst fynbos had the smallest.

The carbon content of the pasture, wetlands and fynbos incubated biochar reduced by 6.23, 3.62 and 1.01% respectively. Contradictory to the decreases seen in the carbon content, natural oxidation of the biochar surface resulted in increases of 6.14, 5.66, 3.71 and 0.33, 0.01, 0.15% in the oxygen and hydrogen content of the incubated biochars respectively. Accordingly, increases occurred in the molar O/C and H/C ratios of the incubated biochars compared to the fresh biochar (Table 4.3).

Spectroscopic results

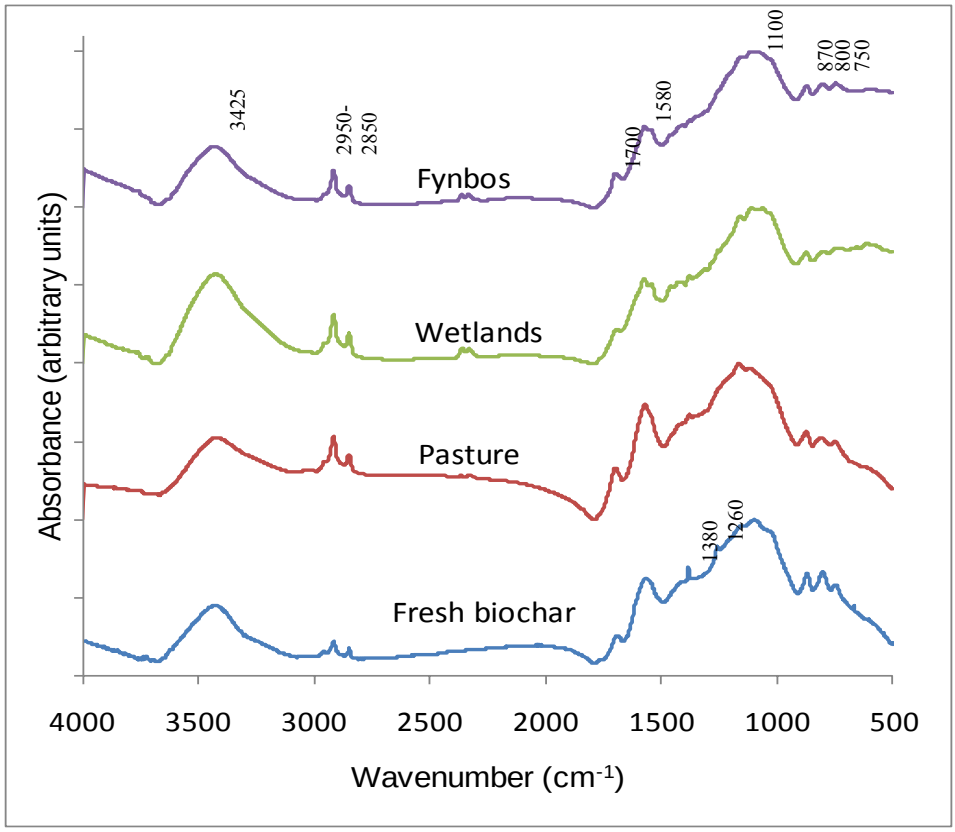

Figure 4.4: FT-IR graphs of biochar incubated under different environmental conditions (Peak assignments also given in Appendix 1.2)

The incubation of biochar for 10 months under different field conditions (pasture, wetland and fynbos) only resulted in small differences between the FT-IR spectra. Changes occurred with respect to the relative proportions of functional groups found at wavenumbers of 3400-3425 cm^{-1} (O-H), 2920-2850 cm^{-1} (aliphatic C-H), 1690-1700 cm^{-1} (C=O), 1550-1580 cm^{-1} (C=C or carboxylate) and 750-850 cm^{-1} (aromatic C-H) (Chen et al. 2005; Bustin and Guo, 1999; Keiluweit et al. 2010; Cheng et al. 2008).

All of the incubated samples had peaks with greater intensity at wavenumbers of 2920-2850 cm^{-1} and 1700 cm^{-1} which is associated with asymmetric and symmetric aliphatic CH stretching (Keiluweit et al. 2010; Pradhan and Sandle, 1999) and carboxylic acid groups (Cheng et al. 2008). Except for the pasture FT-IR spectra, the incubated samples in the wetlands and fynbos showed a decrease in the intensity of the peak at 1575 cm^{-1}, which is associated with aromatic components and carboxylate (Keiluweit et al. 2010; Bustin and Guo, 1999; Cheng et al. 2008).

Discussion

Changes in biochar elemental composition

The data clearly shows that biochar can be substantially oxidized in the natural environment resulting in a loss of carbon content, increases in the oxygen and hydrogen content and accordingly the molar O/C and H/C ratios which resulted in the pH decrease measured from the incubated biochars, compared to the non-incubated biochar. This is in accordance with studies conducted by Cheng et al. (2008) and Cheng and Lehmann (2009). XPS analysis conducted by Joseph et al. (2010) on greenwaste and poultry manure biochar found an increase of more than 0.5 in the O/C ratio after 1-2 years of incubation in soils.

It is generally accepted that biochar oxidizes from the outside inwards resulting in a higher oxidative status on the surface compared to the whole particle (Brodowski et al. 2005; Nguyen et al. 2009). Puri and Sharma (1968) proposed that the oxidation of biochar is initiated by chemisorptions of atmospheric oxygen at unsaturated ring sites, leading to the formation of carboxylic groups at the surface, rather than direct oxidation of C forms (Morterra and Low, 1982; Cheng et al. 2006). Biochar has a redox activity and functions mainly as a reducing agent, with oxygen being the most common electron acceptor, leading to the subsequent formation of O-containing functional groups at the surface (Joseph et al. 2010). Soil minerals with a high oxidative potential can serve as alternative electron acceptors, resulting in the oxidation of aromatic C in biochar (Nguyen and Lehmann, 2009).

From the trial it was clear that all incubated biochars had a higher oxidative status, but to different degrees. The pasture was the most oxidized with an increase in the oxygen content of 6.14% followed by the wetlands and fynbos with of 5.66 and 3.71%, respectively. The H-content of the pasture incubated biochar also increased by 0.33%, whilst the wetland and fynbos showed increases of 0.01 and 0.15%, respectively.This can be attributed to the increase in carboxylic and phenolic functional groups on the surface of the biochar (Cheng and Lehmann, 2009). The variations in the results are a clear indication that external factors do influence the rate of oxidation (abiotic and biotic) which occur at the surface of the biochar at the different sites. These factors include soil physico-chemical properties, soil water regime and microbial activity of the soil, the vegetation and mean annual temperature. This is excluding the biochars own characteristics like charring temperature and feedstock used for the biochar production.

Surface chemistry and characteristics

From the surface acidity and basicity results, pH and the spectroscopic analysis conducted, differences between the surface chemistry of treatments and fresh biochar could be identified. As mentioned previously, all of the incubated samples had higher molar O/C ratios and oxidative status due to biotic and abiotic oxidation. Chemisorption of oxygen to the surface and direct oxidation of aromatic carbon groups results in the formation of various organic functionalities with acid or basic characters (Montes-Morán et al. 2004) and includes carboxylic acids to ketones and esters (Boehm, 2002; Radovic et al. 2001). It is generally accepted that carboxyl groups, lactones, phenol and lactol groups forms the basis for surface acidity on biochar (Montes-Morán et al. 2004).

The pasture incubated soils had the greatest increase in surface acidity compared to the control and any of the other biochars incubated under wetland and fynbos conditions. The oxidation of the pasture incubated biochar led to the formation of new O-containing surface functional groups which contributed to surface acidity. Cheng et al. (2006) found that with progressive surface oxidation of biochar, the oxidation shifted from phenolic groups to cause considerable increases in carboxylic groups. The FT-IR graphs of all the incubated biochars had stronger absorption at wavenumbers 1700 cm^{-1} and 1570 cm^{-1}. Wavenumbers at 1700 cm^{-1} is associated with carboxylic stretching, whilst 1570 cm^{-1} is associated with aromatic components and (C=C) and C=O of conjugated ketones and quinones (Keiluweit et al. 2010). This is confirmed by the increases seen in oxygen and hydrogen contents of the biochars.

However, the degree of oxidation on the fynbos and wetland incubated biochar during the ten month incubation period was less, compared to the pasture biochar. The wetland biochar showed an increase of 0.17 mmol. g^{-1} in surface acidity and a drop in pH of 1 unit compared to the pasture which showed an increase of 0.64 mmol. g^{-1} in surface acidity and a drop in pH of 2 pH units. The FT-IR spectrum also revealed the largest increase in the intensity of the carboxylic peak at wavenumber 1690 cm^{-1}, occurred in the pasture incubated spectra.

However, exactly the opposite trend in terms of surface acidity and basicity was seen in the fynbos incubated sample. A slight decrease in acidity occurred and a considerable increase in surface basicity even though the pH dropped by 1.02 units and the O-content increased by 3.71%. FT-IR also revealed an increase in carboxylic vibrations at 1700 cm^{-1}. Oxygen containing functional groups characterised as basic groups consist of chromene, quinone and pyrone-like groups (Montes-Morán et al. 2004). However, consensus still needs to be reached regarding the strength and contribution of these groups to basicity. Furthermore, an increase

in aliphatic C-H stretching between wavenumbers 2950-2850 cm^{-1} occurred in all of the incubated biochar samples. This is contradictory to Cheng et al (2006) and Nguyen et al. (2009) who found decreases in these peaks which is associated with aliphatic C-H groups. The formation of carboxylic acids like benzoic acid on the surface also shows strong absorption between wavenumbers 2850-2950 cm^{-1} as shown in Chapter 1 (Fig. 1.5.5; pp. 39) and possibly contributed to the increase seen between these wavenumbers.

The fraction DOC extracted from the incubated biochars increased significantly ($p< 0.05$) compared to the fresh biochar and the DOC derived from the pasture incubated biochar was significantly more than the DOC derived from the fynbos and wetland incubated biochar. The increase in surface oxidation resulted in the easier solubilisation of surface compounds which resulted in the increases in DOC obtained from the samples. One can however not discard the possibility of humic acids from the soil, plant- and microbial exudates being adsorbed on the surface and contributing to the increases seen. However, with sampling it was observed that the surface of the biochar particles was much more friable than before incubation.

Effects of soil characteristics on biochar oxidation

Even though the samples were all exposed to in field environmental extremes for ten months, the degree of oxidation differed between treatments and can possibly be ascribed to several factors. Soil texture is a key variable coupled with the degree and rate at which oxidation took place on the biochar surfaces. As shown in table 1, the textural class of the soils from the incubation sites differed. The wetland has a loamy texture, the pasture has a sandy loam texture and the fynbos has a sandy texture. Soil porosity is a function of soil texture and increases from sandy soils to clayey soils. However, the field air capacity of sandy soils are higher compared to more finer textured soils due to the difference in the water holding capacity of the soils (Hillel, 1980). Many studies found that biochar oxidation is initiated by abiotic oxidation with atmospheric oxygen being the most common electron acceptor (Joseph et al. 2010; Cheng et al. 2006). In abiotic incubations studies conducted by Zimmermann (2010) it was found that abiotic oxidation can contribute significantly to biochar oxidation. However, soil texture has a strong influence on gas exchange between the lithosphere and atmosphere, and can, therefore, strongly influence the oxidation rate.

Furthermore, soil texture has a strong influence on the soil water dynamics of the incubation sites and studies conducted by Nguyen and Lehmann (2009) showed that biochar incubated under varying water regimes mineralized and oxidized at different rates. However, this was also influenced by the biochar feedstock used and its quality. Also, the sandy texture of the

fynbos soil would have been exposed to a greater degree of leaching intensity, removing more nutrients and DOC during the drying and wetting cycles.

Texture has also been shown to have an effect on C and N mineralization rates. Hassink (1995) found that C and N mineralization per unit of total microbial biomass were lower in fine-textured soils compared to coarse-textured soils. Furthermore, soil pH has a strong effect on the soils biological properties (Brady and Weil, 2002) and can be considered just as important as soil C and N concentrations (Wardle, 1992). Aciego et al. (2008) revealed a significant increase in CO_2 evolution with increasing pH and that the microbial biomass and -activity stabilised at pH values of 5 to 7.

The results from the trial showed the pasture soil had the greatest microbial biomass and the smallest C/N ratio followed by the wetland and fynbos soils (Table 4.3). This is the result of a higher soil pH, higher carbon inputs in the form of root exudates and dead roots to stimulate microbial activity and also fertilization and cattle manure inputs during the year. During humification or composting of organics the C/N ratio decreases significantly (Thambirajah et al. 1995) due to the loss of carbon through microbial respiration as CO_2 and the recycling of N in the system (Shilesky and Maniotus, 1969; Golueke, 1992). The pasture had the narrowest C/N. This can be ascribed to the manure inputs and higher microbial activity which decomposes the organics at a higher rate and recycles N faster. Due to the higher microbial activity that exists under the pasture conditions, more of the easily available carbon fraction of the biochar would have been utilized by microbes and lost as respiratory CO_2, resulting in a more oxidized surface. Many studies have shown that microbes are able to utilize biochar as a sole carbon source (Hofrichter and Fritsche, 1996; Laborda et al. 1995; Kuzyakov et al. 2009).

Except for the differences in soil texture water regimes and microbial biomass, studies by Cheng et al. (2006) found that temperature accelerated biochar oxidation and that a significant positive relationship exists between mean annual temperature (MAT) and biochar oxidation (Cheng et al. 2008). However, due to the close proximity of the incubation sites and short incubation period, this would have contributed minimally to biochar oxidation.

One has to take into account that the biochar in this trial were not directly incorporated in the soil and was also placed in the top 5 cm of each soil. This would have minimized the effect of texture and soil minerals on the degree of oxidation. Furthermore, even though the wetlands had a much finer texture compared to the fynbos soils, the pH, surface acidity and FT-IR spectra of the fynbos and wetland incubated biochars did not differ considerably and one can

conclude a similar degree of oxidation on the biochars sampled from these sites. However, even though the pasture had finer texture compared to the fynbos soil, considerable differences existed in the pH, surface acidity and FT-IR spectra (in terms of the carboxylic peaks) of the biochars incubated at these sites. The greatest difference between the soils of each site is exhibited in the pH and microbial biomass. Therefore one can conclude that the difference in microbial activity between the incubation sites contributed the most to the differences seen in biochar oxidative status between sites.

Conclusion

The results from this study clearly show that in spite of biochars recalcitrance, biochar has a redox activity and can serve as a reducing agent in redox reactions. It is also clear that differences in the rate of oxidation can occur between different geographic locations with different soil physico-chemical characteristics, climatic conditions, biological activity and vegetation cover. These factors exercise strong influence on soil oxidative potential, soil water regimes, aeration, C/N ratios and C and N-mineralization rates. However, due to the biochar not being mixed with the soils and the samples only being incubated near the surface, the direct effect of soil physico-chemical on biochar oxidation is minimized. The pasture had a considerably higher microbial activity as a result of the grassland vegetation, manure additions from the cattle and higher pH. Accordingly the biochar incubated at these sites exhibited the greatest degree of oxidation as seen from the FT-IR graphs, surface acidity and the increase in oxygen content of the biochar. Therefore one can conclude that the difference in microbial activity between the incubation sites contributed the most to the differences seen in biochar‘s oxidative status.

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CHAPTER 5: POT TRIALS TO ASSESS THE EFFECTS OF BIOCHAR PRESENCE IN A VIRGIN AND FERTILIZED ACIDIC SANDY SOIL ON TOTAL MICROBIAL POPULATIONS, PLANT ROOTS AND ASSOCIATED SYMBIONTS.

Introduction

Biochar can be described as a carbon rich material low in nutrients (Bridle and Pritchard 2004). The nutritional value of biochar is highly dependent on the precursor used, as it has been shown that biochar produced from animal waste has a higher nutrient content when compared to plant derived biochar (Shinogi, 2004; Chan et al. 2008). However, the incorporation of biochar into soils can have many beneficial effects within soils and will improve soil quality through its liming ability, by increasing the CEC and nutrient retention ability of soils and also increasing the water holding capacity of soils (Laird et al. 2010; Glaser et al. 2002; Lucas and Davis, 1961; Atkinson et al. 2010). The addition of biochar, therefore, has the ability to cause major shifts in the chemistry, nutrient levels and their ratios within soils which can influence the availability and solubility of elements such as N, P, Ca, Mg and Mo (Atkinson et al. 2010) and influence the productivity of crops.

These effects of biochar on soils will not only influence the microflora, but will also have an effect on the microfauna populations inhabiting the soils. However the carbon in biochar is mostly stored in the very stable aromatic backbone of the biochar, with a small fraction found to be easily available to microbes termed labile C or volatile matter fraction. The recalcitrant nature of biochar means it serves mainly as a tool to sequester carbon within the soil system (Joseph et al. 2010; Kuhlbusch and Crutzen, 1995; Atkinson et al. 2010) as it shows great resistance to decomposition and utilization by many microorganisms. Therefore, it is questionable whether biochar would be able to serve as the primary carbon source to microbes and be able to promote increases in the microbial biomass C and activity within the soil systems.

Furthermore, biochar is an adsorbent able to adsorb organic molecules (Pignatello et al. 2006; Joseph et al. 2010) and also has a redox activity which can influence the redox potentials within the rhizosphere and possibly influence signalling between roots and microbes and influence the formation of symbiotic relations between microbes and plants (Joseph et al. 2010; Warnock et al. 2007). Even so, the porous structure and large surface area of biochar

can provide beneficial soil microorganisms such as bacteria and mycorrhizae with refugia (Saito, 1998) and provide more suitable conditions (moisture and nutrients) for microbial proliferation.

The objective of this chapter is firstly to assess the effect of Pine wood derived biochar on crop production, secondly the effect it has on total soil microbial biomass and soil microbial activity and thirdly the effect it has on the mycorrhizal associations and the degree of nodulation with plant roots. Three separate pot trials were conducted for these purposes: (Trial 1) Layered biochar wheat pot trial different amendment rates of biochar with wheat and the common green bean (Trial 2 and 3).

Materials and methods: Layered pot trial with wheat *(Triticum aestivum)*

Soil and biochar characterization

The sandy soil used for the wheat pot trials had a low agricultural potential and is very widespread in the Cape Flats region of the Western Cape, South Africa. The sandy soil used in the pot trial was collected from an unused field in Brackenfell, Western Cape, South Africa (S 33.89532˚ and E 18.72346˚) covered with kikuyu grass and weeds. The soil is texturely classified as pure sand of medium grade. It has a low pH, low carbon and nitrogen content and small CEC as shown in table 5.1. The pH of the soil was measured in distilled water in a 1:2.5 soil to water ratio (White 1997). The total C and N content of the soil was determined by the EuroVector Elemental Analyzer using the dry combustion method (Nelson and Sommers 1996). The CEC of the soil was determined using 1 M NH_4OAc (pH 7.0) according to the method of Summer and Miller (1996). All determinations were carried out in duplicate. The biochar was characterized in Chapter 1 (pp. 29).

Table 5.1: Soil and biochar characterization

	pH_{H2O}	% C	% N	C/N Ratio	$CEC_{7.0}$ (cmolc.kg^{-1})
Soil	5.14	0.16	0.03	5.3	1.96
Biochar	9.72	82.71	0.53	156.1	34.30

Pot trial setup

The trial was conducted in a tunnel on Welgevallen experimental farm of the University of Stellenbosch, Stellenbosch, South Africa. It consisted of twelve 7 litre pots. Each pot consisted of three sandy layers at 0-3, 6-9 and 12-17 cm and two biochar layers at 3-6 and 9-12 cm depth respectively.

Ten wheat seeds were planted per pot and thinned out to four seedlings after emergence. The plants were fertilised with macronutrients according to the following fertiliser recommendation: N: 101 ; P: 50 ; K: 75 ; Ca: 30 ; Mg: 23 ; S: 30 kg ha^{-1}. The pots were fertilized in a single application at planting while micronutrients were applied as foliar spray, 2 and 8 weeks after germination, using Omnispoor fertiliser consisting of the following trace elements: B: 22; Cu: 1.25; Fe: 87; Mn: 18; Zn: 10.7; Mo: 2 g/kg at 20 mg l^{-1}. Pots were irrigated on a weekly basis up to field capacity.

Soil and plant analyses

The wheat plants and roots of each layer were collected twelve weeks after germination, washed and oven dried at 50 ˚C for 6 days and their dry root weights recorded. The biochar and sandy layers were carefully separated and a sub sample of soil and roots were collected from each layer. The roots were stored in 50% ethanol solution for the determination of AM fungal colonization.

Mycorrhizal infections were determined using Trypan blue (chlorazol black E) in lactoglycerol to stain the roots after they were cleared in 10% w/v heated KOH (Bevege 1968; Phillips & Hayman 1970; Kormanik & McGraw 1982; Brundrett et al. 1984). Non-woody roots were used for this purpose. Twenty-five 1 cm root segments were placed on a microscopic slide before examining them for mycorrhizal infections under a compound microscope at x200 magnification. Soil microbial biomass was determined using microwave irradation and extraction with 0.5 M K_2SO_4 as described by Islam and Weil (1998). The microbial biomass carbon was then determined via the rapid oxidation spectrophotometric method of Heanes (1984).

The sub samples of biochar and soil taken from each layer were analysed for pH_{H2O} and electrical conductivity (EC) using a 1:20 and 1:2.5 biochar and sand to solution ratio (Cheng and Lehmann 2009; White 1997).

Phosphorus was extracted according to the method described by Soltanpour and Schwab (1979) using NH_4HCO_3-DTPA as the extracting solution, which is suitable for alkaline samples. The phosphorus content was then colometrically determined using the ascorbic acid method. All the data are shown in tables 5.3 and 5.4.

Statistical analyses

Statistical analysis was conducted using SAS 9.1 for Windows. Significant differences amongst the layers were distinguished at the P< 0.05 level using Tukey's Studentized Range test. A single factor ANOVA was used to distinguish between the treatments means of the different layers.

Results

Table5.2: Analysis of separate layers

Analysis	Sand (0-3 cm)	Biochar (3-5 cm)	Sand (5-8 cm)	Biochar (8-10 cm)	Sand (10-17 cm)
Gravimetrical water content at harvest (%)	5.3	123.3	7.4	134.8	8.7
pH_{H2O}	6.67	7.3	7.56	8.27	6.83
Electrical conductivity (mS/cm)	693	356	429	818	739
Phosphorus content (µg $P.g^{-1}$)	17.36	13.91	1.66	10.95	1.61

Table 5.3: Dry root weight and microbial data in separate layers

	Sand (0-3 cm)	Biochar (3-5 cm)	Sand (5-8 cm)	Biochar (8-10 cm)	Sand (10-17 cm)	Difference in means of biochar and sandy layers: P-value
Root dry weight (g/cm)	0.336 a	0.567 a	0.096 b	0.273 b	0.036 b	0.039
Microbial biomass C (mg C/ kg)	54.654 a	130.089 b	52.240 a	161.691 b	62.172 a	<0.001
Mycorrhizal number	64 a	27 a	39 a	74 a	84 a	0.191

[a]Numbers followed by the same symbol are not significantly different at P< 0.05 level based on Tukey's Studentized range test.

Root growth through layered pots

The results shown in Table 5.3 indicated a significant increase in root dry weight within the biochar layers compared to the sandy layers. However, this increase is mostly attributed to the first biochar layer rather than the second biochar layer. The top sandy layer had similar

dry root weights compared to the first biochar layer. The rest of the sandy layers had dry root weights significantly less than the first biochar layer at 3-5 cm depth. The biochar layer at 8-10 cm depth had a greater dry root weight compared to the sandy layer above and below it (89 and 116% respectively), but this increase was not significant (Table 5.3). Overall, the two biochar layers significantly promoted root growth (P= 0.039) compared to the sandy layers as shown in Figure 5.1 below.

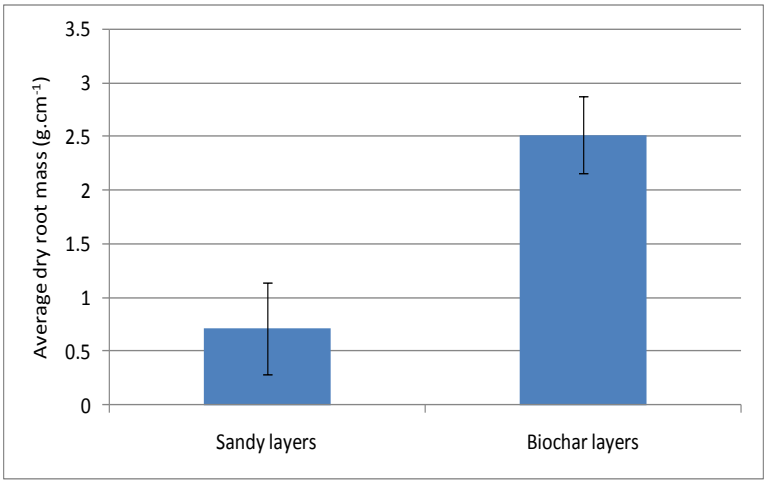

Figure 5.1: Average dry root mass of the biochar and sandy layers (P= 0.039)

Analysis of total microbial biomass C (C_{TMB})

The results shown in Table 5.3 revealed a significant increase (P< 0.05) in the soil microbial population, inherited from the sandy soil, within both the biochar layers and between the average C_{TMB} of the sandy and biochar layers (Fig. 5.2). Microbes, therefore, readily colonized these zones and propagated at a higher rate within the biochar layers. Biochar layer one and two caused an increase in C_{TMB} of 131% and 187% respectively compared to the average C_{TMB} of the three sandy layers.

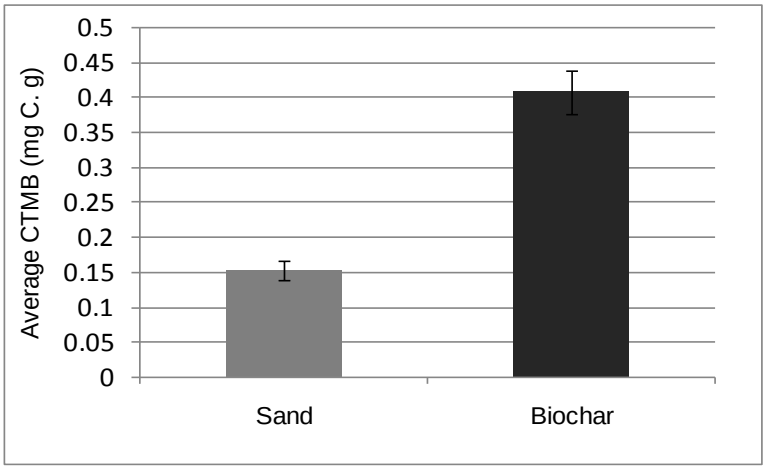

Figure 5.2: Average C_{TMB} sandy and biochar layers (P< 0.001)

Degree of mycorrhizal colonization

From the results shown in Table 5.3 and Figure 5.3, a decrease in the degree of mycorrhizal colonization with the roots of *Triticum aestivum* (wheat) can be seen in the middle sections of the pots (3-8 cm depth) relative to the sandy layers above and below. This decrease however, is not significant due to the large variation within the data. Overall, the natural mycorrhizal population within the sandy soil seemed to be less affected in the soil layers whose own soil chemistry were not strongly influenced by the biochar layers.

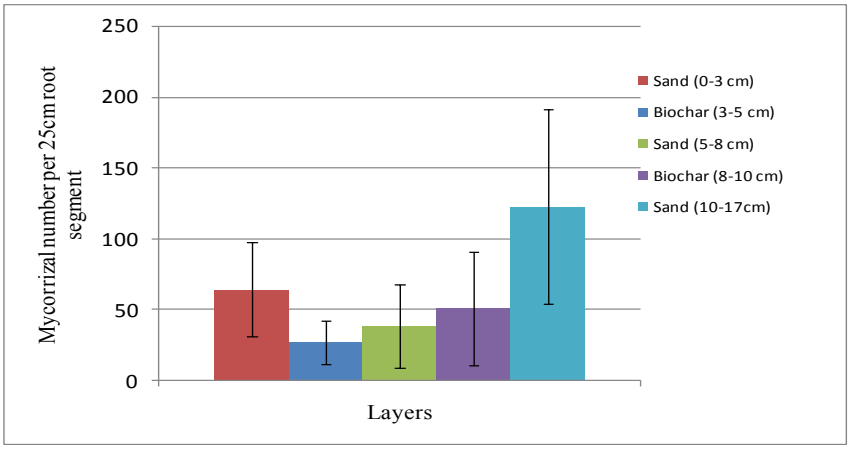

Figure 5.3: Average mycorrhizal number per 25 cm root segment in separate layers

Discussion

Root growth through layered pots

Overall, the biochar layers in the pots promoted root growth compared to the sandy layers. The sandy layer at 0-3 cm had a dry root weight comparable to the first biochar layer. This can be ascribed to the root morphology of *Triticum aestivum* (wheat), with thicker anchor roots growing in the top sandy layer. Below the first biochar layer a peak in dry root weight was again seen in the biochar layer at the depth of 8-10 cm. Several reasons can account for the increase in root growth within the biochar layers.

Firstly at harvesting, both the biochar layers had gravimetrical water contents between 120-135% compared to 5-10% of the sandy layers above and below it. The higher water holding capacity is due to the high porosity and the capillary forces which draw soil solution into the micro-voids of the biochar (Atkinson et al. 2010; Hillel, 1980). Consequently, the biochar layers continuously had higher water contents between irrigation cycles. The increased root growth can also be ascribed to the root hairs being able to enter water filled macro-pores of biochar and in doing so can bind to the biochar particle‘s surface (Joseph et al. 2010).

Secondly, biochar layers provided a higher CEC compared to the sandy layers (Table 5.1), which meant that a higher fraction of the nutrients applied as fertilizer were retained within the biochar layers after irrigation cycles (Lehmann et al. 2003b) compared to the sandy layers. These nutrients could be taken up by plant roots over a longer period of time because of the slower drying out cycle within these layers.

Thirdly, the biochar used in this trail had a volatile fraction of 19.3% and recent studies have shown that this fraction can contain organic molecules which can trigger germination (Dixon 1998) and promote nutrient uptake by roots (Riedlinger et al. 2006). However, this is strongly influenced by the precursor used for the production of the biochar.

Stratification of microbial biomass through pots

Results clearly indicated a significant increase in C_{TMB} within both biochar layers compared to any of the other sandy layers which did not significantly differ from each other. The porous nature of biochar not only increased the water holding capacity of these layers, but also provided suitable micro habitats for microorganisms to colonise (Joseph et al. 2010). These micro-pore habitats provide microorganisms with refuges (Saito, 1998), moisture, and greater protection against predators and climatic extremes and also allow for a wide variety of microbial communities to colonise them (Thies and Rilling, 2009).

In the previous section a significant increase in root growth was seen within the biochar layers and therefore these layers had a larger rhizosphere volume compared to the sandy layers. In the rhizosphere, growing roots can release organic compounds, tissue material and sloughed-off cells (Violante and Gianfreda 2000) which can then be adsorbed on the surface of biochar particles and serve as substrate for microbes within the micro-voids. Nutrient interception by the biochar layers also means that more nutrients are readily available for microbial uptake. Furthermore, the large volatile fraction of the pine wood biochar, provides the microorganism with a readily available carbon source and therefore can stimulate the initial increase in C_{TMB} directly within these layers.

Degree of mycorrhizal colonization

The data collected indicated no significant differences in the degree of mycorrhizal colonization formed with the roots between any of the layers. However, the degree of mycorrhizal colonization did decrease in the two biochar layers and in the sandy layer between them (Table 2). This decrease can be attributed to several factors. Firstly, due to the higher microbial activity in the biochar layers, the aeration requirements of these layers are higher. Biochar has a very strong water holding capacity which decreases the air-filled pore fraction within these layers. This will decrease the rate of gas exchange between the layers and the atmosphere due to the slower rate of diffusion (Hillel, 1980) and would have caused more reduced conditions to reside within these layers.

Secondly, biochar contains few nutrients to benefit mycorrhizae (Lehmann et al. 2003b), but because of its higher CEC, it has the potential to retard the leaching of nutrients (Major et al. 2009). This means that biochar can change the local nutrient balance within soil (e.g. N/P ratio) which can have a strong influence on mycorrhizal colonisation (Miller et al. 2002, Warnock et al. 2007). As shown in Table 3, the plant available phosphorus was higher within the biochar layers (Table 5.2) due to the ability of biochar to retain phosphorus added with fertilization. Previous studies (Vesjadova et al. 1990) have shown a strong negative correlation between plant available phosphorus and the degree of mycorrhizal colonization, with higher available phosphorus levels decreasing the need for mycorrhizal symbiosis. These higher phosphorus levels contributed to the decrease in the degree of mycorrhizal colonization within these layers.

Thirdly, the chemistry (Table 5.2) of the biochar layers and the sandy layers differed a great deal, especially in terms of pH. The soil used in this trial had a low pH and a natural mycorrhizal population suited to these acidic conditions. The pH in the top sandy layer and at the bottom of the pot were least affected by the liming effect caused by the biochar layers and therefore also had the greatest degree of mycorrhizal colonisation. The inherited mycorrhizal population within the middle section (3-10 cm) struggled to adjust to the drastic increase in pH which led to the decrease in mycorrhizal colonisation. Apart from the liming effect of biochar it is also speculated that biochar could interfere with the signalling dynamics of the rhizosphere by serving either as a signalling source or sink (Warnock et al. 2007). The fresh biochar used in the layers possibly adsorbed signalling compounds released by the roots which could have led to the decrease in mycorrhizal colonisation.

Pot trial 2: Different biochar amendment rates with wheat

Materials and methods

Pot trial setup

The trial was conducted in a tunnel on Welgevallen experimental farm of the University of Stellenbosch, Stellenbosch, South Africa. The pot trial consisted of 40 pots amended with sieved biochar (< 2 mm) at concentration rates of 0, 0.05, 0.5, 2.5, and 10 % w/w. The biochar amendment was thoroughly mixed with the sand using a concrete mixer. The plant test specie used was wheat (*Triticum aestivum*). Each treatment consisted of 8 pots of which 4 were fertilized and 4 non-fertilized. Ten wheat seeds were planted per pot and thinned out to four seedlings after emergence. The fertilized pots received fertilization in a split application of 70% at planting and 30% 6 weeks after planting. The plants were fertilized with macronutrients according to the following fertilizer recommendation: N:101 ; P:50 ; K:75 ; Ca:30 ; Mg:23 ; S:30 kg. Ha^{-1}. Micro-nutrients were applied using Chemicult broad spectrum fertilizer. The pots were irrigated up to field capacity on a weekly basis for the duration of the trial and inspected every week for any pathogenic infections until the destructive harvesting after 12 weeks. Pots were ordered according to a complete randomized block design shown in Table 5.4.

Soil analysis

The sub samples of biochar and soil taken from each layer were analysed for pH_{H2O} and electrical conductivity (EC) using a 1:20 and 1:2.5 biochar and sand to solution ratio (Cheng and Lehmann 2009; White 1997).

Phosphorus was extracted according to the method described by Soltanpour and Schwab (1979) using NH_4HCO_3-DTPA as the extracting solution, which is suitable for alkaline samples. The phosphorus content was then colometrically determined using the ascorbic acid method.

Mycorrhizal and plant analysis

The plant and root material were collected at harvesting and thoroughly washed and cleaned before sub-samples of the roots were collected and stored in 50% ethanol for the determination of mycorrhizal infections. The plant material was then oven dried at 80 ˚C for two days and the dry mass of the plant and roots recorded.

Mycorrhizal infections were determined using Trypan blue (chlorazol black E) in lactoglycerol to stain the roots after they were cleared in 10% w/v heated KOH (Bevege 1968; Phillips & Hayman 1970; Kormanik & McGraw 1982; Brundrett et al. 1984). Non-woody roots were used for this purpose. Twenty-five 1 cm root segments were placed on a microscopic slide before examining them for mycorrhizal infections under a compound microscope at x200 magnification.

Table 5.4: Randomized complete block design of the pots in the tunnel.

F0*B3	F1*B4	F0*B2	F0*B1	F1*B2	F1*B1	F1*B3	F0*B0
F1*B0	F0*B0	F0*B4	F0*B3	F0*B1	F0*B0	F1*B4	F1*B2
F1*B2	F0*B1	F1*B0	F1*B4	F0*B3	F0*B2	F1*B1	F1*B3
F1*B4	F1*B1	F0*B4	F1*B3	F1*B2	F0*B1	F0*B0	F0*B4
F0*B2	F1*B3	F1*B0	F0*B4	F0*B2	F1*B0	F0*B3	F1*B1

Results

Dry plant and root biomass

Table 5.5.1: Dry root- and plant biomass attained from unfertilized pots

% Biochar (w/w)	Dry plant biomass (g)	Dry root biomass (g)	Total biomass production (g)
0	1.29b	1.21b	2.49b
0.05	1.24b	1.20b	2.43b
0.5	1.71c	1.50b	3.21c
2.5	1.80c	1.45b	3.25c
10	0.68a	0.57a	1.24a

Table 5.5.2: Dry root- and plant biomass attained from fertilized pots

% Biochar (w/w)	Dry plant biomass (g)	Dry root biomass (g)	Total biomass production (g)
0	18.91b	9.83b	28.73b
0.05	23.08c	15.88c	38.96c
0.5	23.99c	14.17c	38.15c
2.5	19.00b	12.66bc	31.66b
10	4.81a	2.44a	7.26a

[a]Numbers followed by the same symbol are not significantly different at P< 0.05 level based on Tukey's Studentized range test. *Graphs of the seperate shoot and root dry weights are shown in Appendix 4.1 and 4.2. For the purpose of the discussion total biomass production will be the main focus.

Figure 5.4 clearly shows that the total biomass production was significantly enhanced at the biochar amendment rates of 0.5 and 2.5% (w/w). The total biomass production increased by 28.9 and 30.5% respectively compared to the control. A decrease of about 50% in dry weight yield occurred at the biochar amendment rate of 10% (w/w).

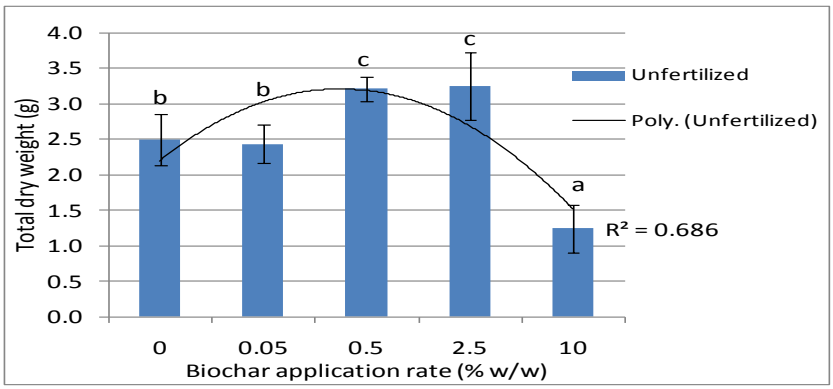

Figure 5.4: Total dry weight production attained from unfertilized treatments

The maximum yield shifted to between the 0.05 and 0.5% (w/w) biochar amendment rates in the fertilized pots (Table 5.5.2) which caused an increase in total biomass production of 32.8 and 35.6% respectively compared to the control. The root growth was significantly enhanced with the application of 0.05-0.5 and 2.5% (w/w) biochar applications, but the greatest increase compared to the control occurred at 0.05% (w/w) biochar application rates. A steady decrease in total biomass production became apparent at biochar amendment rates above 0.5% (w/w). The 2.5% (w/w) amendment rate caused an average increase of about 3g in dry weight when compared to the control but caused a decrease of about 6.5-7g compared to the maximum yields at 0.05 and 0.5% amendment rates. In contrast to these results, the 10% biochar amendment rate caused a significant decrease in dry weight yield of about 74% compared to the control and 81% compared to the 0.05% biochar amendment rate.

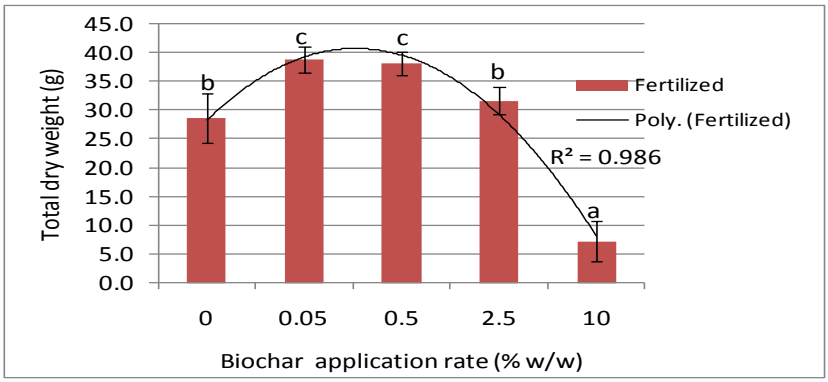

Figure 5.5: Total dry weight production attained from fertilized treatments

Degree of mycorrhizal colonization

Table 5.6: Mycorrhizal number per 25 cm root segment

% Biochar added (w/w)	Fertilized	Unfertilized
0	26.33a	29.67 a
0.05	19.44a	6.44 b
0.5	18.44a	9.00 b
2.5	10.00a	3.67 b
10	4.67a	0.11 b

[a]Numbers followed by the same symbol are not significantly different at P< 0.05 level based on Tukey's Studentized range test.

In both the fertilized and unfertilized pot trials the same trend is followed as the degree of mycorrhizal colonization decreased with an increase in the biochar amendment rate. The wheat roots collected from the unfertilized and fertilized control pots had a higher degree of mycorrhizal colonization compared to pots that received biochar amendments. The unfertilized control pots had significantly more mycorrhizae compared to any of the other treatments within the unfertilized biochar amended pots and even though the fertilized control also showed a higher degree of mycorrhizal colonization, it was not significant due to the large variation connected to the data. Overall, the degree of mycorrhizal root colonization decreased considerably with increasing biochar concentrations in both the fertilized and unfertilized pots (Fig. 5.6).

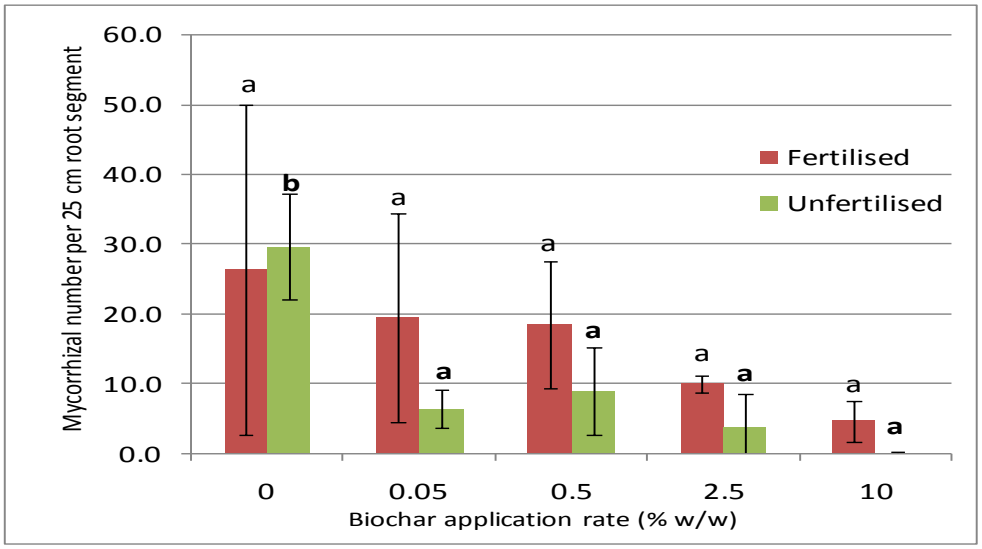

Figure 5.6: Average mycorrhizal number per 25 cm root segment

Discussion

Dry plant and root biomass

The Graphs (Fig. 5.4 and 5.5) shows a definite peak in the dry weight of wheat at biochar amendment rates of 0.05-0.5% and 0.5-2.5% in the fertilized and unfertilized treatments respectively. Pot trials conducted by Chan et al. (2008) also showed increased crop yields when using biochar produced at temperatures below 500˚C and containing a large ash fraction. The addition of biochar to the acidic soil used in this trial would have shifted the nutrient balance by influencing the bioavailability of the elements through the increase in soil pH (Fig. 5.7) caused by the biochar addition. Studies have shown an increase in the plant uptake of elements such as P, K, Ca, Zn and Cu upon biochar application (Major et al. 2010a, b; DeLuca et al 2009). Furthermore, biochar has been shown to improve biological processes such as net nitrification (DeLuca et al. 2006), improve P availability by complexing ions (Al^{3+}, $Fe^{2+/3+}$ and Ca^{2+}) responsible for precipitation reactions (Stevenson and Cole, 1999; Deluca et al. 2009).

In the unfertilized pots, the total biomass production significantly improved up to a optimal biochar application rate previously identified. Since biochar reduced aluminium toxicity, increased soil pH of the acid soil, and inherently contains significant amounts of plant nutrients such as potassium, calcium and magnesium, it is suggested that these are the main reasons for enhanced plant growth seen in this trial (Sika, 2011).

The pH (Fig. 5.7) of soils has a very strong influence on the solubility of many elements. Biochar, can therefore play a significant role in nutrient transformations and bioavailability within the soil through its effect on pH. The soil used in the trial had a medium acidic pH of 5.14, whilst the biochar had a very alkaline pH of 9.39 in water (Table 5.1). In the pot trial the greatest yield in dry weight of the wheat plants was found in pots with a more neutral to alkaline pH in comparison to the control which had an acidic pH. The maximum yield in dry weight occurred at a pH of 7.3 and 6.3-6.8 in the unfertilized and fertilized pots respectively (Table 5.7).

The increase in total biomass production seen in the fertilized pots at biochar amendment rates of 0.05-0.5% compared to the control is a clear indication of the synergistic effect between biochar and fertilizer on plant growth stimulation. This highlighted the beneficial effects of biochar on soil quality (Chan et al. 2008) and the increase in fertilizer efficiency when used in combination with biochar (Lehmann et al. 2003b). Biochar does not serve as a

fertilizer itself, but clearly can increase fertilizer use efficiency if applied at the correct amounts to soil.

Table 5.7: Analysis of pure biochar, sand and biochar amended pots

	pH $_{(H2O)}$	EC (mS/cm)	Plant available P (mg/kg)
Raw biochar	9.39	0.75	89.6
Pure Sand	5.14	0.06	3.94
0.05	6.32	0.071	7.53
0.5	6.8	0.09	7.81
2.5	7.34	0.09	8.82
10	8.42	0.152	11.18

In contrast to the increase seen with lower biochar application rates, both the fertilized and unfertilized pots showed a significant reduction in yield at the 10% (w/w) biochar application rate compared to the control and the maximum yields. The inhibited plant growth at the 10.0 % biochar (w/w) level can be attributed to several factors which includes over liming, waterlogged soil or more reduced conditions, together with nutrient deficiencies of nitrogen, phosphorus, zinc, boron, manganese and copper. (Sika, 2011). Over liming resulted in significant increases in pH (Table 5.6) which could have affected the bioavailability of key plant nutrients especially micro-nutrients such as Mn, Zn and B (Kabata-Pendias, 2011)(Fig. 5.7). The continuous wetter conditions also made the plants more susceptible to pathogenic infections. Plants at the 10% biochar amendment rates showed symptoms of infection and were accordingly analyzed at the disease clinic of the Department of Plant Pathology, and the causal agent of the symptoms was identified as *Pythium aphanidermatum.*

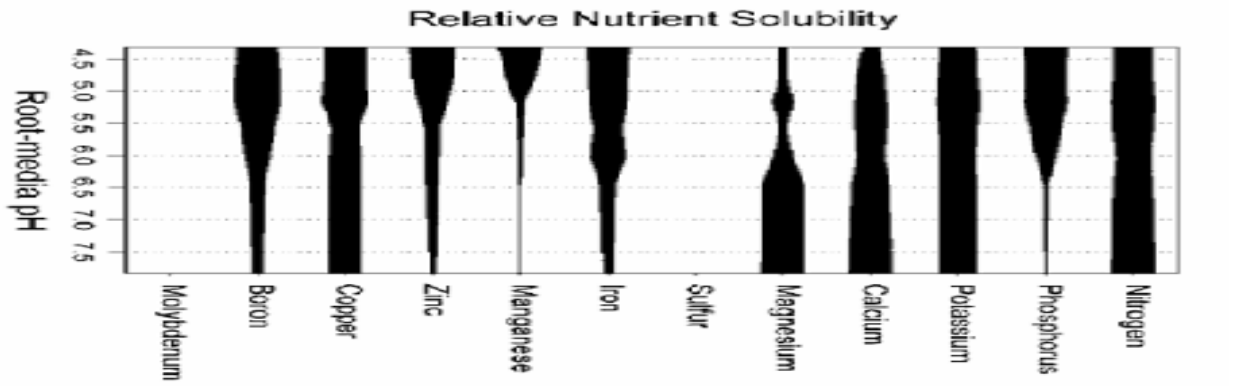

Figure 5.7: Relative solubility of nutrients at different pH levels in one peat-based media (graph based on research by Dr. John Peterson, the Ohio State University)

Degree of mycorrhizal colonization

The decrease in the degree of mycorrhizal colonization (Fig. 5.6) can be attributed to several possible reasons. Firstly, the addition of biochar to the acidic sandy soil has marked effects on the physical (bulk density, aeration, soil water) and chemical (CEC, EC, pH, nutrients) properties of the soil. In the pot trials an increase in pH and EC was recorded with increases in the amendment rate which possibly had a negative impact on the mycorrhizal inhabitants of the soil which was suited to the acidic conditions. The unfertilized control pots had a significantly higher degree of mycorrhizal colonization and the same result was attained for the fertilized control pots which were considerably more compared to the biochar amended treatments. These pots received no biochar which meant their soil chemistry and physical conditions remained the same. The different concentrations also caused differences in the aeration and the soil water regimes. Pots amended with 10% biochar (w/w) had significantly less mycorrhizae compared to the controls due to the continuous wet conditions and *Pythium aphanidermatum* infection.

Biochar contains few nutrients to benefit mycorrhizae (Lehmann et al. 2003b), but because of its higher CEC, it has the potential to retard the leaching of nutrients (Major et al. 2009). This means that biochar can change the local nutrient balance within the soil (e.g. N/P ratio) which can have a strong influence on mycorrhizal colonisation (Miller et al. 2002, Warnock et al. 2007). The addition of biochar caused an increase in plant available P (Table 5.7), which possibly suppressed the need for associations between plant roots and mycorrhizae (Vesjadova et al. 1990). However, this contribution to total available P in the fertilized pots is minimal compared to what the fertilizer added.

Furthermore, it is speculated that biochar could interfere with the signalling dynamics of the rhizosphere as it has a redox activity, serving as a reducing agent and has the ability to adsorb to organic compounds through H-bonding, ligand exchange reactions, direct electrostatic reactions and cation bridging (Joseph et al. 2010; Warnock et al. 2007).

Pot trial 3: Different biochar amendment rates with the common green bean

Materials and methods:

Soil

The soil was characterized again as a new load was fetched for the bean pot trial from the same site. The pH of the soil was measured in distilled water in a 1:2.5 soil to solution ratio (White 1997). The total C and N content of the soil was determined by the EuroVector Elemental Analyzer using the dry combustion method (Nelson and Sommers 1996). The CEC of the soil was determined using 1 M NH_4OAc (pH 7.0) according to the method of Summer and Miller (1996). The biochar was previously characterized in Chapter 1 (Table 1.1; pp. 29). All determinations were carried out in duplicate and are shown in Table 5.8.

Table 5.8: Soil and biochar characterization

	pH_{H2O}	% C	% N	C/N Ratio	Ash Fraction	$CEC_{7.0}$ (cmolc.kg^{-1})
Soil	6.5	0.24	0.014	17.1	*	1.96
Biochar	9.72	82.71	0.53	156.1	3.04	34.30

Pot trial setup

The pot trial was conducted in a tunnel on Welgevallen experimental farm of the University of Stellenbosch, Stellenbosch, South Africa. The pot trial consisted of 60 pots (7 L) with a net weight of 6 kg sand amended with biochar (< 2 mm) at concentration rates of 0, 0.05, 0.5, 2.5, and 10 % w/w. The biochar amendment was thoroughly mixed with the sand using a concrete mixer. Each treatment consisted of 12 pots of which 6 were fertilised and 6 never received any fertilizer additions. The plant test species used was the common green bean (*Phaseolus vulgaris)*. Seeds were prepared by imbibing them over night in distilled water. The seeds were then planted into trays containing pure silica sand as germinating media. Before the seedlings were transplanted, the pots were treated, 16 days before transplanting the seedlings, with 300 ml Xanbac D (fungicide with active ingredient-dichlorophen) for the effective control of *Pythium* spp., previously identified in the wheat trials. A week after germination, one seedling were planted in each of the biochar amended pots. Before transplanting the seedlings, their roots was washed off with water to remove the silica sand and dipped in a suspension containing the live legume bacteria, *Rhizobium leguminosarum* biovar *phaseoli.* Pots were placed according to a randomized block design shown in Table 5.9.

Non-fixing plant control pots containing autoclaved soil were required for applying the isotope dilution method to quantify the amount of nitrogen fixation. The seeds of the non-fixing plant control pots were surface-sterilised in a 3% sodium-hypochlorite solution for 5 minutes and washed 5 times with sterilised distilled water afterwards. Sterilised green bean *(Phaseolus vulgaris)* seeds were planted in a pot containing autoclaved sand, whilst in the other pot sterilised wheat seeds *(Triticum aestivum)* were used as the non-nodulating species.

Each pot was fertilised using Kompel-chemicult hydroponic nutrient powder containing macronutrients (N: 0.065, P: 0.027, K: 0.13, Ca: 0.07, Mg: 0.022, S: 0.075 g/l) and micronutrients (Fe: 0.0015, Mo: 0.00001, Cu: 0.00002, B: 0.00024, Mn: 0.00024, Zn: 0.00005 g/l). Each pot received a basal dosage of 1 L chemicult solution (1 gram chemicult/L) at planting and 250 mL on a weekly basis thereafter. The pots were irrigated on a weekly basis using tap water.

Table 5.9: Complete randomized block design for green bean trial

F0 BC 11	F1 BC 11	F1 BC 35	F1 BC 05	F0 BC 43	F1 BC 14	F0 BC 41	F0 BC 04	FO BC 13	F1 BC 44	F0 BC 44	F0 BC 24	F1 BC 16	F1 BC 02	F0 BC 22	F0 BC 05	F0 BC 01	F1 BC 04	F1 BC 32	F1 BC 46
F1 BC 34	F0 BC 46	F1 BC 26	F0 BC 36	F0 BC 34	F0 BC 14	F0 BC 02	F1 BC 42	F1 BC 21	F0 BC 26	F0 BC 42	F1 BC 35	F0 BC 21	F0 BC 03	F0 BC 25	F1 BC 12	F0 BC 31	F1 BC 24	F0 BC 45	F0 BC 15
F0 BC 32	F1 BC 13	F0 BC 42	F1 BC 36	F0 BC 16	F1 BC 23	F1 BC 01	F0 BC 12	F0 BC 33	F1 BC 43	F1 BC 15	F1 BC 03	F0 BC 23	F1 BC 41	F1 BC 25	F1 BC 06	F1 BC 33	F1 BC 31	F1 BC 22	F0 BC 06

Soil and plant analysis

Harvesting and nutrient analysis of plant materials

Harvesting intervals occurred at day 24 and 39 after transplanting, with the removal of 3 replicates from each treatment. With harvesting the plant shoots, roots and root nodules were separately sampled, dried at 80 ˚C for two days where after the dry weights were recorded. A sub-sample of root segments was stored in 50% ethanol solution for the determination of AM fungal colonization. Plant analysis was however only conducted on the bean plants harvested after 39 days. The dried shoots and roots were milled to a size fraction smaller than 0.5 mm and mixed together. The milled samples were sent to a commercial laboratory (Bemlab, De Beers Rd, Somerset West, South Africa) and analyzed for total N, P, K, Ca, Mg, Na, Mn, Fe, Cu, Zn and B concentrations using inductively coupled mass spectrometry (Varian ICP-OES) and a LECO-nitrogen analyzer with suitable standards.

Isotopic analysis (δ15N) was carried out by Archeometry Department, University of Cape Town. The isotopic ratio of δ15N was calculated as $\delta = 1000‰ (R_{Sample}/R_{Standard})$. R is calculated as the molar ratio of the heavier to the lighter isotope of the samples and standards as defined by Farquhar et al. (1989). A sub-sample of the oven dried leave material was used for the purposes of these calculations. Between 2.100and 2.200 mg of each milled sample was weighed on a Sartorius microbalance (Goettingen, Germany) and placed into 8mm-5mm tin capsules (Elemental Microanalysis Ltd., Devon, UK). The samples were then combusted in a Fisons NA 1500 (Series 2) CHN analyzer (Fisons Instruments SpA, Milan, Italy). The δ15N values for the nitrogen gases released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyzer by a Finnigan MAT Conflo control unit. Three standards were used to correct the samples for machine drift: two in-house standards (Merck Gel and Nasturtium) and one IAEA (International Atomic Energy Agency) standard—$(NH_4)_2SO_4$ (Mortimer et al. 2008).

Microbial analysis

Mycorrhizal infections were determined to confirm whether the Xanbac D addition had a negative influence on mycorrhizal root colonization. Mycorrhizal infections were determined using Trypan blue (chlorazol black E) in lactoglycerol to stain the roots after they were cleared in 10% w/v heated KOH (Bevege 1968; Phillips & Hayman 1970; Kormanik & McGraw 1982; Brundrett et al. 1984). Non-woody roots were used for this purpose. Twenty-five 1 cm root segments were placed on a microscopic slide before examining them for mycorrhizal infections under a compound microscope at x200 magnification.

The total microbial biomass C (C_{TMB}) was determined using microwave sterilisation and extraction with 0.5 M K_2SO_4 as described by Islam and Weil (1998). The organic carbon content (microbial biomass C) was then determined via the rapid oxidation spectrophotometric method of Heanes (1984).

The cumulative microbial respiration over 45 days of incubation was determined directly after the second harvest (after the complete removal of the shoots and roots) using the chamber technique (Monteith et al. 1964; Edwards 1982). The microbial respiration was determined on the same fertilized and unfertilized pots used for the bean trial. The fertilized pots were fertilized once at the start of incubation with 250 ml of the fertilizer solution. The incubation chambers covered an area of approximately 95 cm^2. Soda lime was dried at 105 ˚C for 24 hr before incubation. Pre-weighed soda lime in perforated plastic containers was placed in the chambers to adsorb CO_2 released by microbial respiration. The soil moisture

content was kept between 60-80% for the duration of the trial. The soda lime was collected after the 45 days of incubation, dried at a 105˚C and weighed to record the weight increase. Due to the absorption of CO_2 during the drying process and possible leakage of the chambers, blank chambers were used to correct for the CO_2 which does not form part of the soil CO_2 efflux (Keith and Wong, 2006). The CO_2 flux was then calculated using the correction factor proposed by Grogan (1998).

Soil chemical and nutrient analysis

The sub samples of the biochar amended soil taken from each pot were analysed for pH_{H2O} and electrical conductivity using a 1:20 (Cheng and Lehmann, 2009) and 1:5 biochar-sand to solution ratio.

The total C and N content of the soils with different biochar amendment rates, were determined using a EuroVector Elemental Analyzer using the dry combustion method (Nelson and Sommers 1996)

The exchangeable cations were determined using the ammonium acetate method. Exchangeable cations were extracted with 1 M NH_4OAc for 60 minutes at pH= 7. The extractant was analysed at the CAF lab, Soil Science Department, Stellenbosch University, for Ca^{2+}, K^+, Na^+, and Mg^{2+} using a Varian atomic absorption spectrometer.

The total macro and micro-elements were extracted using NH_4HCO_3-DTPA as the extracting solution, according to the method described by Soltanpour and Schwab (1977) which is suitable for alkaline samples. Extractants were analyzed for macro-elements (Ca, Mg, K and Na) and micro-elements (B, Cu, Mn, Fe, Cu, Zn) at the Departmant of Geology, Stellenbosch University, using ICP-AES and ICP-MS analyzers respectively.

The phosphorus was extracted using NH_4HCO_3-DTPA extracting solution according to the method described by Soltanpour and Schwab (1979) which is suitable for alkaline soils. The P content was determined by the ascorbic acid method.

The ammonium (NH_4^+) and nitrates (NO_3^-) were extracted using 2 M KCl (Bremner & Keeney, 1966) solution. The nitrates were analysed by a commercial laboratory (Bemlab, De Beers Rd, Somerset West, South Africa) using Auto Analyser, Technicron III. The NH_4^+ extracted was determined by treatment of an aliquot of the extract with salicylate and hypochlorite at high pH in the presence of the catalyst sodium nitroprusside and chelating agent ethylenediaminetetraacetic acid (EDTA) to develop an emerald green colour. The NH_4^+

concentration was then determined colometrically by measuring the absorbance at 667 nm against calibrated absorbance measurements (Mulvaney, 1996).

Statistical analysis

The difference between several factors in response to different rates of biochar application was tested with an analysis of variance (ANOVA) (Super-Anova, Statsgraphics Version 7, 1993, Statsgraphics corporation, USA). Where the ANOVA revealed significant differences, the means were separated using a *post hoc* Tukey LSD Compromise, multiple comparisons test ($p \leq 0.05$). Different alphabetical letters were used to indicate significant difference between treatment means (n= 3 for each treatment).

Results

Changes in soil quality

Table 5.10: Soil quality parameters

% Biochar added: Unfertilized	pH_{H2O} before	pH_{H2O} after	EC before µS/cm	EC after µS/cm	% C (wt %)	% N (wt %)	C/N Ratio	Exchangeable cations ($cmol_c.kg^{-1}$ soil)			
								Ca	Mg	Na	K
0	6.3	6.2	61.2	45.9	0.24	0.014	17.1	4.2	1.4	2.6	1.0
0.05	6.8	6.8	46.3	53.2	0.24	0.012	20.0	4.6	1.2	2.5	1.7
0.5	7.1	7.1	39.9	40.3	0.38	0.01	38.0	4.3	1.5	2.3	2.9
2.5	7.9	8.3	67.2	46.5	1.21	0.02	60.5	3.9	2.1	3.0	3.0
10	9.5	9.6	183.3	86.9	7.5	0.06	125.0	11.0	3.2	3.9	7.1
Fetilized											
0	6.5	6.0	59.1	89.5	0.37	0.01	37.0	6.3	1.6	2.4	2.1
0.05	6.6	6.6	126.4	108.5	0.25	0.01	25.0	10.0	2.3	3.0	2.6
0.5	6.8	6.8	97.1	95.0	0.42	0.01	42.0	8.1	1.9	2.4	2.7
2.5	7.8	7.7	124.7	115.6	1.36	0.02	68.0	8.6	3.0	2.9	3.8
10	9.3	9.0	215.2	185.1	7.82	0.08	97.8	12.9	4.2	3.9	7.3

*The analysis was conducted after the first irrigation cycle and after the basal dosage of fertilizer was applied.

Biochar caused a significant increase in pH of the soil and also increased the electrical conductivity (EC) of the soils. It is also clear that the pH decreased over the 39 days in most treatments. The same trend is followed in the EC of the treatments which decreased in most treatments over the 39 days even though fertilizer was added to the pots and no leaching was allowed. The addition of biochar not only limed the soils, but also increased the carbon content of the soils and as a result increased the C/N ratio of the soils with increasing application rate.

The addition of biochar also increased the exchangeable cations in both the fertilized and unfertilized pots. Increases in the Ca^{2+} and K^{+} content of the soils were the most prominent, which explains the liming effect of biochar on soil pH. The Na^{+} and Mg^{2+} content also

increased considerably as the biochar application rate increased, indicating that biochar contributes to the nutritional status of the pots. The increase in these cations resulted in the increase seen in the EC of the pots. The biochar also provides a larger CEC* compared to the sand alone (Table 1) and therefore provides a larger surface area for nutrient retention as the biochar amendment rates increased.

Plant elemental composition and growth response

* For the purpose of the discussion, focus will mainly be given to the total biomass production. The seperate mass of the roots and shoots are shown in Appendix 4.3 en 4.4.

Table 5.11: Total plant elemental composition grown under different biochar application rates

% Biochar added: Fertilized	N	P	K	Ca	Mg	Na	Mn	Fe	Cu	Zn	B
	mmol. g^{-1} dw					mmol. kg^{-1} dw					
0	1.74b	0.16a	1.08a	0.20ab	0.20ab	56.69a	1.55c	5.37b	0.10a	2.53c	3.76b
0.05	1.63ab	0.15a	1.19a	0.34c	0.21b	67.97a	0.95abc	5.40b	0.10a	2.04bc	3.61ab
0.5	1.57ab	0.15a	1.25a	0.30bc	0.20ab	71.69a	1.24bc	6.88b	0.11a	1.43ab	3.51ab
2.5	1.38a	0.17a	1.36a	0.23ab	0.19ab	71.43a	0.55ab	4.75b	0.12a	0.95a	2.99ab
10	1.44ab	0.14a	1.28a	0.16a	0.15a	92.70a	0.30a	2.21a	0.10a	0.85a	2.44a
Unfertilized											
0	1.67c	0.08ab	0.45a	0.16a	0.20a	106.40ab	0.70b	8.21a	0.18a	3.53a	2.81a
0.05	1.53bc	0.06ab	0.40a	0.27bc	0.20a	147.10bc	0.61b	7.97a	0.21a	3.43a	2.50a
0.5	1.44b	0.06a	0.71b	0.31c	0.20a	67.96a	0.66b	6.36a	0.17a	2.73a	2.74a
2.5	1.26a	0.07ab	1.12c	0.22ab	0.17a	106.36ab	0.37ab	7.03a	0.24a	3.35a	2.56a
10	1.19a	0.10b	1.53d	0.17a	0.16a	186.45c	0.26a	5.49a	0.21a	2.88a	2.53a

* Different letters indicate significant differences between treatments means ($p \leq 0.05$) and are ranked from the lowest value

The uptake of several macro- and micronutrients was promoted or adversely affected. In the fertilized treatments the uptake of nitrogen were adversely affected with increasing biochar application rates. The plant material of the control contained significantly more N compared to any of the treatments which received biochar additions, were no significant differences in N content existed between treatments, but a decreasing trend with increasing biochar application rate was followed. The uptake of both Na and K were promoted with increasing biochar application rates, whilst Mg uptake decreased significantly between 0.05 and 10% (w/w) biochar application rates. The plant material from the 0.05 and 0.5% (w/w) biochar application rates contained significantly more Ca compared to the 10% biochar application rate and considerably more compared to any of the other treatments in both the fertilized and unfertilized pots. The uptake of micro-nutrients was mostly inhibited. The uptake of Mn, Zn, and B decreased significantly from the control to the 10% (w/w) biochar application rates, whilst the Fe content of the plant materials increased up to 0.5% (w/w) biochar application rate, where after a decrease occurred with increasing biochar application rates.

In the unfertilized treatments, exactly the same trends with respect to the macro-nutrients were followed as discussed in the fertilized treatments. Significant decreases occurred in the N uptake with increasing biochar application rates and a decreasing trend were also identified in the uptake of Mg. The uptake of P, K and Na were promoted with significant increases occurring in the P and K content of the plant with increased biochar additions. The Ca content of the plant materials were again the highest at the 0.05 and 0.5% (w/w) biochar application rates which is associated with the highest total biomass production. The uptake of most micro-elements was again adversely affected with increasing biochar application rates especially manganese which showed a significant decrease from the control to the 10% biochar application rate. Considerable decreases amounting to 2.72, 0.65, and 0.28 mmol.kg^{-1} dw occurred in the uptake of Fe, Zn and B, respectively, between the control and the 10% biochar amendment rate, whilst the uptake of Cu was slightly enhanced (Table 5.11).

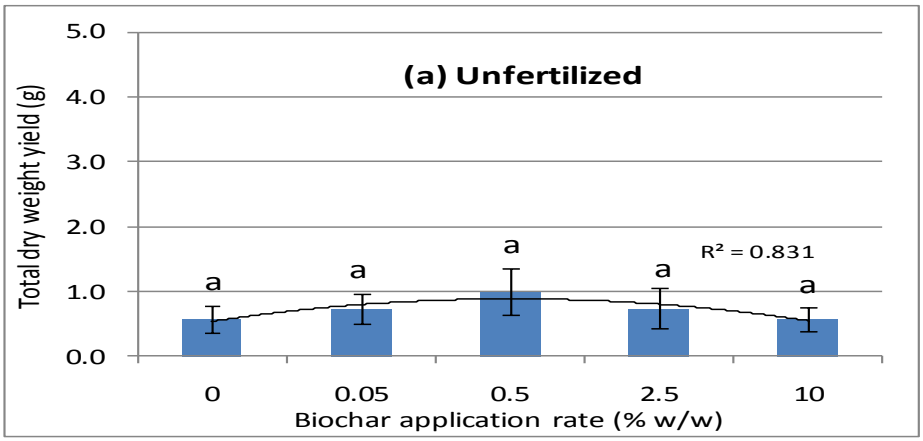

Figure 5.8: The total biomass production from the (a) unfertilised and (b) fertilised treatments. Different letters indicate significant differences between treatments means ($p \leq 0.05$) and are ranked from the lowest value.

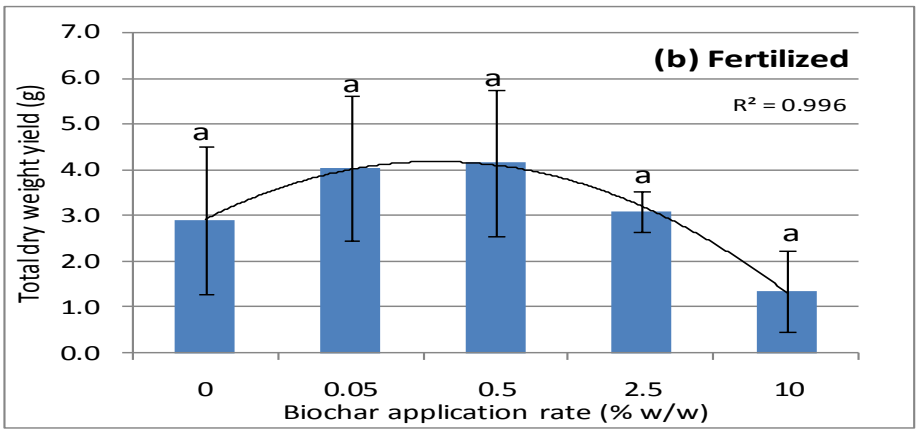

Figure 5.9: The total biomass production from the (a) unfertilised and (b) fertilised treatments. Different letters indicate significant differences between treatments means ($p \leq 0.05$) and are ranked from the lowest value.

Analysis of the total biomass production (shoot and roots) revealed a definite positive response up to 0.5 % (w/w) biochar amendment rate at which the maximum biomass production was obtained in both unfertilized and fertilized pots. A decline in biomass

production followed thereafter. In both the unfertilized and fertilized treatments a polynomial trend is followed (R^2= 0.83; 0.99) (Fig. 5.8 and 5.9). In both trials the amendment rates between 0.05- 2.5% (w/w) did cause increases of different magnitudes in the dry weight of the beans compared to the control. At the 10% (w/w) biochar amendment rate in the unfertilized pots, the dry weight production only increased by 1.4% compared to an increase of 77% at the 0.5% (w/w) biochar amendment rate with regard to the control. As in the case of the unfertilized pots, the fertilized pots also produced the greatest increase in dry weight at 0.5% (w/w) biochar amendment rate of 43% (Fig. 5.9). However, in contrast to the unfertilized pots, the 10% biochar amendment rate caused a decrease of 53.5% in dry weight compared to the control.

Biochars effect on the processes of nitrification and ammonification

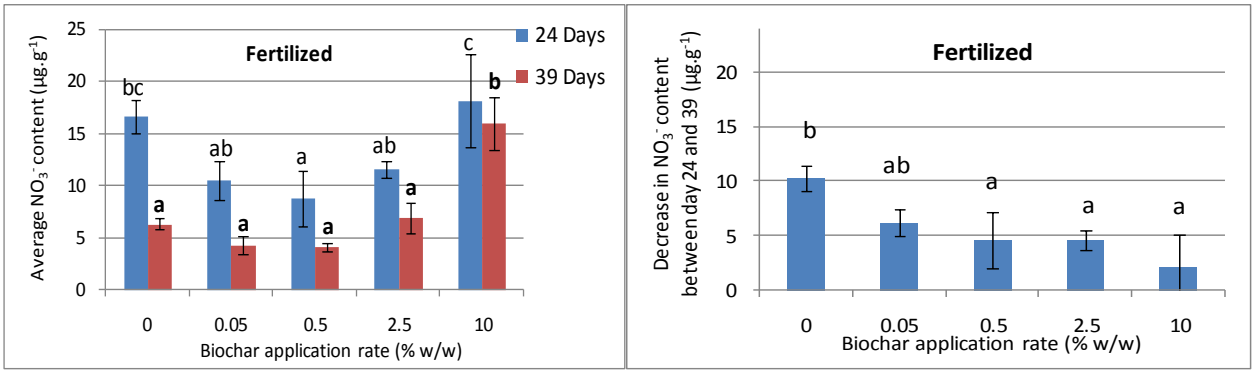

Figure 5.10: (a) Average NO_3^- content of fertilized pots at harvesting intervals and (b) change in the NO_3^- content over time interval of 15 days. Different letters indicate significant differences between treatments means corresponding to 24 and 39 days ($p \leq 0.05$)

The Kompel-Chemicult fertilizer used in the trial provided nitrogen in the form of nitrates (NO_3^-). In figure 1a the biochar amendment rates between 0.05-2.5% (w/w) had the lowest yet similar amounts of mineral NO_3^-, whilst the 10% (w/w) biochar applications rate had the highest amount of mineral NO_3^- compared to any of the other treatments at both sampling periods. The magnitude of the decrease in mineral NO_3^- content over the time interval of 15 days (Fig. 5.10b) clearly indicated that smaller decreases occurred in the mineral NO_3^- content with increasing biochar application rates. The magnitude of the decrease in the control pots were significantly more compared to any of the other treatments except the 0.05% (w/w) biochar amended treatments.

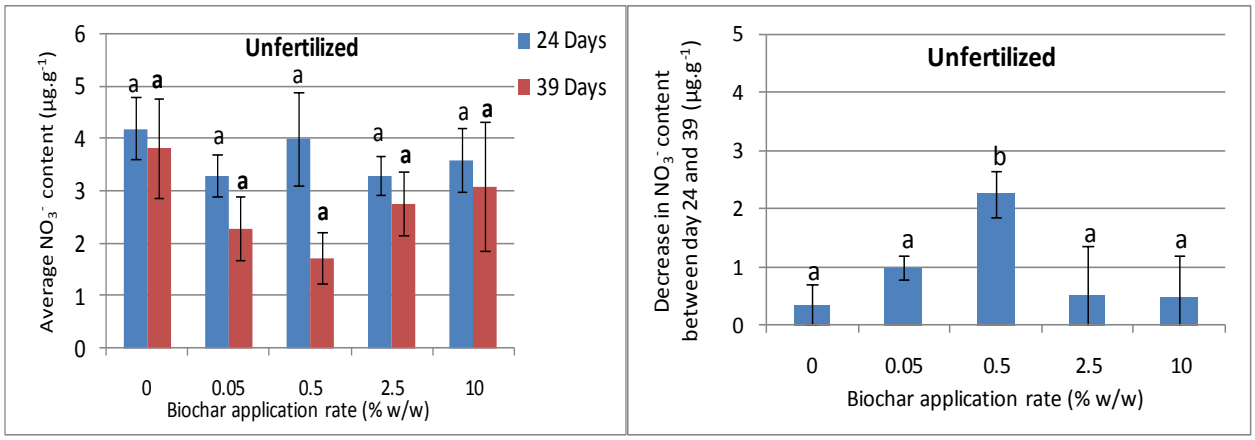

Figure 5.11: (a) Average NO_3^- content of unfertilized pots at harvesting intervals and (b) change in the NO_3^- content over time interval of 15 days. Different letters indicate significant differences between treatments means corresponding to 24 and 39 days ($p \leq 0.05$)

In the unfertilized treatments all the pots had similar mineral NO_3^- contents with no significant difference occurring between any of the treatments at day 24. The highest amounts of mineral NO_3^- were extracted from the control and 0.5% (w/w) biochar amended pots at day 24 (Fig. 5.11a). However, decreases in the mineral NO_3^- content occurred in all pots over the time interval of 15 days (Fig. 5.11b). The magnitude of these decreases were the highest in the 0.05 and 0.5% (w/w) biochar amended pots which led to these pots also containing the lowest mineral NO_3^- content at day 39. The magnitude of the decrease in mineral NO_3^- content were less and similar in the control, 2.5 and 10% (w/w) biochar application rates, which led to the control containing considerably more mineral NO_3^- compared to the pots that received biochar additions.

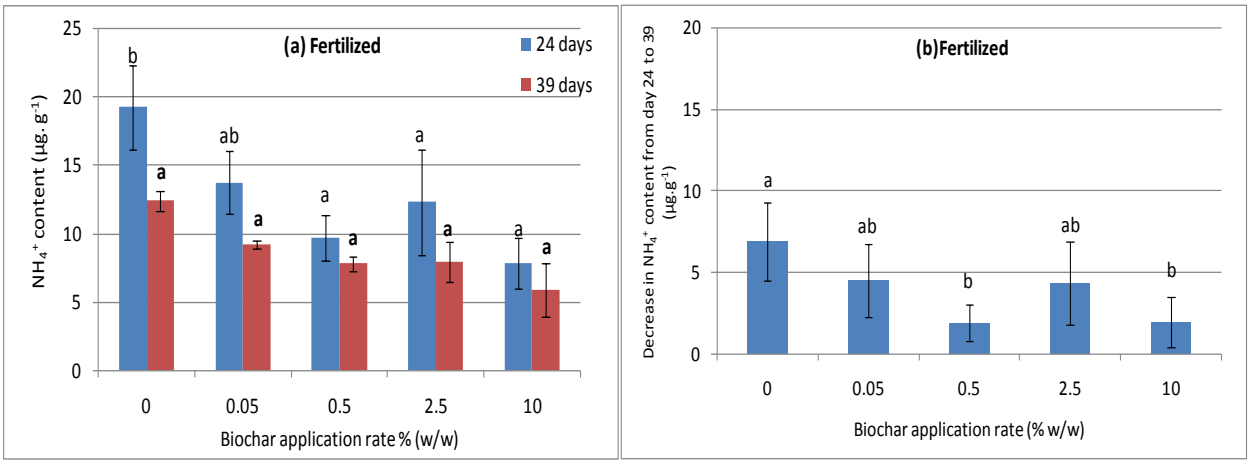

Figure 5.12: (a) Average NH_4^+ content of fertilized pots at harvesting intervals and (b) change in the NH_4^+ content over time interval of 15 days. Different letters indicate significant differences between treatments means corresponding to day 24 and 39 ($p \leq 0.05$).

Figure 5.12a clearly shows that the increased application of biochar significantly reduced the mineral NH_4^+ content. The biochar application rates of 0.5% up to 10% (w/w) had significantly less mineral NH_4^+ compared to the control after 24 days. However, the reduction in mineral NH_4^+ content (Fig. 5.12b) in the following 15 days was significantly more in the control compared to the reduction seen at the 0.5 and 10% (w/w) biochar application rates and considerably more than in any of the other treatments. This resulted into no significant differences existing in mineral NH_4^+ content of the treatments at day 39.

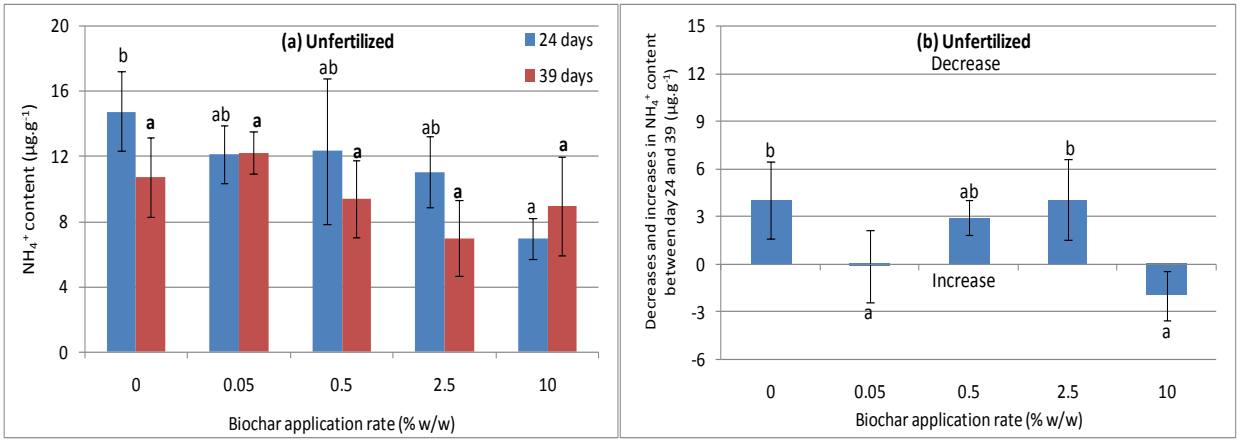

Figure 5.13: (a) Average NH_4^+ content of unfertilized pots at harvesting intervals and (b) change in the NH_4^+ content over time interval of 15 days. Different letters indicate significant differences between treatments means corresponding to day 24 and 39 ($p \leq 0.05$).

The same trend was followed in the unfertilised pots with the 10 % (w/w) biochar application rate significantly reducing the amount of mineral NH_4^+ compared to the control. Each of the biochar amended treatments had considerably lower amounts of mineral NH_4^+ compared to the control treatment (Fig. 5.13a) at day 24. However, the mineral NH_4^+ content decreased considerably in the control, 0.5 and 2.5 % (w/w) biochar application rates between days 24 to 39, whilst a considerable increase in the mineral NH_4^+ content occurred at the 10% (w/w) biochar application rate. As a result no significant differences existed between the treatments at day 39.

Changes in total microbial biomass C between treatments and over time

Table 5.12: Change in C_{TMB} over time in fertilized and unfertilized treatments

% Biochar applied	Fertilized (mg C. g^{-1})	Non-fertilized (mg C. g^{-1})
C_{TMB} at 24 days		
0	0.08a	0.18a
0.05	0.06a	0.18a
0.5	0.11a	0.18a
2.5	0.19a	0.12a
10	0.22a	0.12a
C_{TMB} at 39 days		
0	0.22a	0.16b
0.05	0.16a	0.12ab
0.5	0.22a	0.039a
2.5	0.20a	0.040a
10	0.24a	0.051a

* Different letters indicate significant differences between treatments means ($p \leq 0.05$) and are ranked from the lowest value.

Contrasting trends were seen in the change of the C_{TMB} over time in the fertilized and unfertilized pots. In the fertilized treatments, biochar additions had a significant effect on C_{TMB} which led to significant increases at biochar application rates of 2.5 and 10% (w/w) and a considerable increase at 0.5% in the fertilized pots after 24 days compared to the control (Table 5.12). However, these results were not repeated at day 39 as no significant difference existed between treatments due to considerable increases in C_{TMB} in the control, 0.05 and 0.5% (w/w) biochar amended treatments over the 15 day interval (Fig. 5.14). The magnitude of these increase in C_{TMB} decreased as the biochar amendment rate increased with only marginal increases occurring in C_{TMB} at the 2.5 and 10% (w/w) biochar amended treatments between days 24 to 39.

No significant differences existed between any of the unfertilized treatments at day 24. However, in contrast to the increases in C_{TMB} over time and with increasing biochar application rate, significant decreases occurred in C_{TMB} of the 0.5, 2.5 and 10% (w/w) biochar application rates compared to the control (Table 5.12) at day 39, due to the considerable decreases that occurred in C_{TMB} over the two intervals (Fig. 5.14).

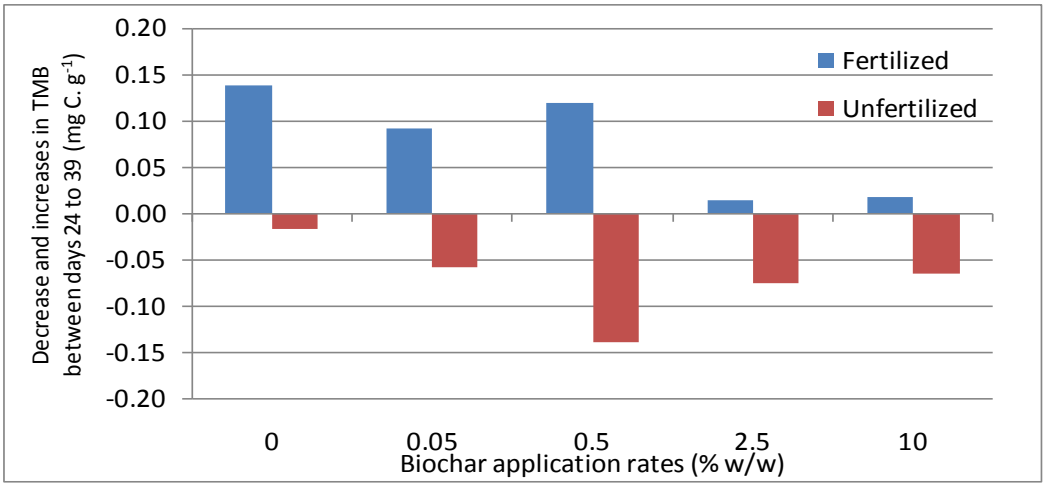

Figure 5.14: Change in microbial biomass C between days 24 and 39

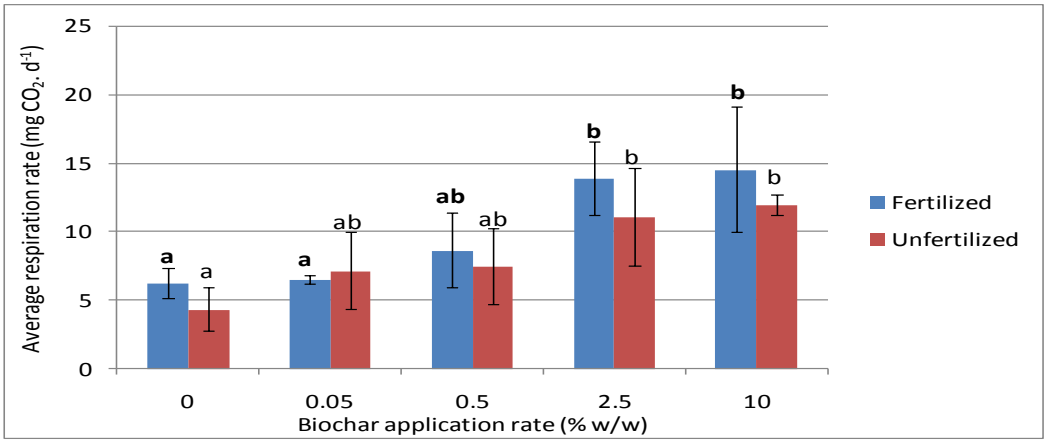

Figure 5.15: Average microbial respiration rate over 45 days. Different letters indicate significant differences between treatments means corresponding to the fertilized and unfertilized treatments ($p \leq 0.05$) and are ranked from the lowest value.

In contrast to the decline seen in C_{TMB} of the unfertilized pots between day 24 and 39, an increase can be seen in microbial respiration following 45 days after the beans have been harvested in both the fertilized and unfertilized pots (Fig. 5.15).

It is clear that increased biochar additions promoted microbial activity as seen in the significant differences existing between treatments in the fertilized and unfertilized pots due to the increasing trend seen in CO_2 evolution with increasing biochar applications. Furthermore, the addition of fertilizer also promoted microbial respiration compared to the unfertilized treatments (Fig. 5.15).

Biological nitrogen fixation (BNF)

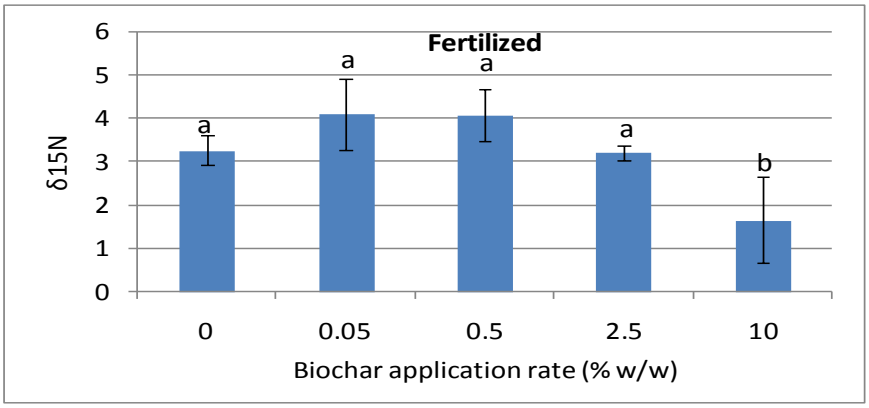

Figure 5.16: Biological nitrogen fixation after 39 days in fertilized bean plants. Different letters indicate significant differences between treatments means ($p \leq 0.05$) and are ranked from the lowest value.

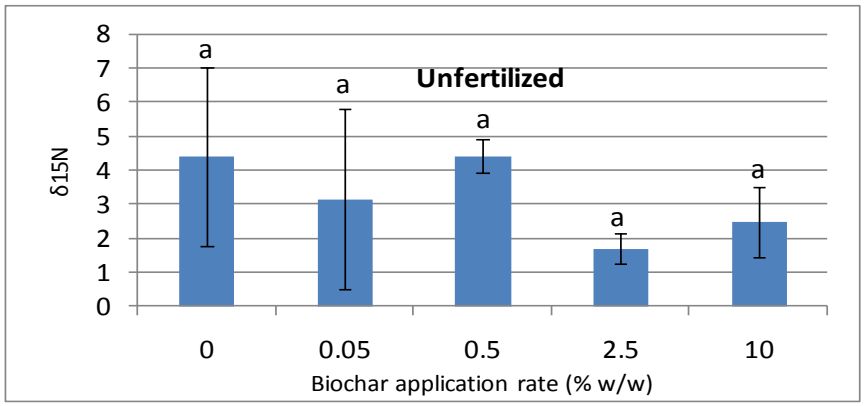

Figure 5.17: Biological nitrogen fixation after 39 days in unfertilized bean plants. Different letters indicate significant differences between treatments means ($p \leq 0.05$) and are ranked from the lowest value.

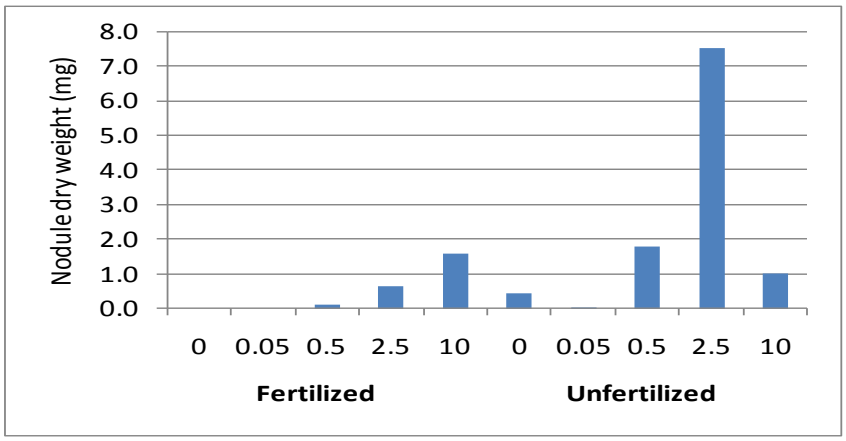

Figure 5.18: Degree of nodulation with increasing biochar application in fertilized and unfertilized treatments

In both fertilized and unfertilized treatments the addition of low rates of biochar to soil had no significant effect on BNF. In the fertilized pots no difference in BNF existed between treatments up to the 2.5% (w/w) biochar application rate. However, a significant increase in

BNF occurred at the 10% (w/w) biochar application rate compared to the control and the other treatments (Fig. 5.16). This corresponds to the beans which had the highest degree of nodulation. When viewing Figure 5.16 from the 0.05 to the 10% (w/w) biochar application rates a relatively high linear correlation (R^2= 0.73; Fig. 5.19) between BNF and increasing biochar application could be identified, clearly indicating a strong positive response in terms of BNF and increased biochar additions.

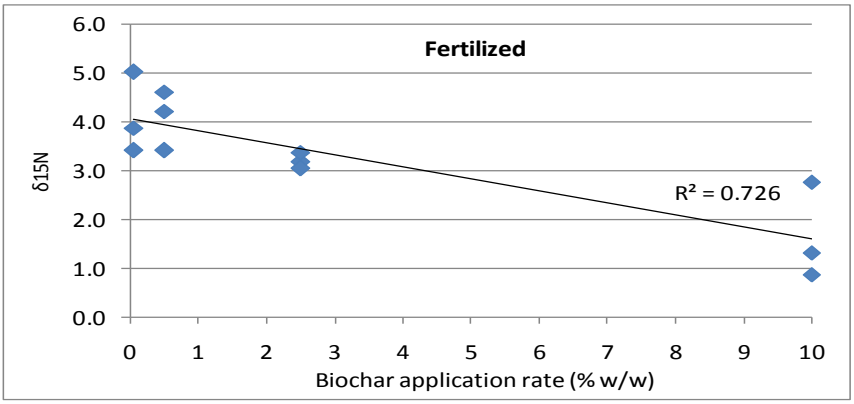

Figure 5.19: Linear correlation between biochar application rate and δ15N

Also, no significant difference existed in BNF between the treatments of the unfertilized bean plants. However, in both cases an overall decrease in the nitrogen deriving from the soil can be seen as a result of increased biological N_2 fixation from the atmosphere which contains considerably less ^{15}N compared to soils. As a result, the 10% (w/w) biochar application rate in both the fertilized and unfertilized treatments as well as the 2.5 % (w/w) biochar application rate of the unfertilized biochar amended pots promoted BNF relative to the lower biochar application rates. However no specific trend could clearly be identified from the data.

Discussion

Plant elemental composition and growth response

The addition of biochar at different application rates had considerable effects on both the total dry weight yields and the elemental composition of the green beans. In unfertilized and fertilized treatments the 0.5% and 0.05-0.5% (w/w) biochar amendment rates produced the highest dry weight yields, respectively, compared to the control and other biochar application rates. Even though the N uptake decreased compared to the N uptake of the control bean plants, it did not result in loss of biomass production. The decrease in N uptake is in accordance with the research of Chan et al. (2007b) who also found decreased N uptake by plants when biochar from plant origin, low in N, is supplied to plants.

Due to the liming effect biochar caused significant increases in pH and as a result also increased the alkali-cations, Ca, Na and K on the cation exchange sites (Table 5.10). This translated into an increased uptake of these macro-elements as the biocar application rate increased. However, in both the fertilized and unfertilized treatments the highest Ca content obtained (Table 5.11) from the elemental analysis of the bean material were associated with the greatest total biomass production (Fig. 5.8 and 5.9). Ca deficiency has been shown to poses a serious yield and quality limitation for several crops (Naeem et al. 2009) as it is a critical component of both cell walls and membranes, where it cross-links pectic chains and affects the mechanical properties of pectic gel (Matoh and Kobayashi, 1998; Epstein and Bloom, 2005). Furthermore, Khan et al. (2001) observed that the leaves of calcium treated plants trapped more sunlight to increase the rate of photosynthesis as compared to the control plants. Calcium could therefore have contributed to the greater biomass production seen at the 0.05 and 0.5% biochar application rates.

The liming effect of biochar also improved the soil quality as it contributed to the nutritional status of the soils and can affect the availability of many micro-nutrients (Fig. 5.7, trial 2). Studies have shown an increase in the plant uptake of elements such as P, K and Ca upon biochar application (Major et al. 2010a, b; DeLuca et al. 2009), which is in accordance with our results. The bio-availability of micronutrients such as Mn, Zn, B and Fe are pH dependent and decreases with increasing pH (Kabata-Pendias, 2010). Depending on the initial pH of the soil, biochar applications and the liming effect caused by the biochar, may increase or decrease the uptake of micro-nutrients. In this trial the uptake of all micro-nutrients tested for (except Cu) were adversely affected (Table 5.11), mainly due to the increase of pH above optimal levels. Especially Mn and Zn uptake was inhibited because the availability of these micro-elements decreases considerably at pHs above 4.5 and 5 respectively according to Figure 5.7 (Trial 2). In well drained soils at a pH below 5.5, Mn is easily available to plants (Kabata-Pendias, 2010). Zinc is mostly available in the acid pH range and least available in alkaline or recently limed soils (Plaster, 2009). In the pot trial the greatest total biomass production of the bean plants was found in pots with a more neutral to alkaline pH in comparison to the control which had a more acidic pH. The maximum total biomass production occurred at a pH_{H2O} of 7.1 and 6.6-6.8 in the unfertilised and fertilised pots, respectively. A considerable reduction occurred in the total biomass production of the beans at the 2.5 and 10% (w/w) biochar application rates compared to the maximum yield attained at biochar application rates of 0.5 % in both the unfertilized and fertilized pot trials (Fig 5.8 and 5.9). The decreases coincide with a decrease in the uptake of Ca at biochar application rates above 0.5% (Table 5). Furthermore, the pH of the soil amended at biochar application

rates of 10% (w/w) were significantly higher and resulted in decreased uptake of all micro-nutrients, especially Zn and Mn.

Several biological reactions in the plants utilize Fe, Zn and Mn of which chlorophyll production and enzymatic activities is the most important (Plaster, 2009; Epstein and Bloom, 2005). In 1844, Eusébe Gris showed that the addition of iron salts to plants with chlorosis was effective in relieving chlorosis. Furthermore, iron is mostly localized within the chloroplast of the leaves (Whatley et al. 1951; Seckbach, 1972). The decrease in the uptake of these micro-elements therefore, resulted in a decrease in the chlorophyll content index measured, with increasing biochar application rates (Appendix 4.5). The photosynthetic efficiency was found to be the best at biochar application rate of 0.5% in the fertilized pots after three weeks (Zetler, 2011) which is associated with the plant material containing the highest Fe content. This resulted in an increase in C fixation from the atmosphere which resulted in the yield increase.

Changes in total microbial biomass C between treatments and over time

The addition of biochar to the infertile sandy soil would have had a strong influence on the soil chemical properties as it would have caused an increase in pH, CEC, exchangeable cations and base saturation (Ogawa and Okimori, 2010). This resulted in increases and shifts in the nutrient status of the soil, especially in terms of the macro-nutrients as seen in the increases of the exchangeable cations (Table 5.10). Biochar also promotes carbon sequestration and increases the carbon content of soils (Lehmann et al. 2006). The soil physical properties such as water retention capacity and porosity increased (Ogawa and Okimori, 2010) and therefore, strongly influenced the soils drying cycles. Overall, biochar improved the soil quality and fertility of the soils as can be seen in the increased dry weight yields of the beans at 0.5% w/w application rate and one would expect that this would also result in a positive response in microbial growth.

From our data it was clear that the microbial community responded mostly negatively to the addition of high concentrations of biochar. In the unfertilized treatments low application rates of between 0.05-0.5% (w/w) did not have any effect on C_{TMB} after 24 days, but after 39 days a considerably larger decrease in C_{TMB} occurred in all biochar amended pots compared to the control (Fig. 5.14), which resulted in a significant difference in C_{TMB} of the 0.5 % and above biochar amended pots, compared to the control (Table 5.12). In contrast to the trend seen in the unfertilized treatments, the fertilized treatments showed considerable increases in C_{TMB} after 24 days at the higher biochar application rates of 0.5-10% (w/w). However, even though

C_{TMB} increased in all the fertilized treatments over time (Fig. 5.14), the increases were considerably higher in the control and in treatments with lower biochar additions (0.05-0.5% w/w). This led to similar C_{TMB} in all the treatments at the end of the trial.

Our findings contradicts the findings of Chan et al. (2008), who found that poultry produced biochar pyrolysed at 450 ˚C caused significant increases in C_{TMB} in the presence of N fertilizer, whilst poultry biochar produced at 550 ˚C increased the C_{TMB} significantly at higher application rates in the absence of N fertilizer. Furthermore, it has been shown that extracts from poultry produced biochar can promote microbial growth. However, extracts deriving from pine timber tended to inhibit microbial growth as it did not contain any protein derived compounds as was found in the poultry derived biochar extract (Van Zwieten et al. 2010; Das et al. 2008). Van Zwieten also found that biochar produced from feedstocks differing in compositions, have variable effects on microbial activity of different soils with different crop species, and showed both increases and decreases in microbial activity.

The greatest difference between the control and the treatments exists in the pH and C/N ratio (Table 5.10) of the soil and the decrease in C_{TMB} can therefore possibly be correlated to an increase in both these parameters. Studies done by Aciego Pietry and Brooks (2008) and Steiner et al. (2004) both revealed a positive correlation between increasing pH and the stimulation of microbial reproduction (Lehmann et al. 2011). However, each soil‘s fungal and bacterial community react differently upon increase in pH with increasing biochar addition (Rousk et al. 2009), which can result in shifts in the microbial community composition (Anderson et al. 2011). The increase in pH caused by the continuous increase in biochar application rate did not stimulate an increase in microbial biomass of the unfertilized treatments. An increase in microbial biomass was seen with increasing biochar applications in the fertilized treatments after 24 days (Table 5.12), but this effect diminished after 39 days, with all of the treatments having similar TMB carbon. Therefore, the effect of pH in this trial was secondary.

The addition of biochar (C/N ratio= 156) resulted in a significant increase in the C/N ratio between the controls to the 10% (w/w) application rate before the trial started (Table 1). The soil used in this trial contained very small amounts of C (0.24%) and N (0.014%) and had a C/N ratio of 17:1 (Table 1). Microbes need N in a C/N ratio of 8:1 (Havlin et al. 2005; Pastel, 2009) in their bodies and therefore needs to incorporate large amounts of nitrogen. The wide C/N ratios resulting from the addition of biochar would have increased the amount of readily available C (labile C fraction of biochar) available to microbes and induced increased microbial catabolic and metabolic activities. As a result it caused an increase in the nutrient

uptake (Qui et al. 2008), especially N from the soil, to attain a C/N ratio of 8:1. However, as the soil is already very low in total N (Table 5.8) and never received any fertilizer inputs (unfertilized treatments), N would be the limiting factor for microbes to maintain their cell growth and maintenance. Furthermore, microbes had to compete with the green beans, planted in each pot, for nutrients and with other microbes in the soil. It is clear that the C_{TMB} of the controls only decreased marginally, possibly as a result of competition for nutrients, specifically N, with the growing plant and was considerably less compared to the soils amended with biochar (Fig. 5.12) due to a more preferred C/N ratio.

The fertilized pots showed only increases in C_{TMB} of all the treatments over the period between day 24 and 39. This is mainly attributed to the addition of the fertilizer, Kompel-chemicult hydroponic nutrient powder, which supplied the pots with macronutrients (N: 0.065, P: 0.027, K: 0.13, Ca: 0.07, Mg: 0.022, S: 0.075 g/l) and micronutrients (Fe: 0.0015, Mo: 0.00001, Cu: 0.00002, B: 0.00024, Mn: 0.00024, Zn: 0.00005 g/l). The addition of fertilizer alleviated the negative effect of the wide C/N ratio brought about by biochar additions and decreased the competition between the plants-microbes and between microbes-microbes for available N and other nutrients in the soil. The increase seen in C_{TMB} with increasing biochar application at day 24 is possibly due to an immediate positive response of the soil‘s microbial population to the increased available C source and N supplied with the addition of the basal fertilizer dosage at the start of the trial, which promoted microbial proliferation relative to the control which is very low in carbon (Table 5.12). Qui et al. (2008) suggested that the immediate availability of C, N and P and their relative supply compared to that of the cell physiological demands may underlie microbial responses in the short-term. However, the wide C/N ratios of the 2.5 and 10% (w/w) and stronger competition by the common green bean for nutrients later in the trial resulted in a significantly smaller response in C_{TMB} between day 24 and 39 compared to the treatments with smaller C/N ratio. It was clear that the increase in C_{TMB} of the 2.5 and 10% (w/w) biochar application rates was purely because of the large supply available C and N from the basal dosage fertilizer the pots received. This is in accordance with the column trial conducted in chapter 2, were we came to the conclusion that the utilization of biochar as a carbon source is promoted by the addition of a fertilizer.

In contrast to the C_{TMB} analysis done during the trial, the determination of cumulative microbial respiration over a 45 day period after the bean plants were harvested revealed that biochar additions caused small increases in respiration at the lower biochar application rates of 0.05-0.5% w/w and significant increases at the 2.5 and 10% (w/w) application rates

compared to the control, even though the C_{TMB} in the unfertilised treatments decreased at the higher biochar applications rates. However, one has to keep in mind that the trial was conducted after the green beans were harvested and stretched over 45 days. The absence of actively growing plants reduced the competition for nutrients. A few studies have reported increased microbial activity measured as increased CO_2 production upon the addition of biochar to soils (Bruun et al. 2008; Kuzyakov et al. 2009; Smith et al. 2010). Biochar has been shown to have a priming effect on the mineralization of native C pool of soils which could have contributed to the increase in microbial respiration. However, the average CO_2 production in both the fertilized and unfertilized treatments increased linearly (R^2= 0.80; 0.79, respectively) with increasing biochar application rate and is possibly related to the increase in the labile C pool of the soil related to the distinct labile C pool of the biochar itself (Smith et al. 2010). Microbial respiration was higher in the fertilized pots compared to the unfertilized pots and once again confirms the priming effect of fertilizer on biochar utilization by microbes.

Other factors that could possibly have an effect on microbial community and symbiotic relations between microbes and plants include the following: Firstly, the effect of biochar on redox reactions within the soils, as it mainly functions as a reducing reagent (Joseph et al. 2010) and will therefore, possibly have an influence on nutrient transformations (Lehmann et al. 2011). Secondly, it is speculated that biochar could interfere with the signalling dynamics of the rhizosphere as it has a redox activity and lastly the ability of biochar to adsorb to organic compounds through H-bonding, ligand exchange reactions, direct electrostatic reactions and cation bridging (Joseph et al. 2010; Warnock et al. 2007) possibly rendering it less available to microbes.

Biochar effects on biological nitrogen fixation (BNF)

From Figure 5.16 a positive relationship was found between increasing biochar application rate (0.05-10% w/w) of the fertilized treatments and BNF from the atmosphere as evidenced by the correlation between δ15N and biochar application rates without taking the control into account (R^2= 0.73) (Fig. 5.18). A positive response was again seen in BNF at the higher biochar application rates of 2.5 and 10% (w/w). In each of the fertilized and unfertilized treatments, this corresponded to the beans with the highest degree of nodulation (Fig. 5.18). The 2.5 had the lowest δ15N value of 1.7 followed by the 10% which had a δ15N value of 2.5. Clearly the higher biochar rates are associated with the highest BNF (lowest δ15N) in both the fertilized and unfertilized treatments.

Increased biochar additions therefore promoted nodulation and also BNF only at biochar application rates above 0.5% in both trials, whilst lower biochar application rates (0.05- 0.5 %) were associated with the smallest amount of N deriving from the atmosphere. Rondon et al. (2007) also found that biochar additions significantly increased BNF of common beans except that they also showed significant increases in BNF at the lower biochar amendment rates. Nishio and Okano (1991) also revealed increases in BNF for alfalfa *(Medicago sativa* L.*)* with biochar additions.

The increase in BNF at the higher biochar application rates identified previously can be related to several factors which include the increase in pH, C/N ratio caused by the biochar additions, increase in nutritional status of the soils and the effect it had on the N availability and cycling. Soil acidity (pH< 5.5-6) associated with Al^{3+}, Mn^{2+} toxicity accompanied by low amounts of Ca^{2+} and $H_2PO_4^-$ can inhibit rhizobial infection (Havlin et al. 2005). Liming of acidic soils has been shown to increase BNF (Giller, 2001) and the dependency of alfalfa growth on *Rhizobium melitoti* (Havlin et al. 2005) Lime-pelleting of inoculated seeds has widely been used in acid soils, to improve the degree of nodulation (Ruschel et al. 1970; Morales et al. 1973). However, BNF at the biochar application rates of 0.05 and 0.5% caused significant increases in the soil pH (Table 5.10), which did not result in increased BNF of these treatments compared to the control. The degree of nodulation only increased when the pH of the soil increased to above 7. Further increases in pH led to an increase in BNF compared to the lower biochar additions, but did not cause a significant difference in BNF compared to the control. The increase in pH therefore contributed to a lesser extent to BNF.

The addition of biochar caused a significant increase in the C/N ratio of both the fertilized and unfertilized treatments. It has been shown that a C/N ratio greater than 30:1 favor immobilization of N and less than 20:1 favor mineralization (Havlin et al. 2005; Plaster, 2009). The addition of biochar with a wide C/N ratio of 156:1 possibly resulted in the net immobilization of mineral N. The addition of 0.5% (w/w) biochar to the soil increased the C/N ratio to above 30:1 and at 10% (w/w) biochar application rate the C/N ratio of the fertilized and unfertilized treatments had an average of 98:1 and 125:1 respectively, which could have induced severe immobilization of the mineral N.

The total plant N of the fertilized treatments decreased from 1.74 $mmol.g^{-1}$ dw in the control to 1.44 $mmol.g^{-1}$ dw at the 10 % (w/w) biochar application rate and the same trend was seen in the N content of the plants in the unfertilized treatments (Table 5.11) which revealed significantly lower amounts of plant N in the 0.5 -10% (w/w) biochar application treatments. The chlorophyll content index and foliar N also decreased with increasing biochar application

rate (data not shown). Furthermore, in both the unfertilised and fertilised treatments the decrease in mineral NO_3^- content between day 24 and 39 was the smallest at the 2.5 and 10% (w/w) biochar application rates and significantly less compared to the decrease seen in the control pots and 0.05-0.5% (w/w) biochar application rates. The microbial biomass increased considerably in the latter between day 24 and 39 (Fig. 5.14) and possibly contributed to the decrease of mineral NO_3^{-1} as it can compete effectively with plants for NO_3^- (Havlin et al. 2005). Plant uptake of NO_3^- was also the highest at the lower biochar application rates of both the unfertilized and fertilized treatments resulting in significantly higher plant N contents (Table 5.11) in the pots that received no biochar additions.

This is a clear indication of greater N availibility in the soils that received no biochar additions despite mineral NO_3^- content being the highest at the 2.5 and 10% (w/w) biochar application rates of the fertilized treatments at day 39 (Fig. 5.11 and 5.12) compared to the other treatments. Regardless of this, the BNF and degree of nodulation was also the greatest at the higher biochar application rates. Nitrates have been shown to block nodulation at various stages as legumes will preferably utilize NO_3^- as an N source, before forming N-fixing symbiosis (Carrol and Mathews, 1990; Forde and Lorenzo, 2001). We postulate that since the mineral NO_3^- content of the fertilized treatments was the highest at the 10% (w/w) biochar application rate, but still caused a decrease in the uptake of N by the green beans, that biochar absorbs large fractions of the fertilizer added, into its microporous structure. The capillary forces of the micropores draws a lot of the soil solution into these microvoids, essentially removing the nutrients from the soil solution and then serving as a physical barrier between the plant roots and nutrients trapped inside these microvoids. As a result less N was available to the plants and initiated the formation of symbiosis between the legume and *Rhizobium leguminosarum* and resulted into higher BNF in these plants.

Even though biochar promoted nodulation and BNF at the 2.5 and 10% (w/w) biochar application rates, it did not result in higher plant N contents, excepts in the fertilized pots were the 10% (w/w) biochar application rate had a higher plant N content compared to the 2.5% (w/w) biochar application rate. This might indicate low activity within the nodules, possibly due to the complete removal of mycorrhizae, with the addition of the fungicide, Xanbac D to prevent infection of the common green bean by *Pythium* sp. The complete absence of mycorrhizae was confirmed with microscopic inspection of the roots. Mycorrhizae has been shown to improve nodular growth and N_2-fixation (Mortimer et al. 2008) through increased P uptake by the plants, as nodules has a high demand for P (Vadez et al. 1997).

Conclusions made in pot trials

Pot trial 1: Layered pot trial with wheat (Triticum aestivum)

The biochar layers served as local hot spots for root and microbial proliferation and were able to significantly promote root growth and increase total microbial biomass C. This can be attributed to biochar‘s ability to retain soil moisture and nutrients which provided the plant with readily available nutrients over longer period of time from these zones. The inherited microbial population were able to adapt to biochar as a new carbon source and use these layers as refuge because of the constant and more favourable conditions created by the biochar. However, a decrease (though not significant) in mycorrhizal colonisation was seen within the biochar layers which can mainly be attributed to changes in the local nutrient balances caused by biochar and the higher more alkaline pH within these zones compared to the acidic soil from which the mycorrhizae were inherited. However, due to the mycorrhizal data having a large standard deviation as it was only conducted in triplicate, one should be very carefull in making strong statements and conclusions regarding this.

Pot trial 2: Different biochar amendment rates with wheat (Triticum aestivum)

The wheat dry weight data followed a polynomial trend (R^2= 0.98) and it was clear that there is an optimum amendment rate for biochar at which yield will be maximized and after which decreases will occur. The increase in dry weight in the unfertilized and fertilised pots can be attributed to several factors which include soil moisture, increases in the bioavailibity of key nutrients, alkalisation of soil pH, increases in CEC of the soil and an increased carbon content (Atkinson et al. 2010; Glaser et al. 2002). Mycorrhizal root colonization decreased with increasing biochar levels due to changes in the soil physico-chemical properties, changes in bioavailability of nutrients which causes shifts in nutrient balances of the soil and possible interference in the signalling dynamics of the soil.

Pot trial 3: Different biochar amendment rates with the common green bean (Phaseolus vulgaris)

From the present study it was clear that biochar did improve the nutritional status of the soil mainly in terms of the increase in the exchangeable cations. This resulted in an increased uptake of most of the nutrients and led to an increase in the total biomass production of the common green bean plants. An optimal biochar amendment rate of between 0.05-0.5% (w/w) biochar application rates was identified in both the fertilized and unfertilized treatments. The elemental analysis of the plants from the 0.5% biochar application rate indicated Ca to be

significantly or considerably higher compared to the control and other treatments which as mentioned before improves the physiological, biochemical and yield performance of crops as it is a very important structural unit of membranes and cell walls. Higher levels of biochar additions resulted in decreased uptake of various elements which include N, Mg, Mn, Fe, Zn and B which contributed to the decrease in total biomass production.

The determination of microbial biomass during the trial and microbial respiration for 45 days after the trial produced contrasting results. Total microbial biomass C at the end of the trial showed no significant difference between the different biochar amended soils which received fertilizer. The unfertilized biochar amended treatments however, showed a significant decrease in C_{TMB} at the 0.5% (w/w), which is associated with the highest plant biomass production. From the trial, we concluded that the low N content of the agricultural soil used, together with a widening of the C/N ratio, resulted in much higher competition for N in a soil already associated with low amounts of N. As a result the C_{TMB} decreased at the higher C/N ratios compared to the control as microbes could not easily take C and N up in a C/N ratio of 8:1. The addition of fertilizer alleviated N as the limiting factor and together with the significant increase in the available C promoted the initial increase in C_{TMB} at day 24.

The average CO_2 production in both the fertilized and unfertilized treatments increased linearly (R^2= 0.80; 0.79, respectively) with increasing biochar application rate and is possibly related to the increase in the labile C pool of the soil related to the distinct labile C pool of the biochar.

The effect of the wide C/N ratio on the uptake of N was clearly evident in the lower N content of the plants associated with higher biochar application rates. As the average mineral NO_3^-, extracted from the fertilized 10% (w/w) biochar amended treatments, was significantly higher at day 39 compared to the control and other treatments, it was clear that immobilization via microbes as a result of the wide C/N ratio did not contribute considerably to the removal of NO_3^- nor did plant uptake. We postulate that biochar acts as a ―sponge", sucking up a lot of the soil solution into its microvoids, effectively removing it from the soil and then acting as a physical barrier between plant roots and the mineral N aborbed.

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CHAPTER 6: GENERAL CONCLUSIONS

Basidiomycetes such as white- and brown rot fungi have been shown to solubilise coal through the catalytic activity of H_2O_2 and in this trial, hydrogen peroxide was proven to be able to oxidize biochar through hydroxylation and carboxylation reactions to first form phenol which is then oxidized to intermediary carboxylic acids and eventually CO_2. Hydrogen peroxide were able to degrade ±90% of the biochar at a relatively constant rate before the refractory fraction of biochar left over decreased the rate at which oxidation occurred. FT-IR done on the biochar oxidized with 0.333 M H_2O_2 revealed refractory peaks (and new peaks) in the range of 1600-1000 cm^{-1}. These include FT-IR peaks at 1440-1460 cm^{-1}, 1380 cm^{-1}, 1270 cm^{-1} and 1070-1040 cm^{-1} and also at 1580 cm^{-1}. The FT-IR peak assignments are mostly associated with vibrations from transformed lignin and cellulose products (guaiacyl, syringyl and methoxy groups) and aromatic C=C vibrations (1580 cm^{-1}) formed from the components of lignin and cellulose during pyrolysis. Therefore, we hypothesize that the more recalcitrant compounds (lignin etc.) in the pine wood used for the production of this biochar has not fully been transformed and also forms the backbone of the recalcitrant fraction in the biochar.

This is the first study (to our knowledge) showing biochar can almost completely be degraded by H_2O_2 and describing the changes occurring during the degradation. Chemical oxidation using 0.333 M hydrogen peroxide (H_2O_2) successfully degraded 92% of the biochar. About 8% of the biochar (3% ash content) showed a high degree of resistance against oxidation.

The main reactions between H_2O_2 with the biochar‘s surface is considered to be hydroxylation and carboxylation reactions, which explains the increase in the intensity of the FT-IR peaks associated with C=O vibrations (1730 cm^{-1}) and phenolic acid and COOH vibrations (1270 cm^{-1}). Also the reaction between biochar and H_2O_2 led to the formation of various carboxylic acids of which oxalic and maleic was the most prominent at high H_2O_2 concentrations. The clear linear correlation between maleic acid and the concentration of H_2O_2 clearly shows that maleic acid can be considered as the primary product formed from ring cleavage of the aromatics in biochar. Also, the oxalic acid are clearly the most refractory carboxylic acid possibly as a result of the formation of Fe^{2+}/Fe^{3+} complexes which resulted in the accumulation with progressive oxidation. This led to the decrease seen in the peak intensity at 1580 cm^{-1} and in the elemental C content of the biochar. The FT-IR peaks at

2850-2950 cm^{-1} also increased in intensity due to the formation of benzoic acid and p-benzoquinone on the surface of the biochar.

The changes in surface chemistry with progressive oxidation clearly showed a decrease in pH and surface basicity, whilst the surface got saturated with acidic functional groups mainly as a result of extensive hydroxylation and carboxylation reactions. The maximum surface acidity that can be attained on the pine wood biochar was shown to be at ±50 mmol. g^{-1}. The increase in surface acidic O-containing functional group was also confirmed by the increase in elemental oxygen of the biochar.

The aerobic digestion trial of biochar in incubation columns using a wood rotting consortium confirmed that the biochar was unable to activate the microbial consortium and the microbial consortium could not utilize biochar as a sole carbon source in the absence of nutritional inputs or glucose. Abiotic oxidation with the atmospheric oxygen did not contribute or facilitate the oxidation of biochar. However the addition of birnessite strongly promote the solubilisation of biochar and produced increased amounts of leached solubilites. Therefore, when biochar degradation models are developed one should consider the oxidative potential (oxidative soil minerals) of the soils, nutrient status and the composition and activity of the microbial population of the soil system.

All the studies so far focused on the in-situ degradation of biochar. We have attempted to analyze in-vitro the decomposition of biochar in strictly anaerobic conditions dominated by methanogenic consortium. Anaerobic digestion of biochar was found to be quite comprehensive compared to aerobic digestion, even though COD removal efficiencies of 30% are low compared to removal efficiencies of other organics. The low COD removal efficiencies are ascribed to the recalcitrant nature of biochar against hydrolytic depolymerisation, which is the rate limiting step in the COD removal efficiencies attained. The acido- and acetogens were not inhibited by the organic substance provided at low or higher COD inputs or by fluctuations in pH and alkalinity. However COD removal efficiencies were the highest in treatments with starting pHs equal and above 7. Methane production was the greatest at a starting pH= 7, however due to weak control over the system pH, alkalinity and the biochar possibly containing inhibiting compounds (phenols, benzene and toluene) the production of methane was erratic and mostly inhibited, However, when methane production did occur the gas produced consisted of 70% methane and 26% CO_2. Higher COD removal efficiencies will be attained with better control over pH and alkalinity and also a longer HRT.

The *in-situ* - in field incubation of biochar under three different environmental conditions: pasture, fynbos and wetland in close proximity to each other for ten months. These sites were characterized by differences in soil physico-chemical characteristics (texture, soil water regimes, C and N content), climatic conditions, biological activity (C and N mineralization rates) and vegetation cover. The degree of oxidation under each environmental extreme was assessed and it was found that the pasture-incubated biochar underwent the greatest degree of oxidation as it showed the highest increase in elemental oxygen and in acid surface functional groups as confirmed by the increase in the FT-IR peak at 1700 cm^{-1}. Dissolved organic carbon leached from the pasture incubated biochar was significantly higher compared to the other incubated biochar. The pasture had the highest microbial biomass C and the smallest C/N ratio. It was clear that significant differences in the rate of oxidation can occur between different geographic locations with different soil physico-chemical characteristics, climatic conditions, biological activity and vegetation cover. We concluded that the difference in microbial activity (C and N mineralization) between the incubation sites contributed the most to the differences seen in biochar‘s oxidative status.

Biochar alone did cause considerable increases in total plant biomass production at the 0.5-2.5% in both the unfertilized wheat and bean trials. However, it was clear that biochar alone do not serve as a fertilizer and that the biochar amendment should be applied together with fertilizers. Wheat and bean pot trials both confirmed the optimal rate of biochar application in the fertilized treatments to be between 0.05-0.5 % (w/w) which corresponds to 1-10 tons per ha up to 15 cm soil depth. Biochar at the correct application rates considerably increased the efficiency of fertilization by considerably promoting plant biomass production compared to the control. The positive effect of biochar was mainly ascribed to the liming ability of biochar which resulted in increases in the exchangeable Ca, Mg, Na and K of the soils and also increased the pH of the soils. The highest Ca uptake in the bean trial related to the highest yields in the green bean trial.

Biochar application rates of 10% (w/w) caused significant decreases in total biomass production in the wheat trial and also resulted in considerable decreases in the total biomass production in the bean trials. Over liming of the soil by biochar resulted in a significantly higher pH which affected the bioavailability of key plant nutrients. In the bean trial a decrease in the uptake of various elements which include N, Mg, Mn, Fe, Zn and B occurred which contributed to the decrease in total biomass production. Especially N uptake significantly decreased with increasing biochar application as a result of the wide C/N ratio which immobilized N. We hypothesized that biochar physically immobilized N into its

microvoids through capillary suction and then served as a physical barrier between plant roots and absorbed N. However, immobilization of N by microbes could also have contributed to the decrease in N uptake if one takes into account that microbial activity was higher (respiration data) at the higher biochar application rates. Further investigations are however needed to warrant this hypothesizes.

Mycorrhizal colonization of roots decreased with increasing biochar application rates and within the biochar layers in the layered pot trial. In fertilized and unfertilized trials the treatments which received no biochar had the highest number of mycorrhizae. The main difference between treatments is the changes that biochar caused in soil chemistry. The increase in soil pH, EC and changes in the soil nutrient balance (especially C/N ratio) could have had a negative impact on mycorrhizae. Furthermore, it is speculated that biochar could interfere with the signalling dynamics of the rhizosphere as it has a redox activity, serving as a reducing agent and has the ability to adsorb to organic compounds through H-bonding, ligand exchange reactions, direct electrostatic reactions and cation bridging.

In pot trial 3 (bean), a positive correlation (R^2= 0.73) was found between biological nitrogen fixation (BNF) and increasing biochar application rates from the fertilized treatments. In the unfertilized and fertilized pot trials, BNF was significantly enhanced at the 2.5-10% and 10% (w/w) biochar application rates respectively. This trend was ascribed to the liming effects which increased soil pH and a widening of the C/N ratio of the soils which resulted in the immobilization and possible physical removal of N into the microvoids of biochar as previously mentioned.

Biochar did cause a significant increase in total C_{TMB} in the fertilized pots after 24 days due to the higher amounts of available C at the higher biochar application rates and the addition of the basal fertilizer dosage. However, no significant differences existed between treatments after 39 days. In contrast in the unfertilized treatments a progressive decrease in C_{TMB} was seen with increasing biochar applications rates, with a significant decrease occurring at the biochar application rates above 0.5% (w/w) compared to the control. This decrease was ascribed to the wide C/N ratio of these pots and low N availability. As a result the C_{TMB} decreased at the greater C/N ratios compared to the control as microbes could not easily take up C and N in a C/N ratio of 8:1. The addition of fertilizer alleviated the effect of a wide C/N ratio and along with an increased in the available C, promoted the increases seen in C_{TMB} at day 24. This also resulted that biochar layers formed preferred zones for root growth and microbial communities as it retained more of the fertilizer and had large amounts of available carbon.

The average CO_2 production in pots without plants in both the fertilized and unfertilized treatments increased linearly (R^2= 0.80; 0.79 respectively) with increasing biochar application rates. The microbial respiration was significantly more at the 2.5 and 10% (w/w) biochar application rates compared to the control in both the unfertilized and fertilized treatments. Due to the strong correlation between microbial respiration and biochar application rate the increase in microbial respiration may be directly related to the increased availability of labile C with increased rates of biochar application, which resulted in the strong linear relationship between biochar application rate and microbial respiration. Furthermore, the fertilizer application resulted in a higher microbial activity compared to the unfertilized treatments.

APPENDICES

Appendix 1: In vitro oxidation of biochar using hydrogen peroxide

Appendix 1.1: Total oxidation of biochar with 0.333 M hydrogen peroxide

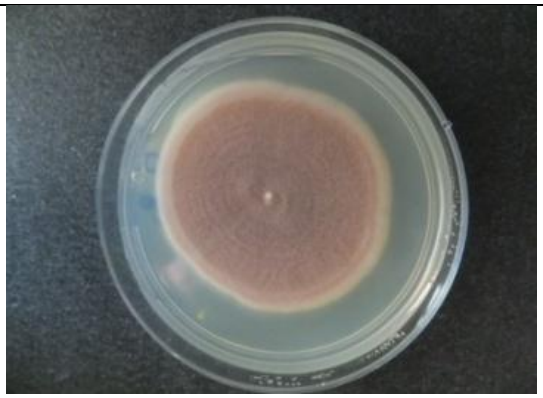
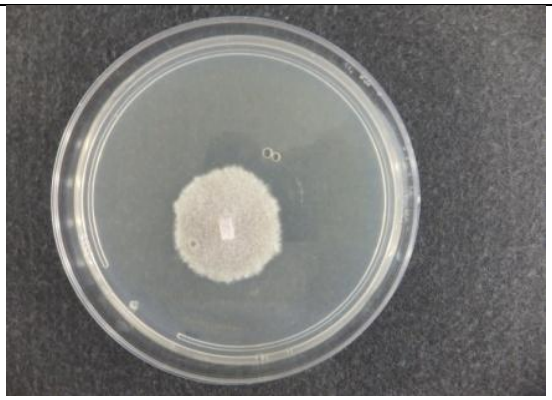
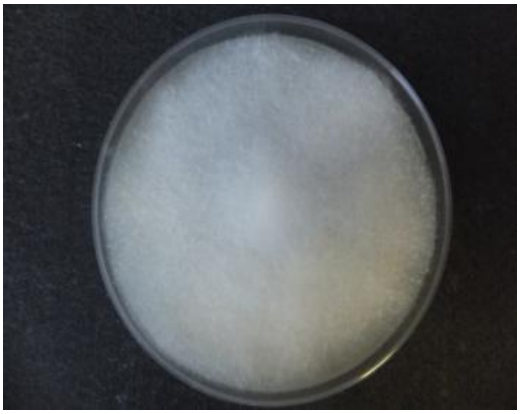
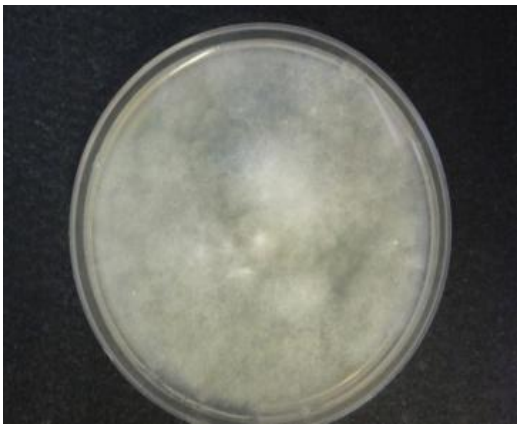
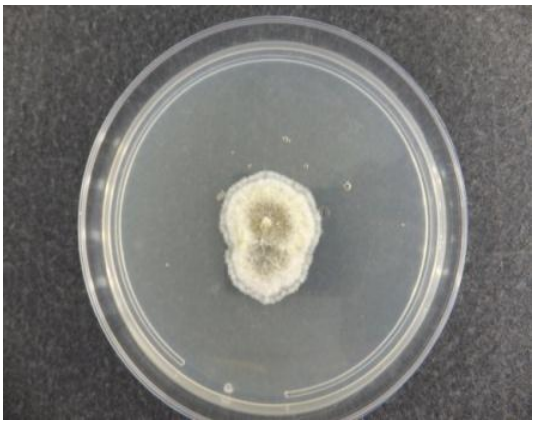
Total H_2O_2 added	Weight loss (g)			Average weight loss (g)	Standard deviation	Total weight loss (g)	Substrate/ Volume H_2O_2 ratio
	Sample A	Sample B	Sample C				
0	0	0	0	0	0	0.000	0.000
50	-0.422	-0.46	-0.444	-0.442	0.019	-0.442	0.200
100	0.178	0.219	0.041	0.146	0.093	-0.296	0.209
150	0.478	0.474	0.522	0.491	0.027	0.195	0.206
200	0.483	0.445	0.467	0.465	0.019	0.660	0.196
250	0.529	0.519	0.522	0.523	0.005	1.184	0.187
300	0.304	0.302	0.287	0.298	0.009	1.481	0.176
350	0.567	0.597	0.572	0.579	0.016	2.060	0.170
400	0.774	0.744	0.749	0.756	0.016	2.816	0.159
450	0.797	0.84	0.829	0.822	0.022	3.638	0.144
500	0.887	0.89	0.88	0.886	0.005	4.523	0.127
550	0.333	0.551	0.476	0.453	0.111	4.977	0.110
600	0.708	0.365	0.515	0.529	0.172	5.506	0.100
650	0.558	0.586	0.564	0.569	0.015	6.075	0.090
700	1.131	1.167	1.08	1.126	0.044	7.201	0.078
750	1.248	1.301	1.277	1.275	0.027	8.477	0.056
800	0.419	0.427	0.526	0.457	0.060	8.934	0.030
850	0.133	0.132	0.165	0.143	0.019	9.077	0.021
900	0.051	0.028	0.046	0.042	0.012	9.119	0.018
950	0.08	0.077	0.078	0.078	0.002	9.197	0.018
1000	0.02	0.009	0.012	0.014	0.006	9.211	0.016

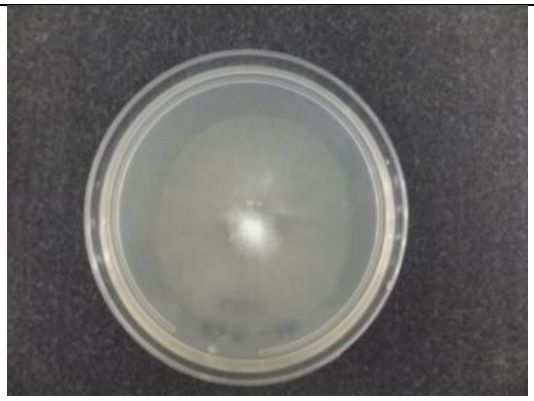
Appendix 1.2: Basic FT-IR peak assignments from biochar (Keiluweit et al. 2010)

3400	phenolic -OH stretching
2850-2950	Aliphatic CH stretching vibration
1690-1730	Aromatic carbonyl/carboxylic C=O stretching
1570-1580	Aromatic C=C ring stretching
1460	Aromatic C=C ring stretching
1380	In plane bending of phenolic -OH related to ligneous syringyl units
1270	Aromatic CO- stretching (Phenolic CO- indicative of guaiacyl units in lignin)
1030	Aliphatic C-O-C and -OH stretching/ Methoxy groups of lignin
870-750	Aromatic CH out of plane deformations/ less substituted rings

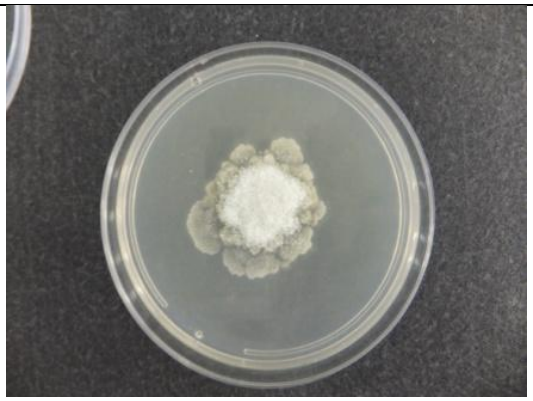
Appendix 2: In vitro aerobic digestion of biochar in sand columns

Appendix 2.1: Illustrations of fungi and yeast collected before and after the incubation period

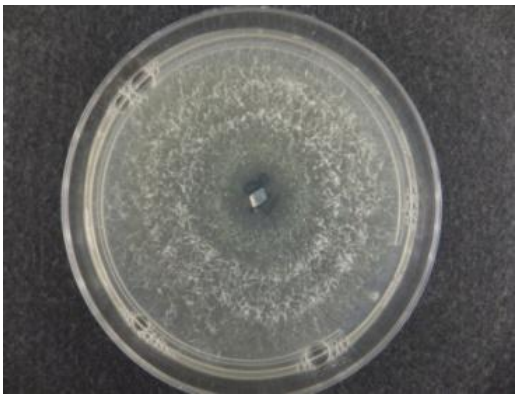
Fungi collected from the inoculant	Fungi collected after incubation
 *Umbelopsis isabellina*	 *Fusarium oxysporum*
 *Mortierella sp.*	 *Fusarium oxysporum*
 *Mortierella sp.*	 *Fusarium equiseti*



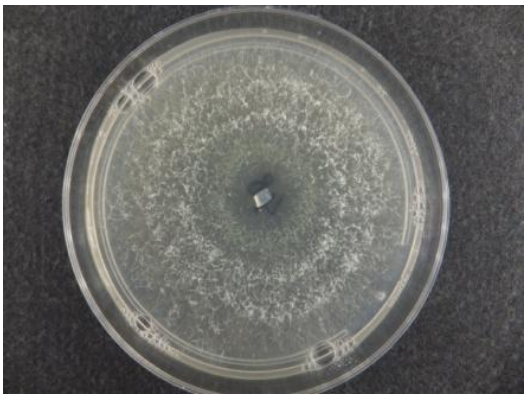
Mortierella sp.



Unidentified species



Unidentified species

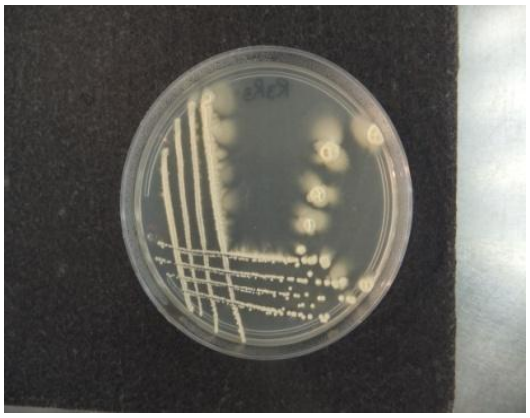


Unidentified species

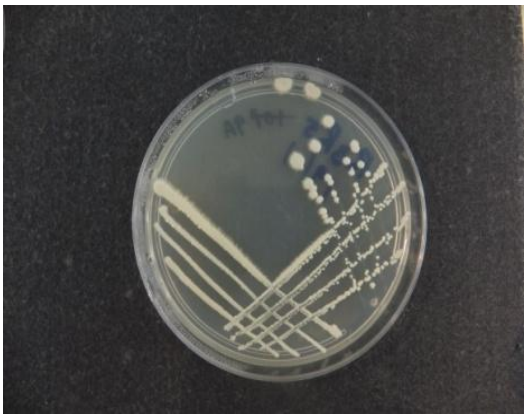
Yeast collected from inoculant

Candida sp.

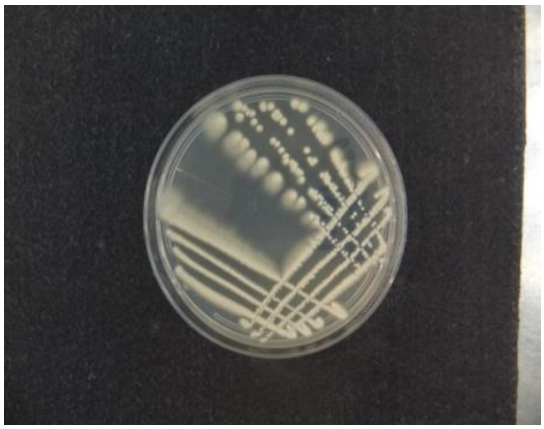
Rhodotorula sp.



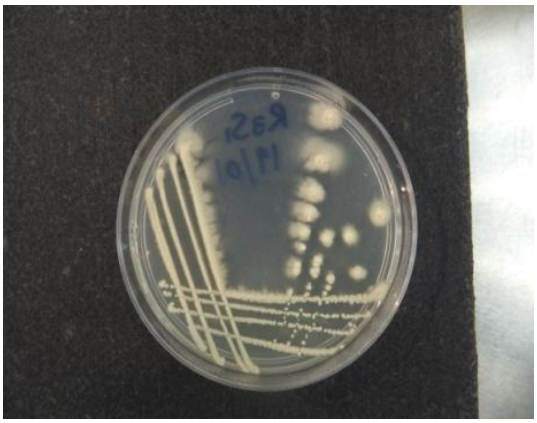
Trichosporon porosum



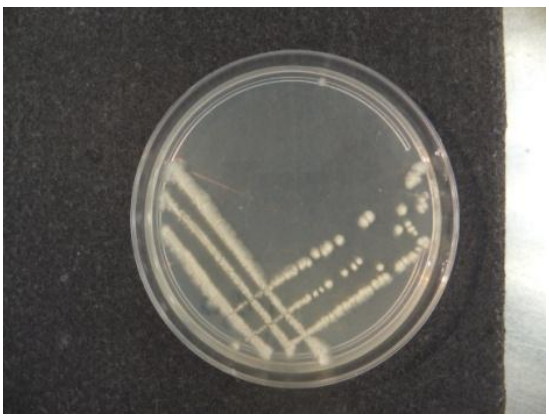
Pichia guilliermondii



Unidentified species



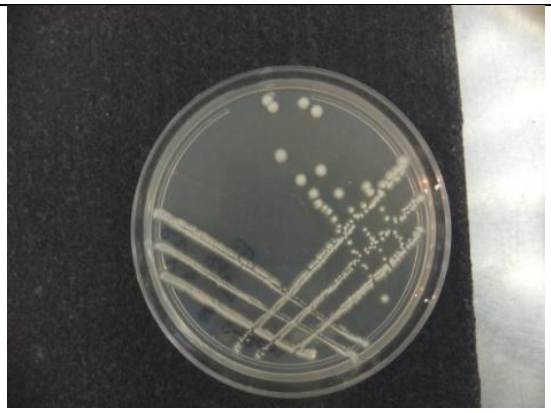
Unidentified species



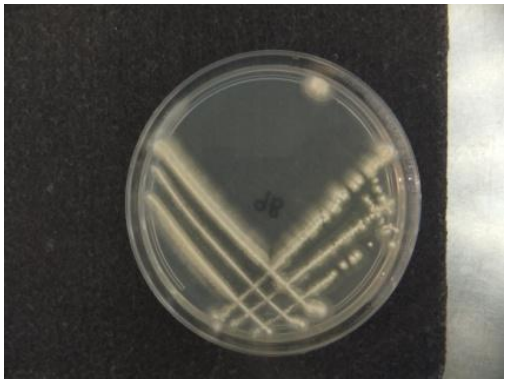
Unidentified species

Yeast collected after incubation

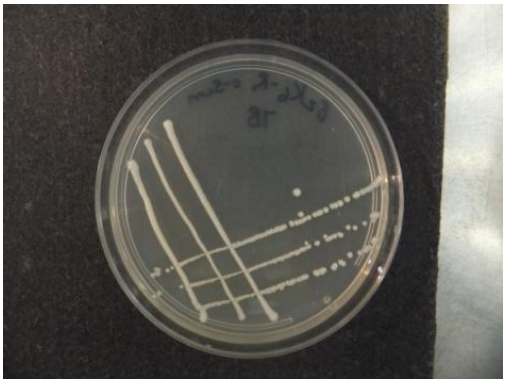
Pichia guilliermondii



Trichosporon porosum



Unidentified species



Unidentified species

Appendix 2.2: Composition of the inoculums microbial consortia

Color code	COLONY MORPHOLOGY			Counts CFU/ml	Genus/species associated with colony
	Form	Elevation	Margins		
Blue	Filamentous	Flat	Eroded/ filamentous	230	*Mortierella sp.* *Umbelopsis isabellina.* *1 Unidentified sp.*
Red	Circular	Convex	Entire	460	*Trichosporon porosum* *Rhodotorula sp.*
Black	Punctiform	Flat	Entire	2510	*Pichia guilliermondii* *Candida sp.* *3 Unidentified sp.*

Composition of the microbial consortium collected from the columns after incubation

Treatment	Color code	COLONY MORPHOLOGY			Counts CFU/g	Genus/species associated with colony
		Form	Elevation	Margins		
Treatment 4	Blue	Filamentous	Flat	Filamentous	13	*Fusarium oxysporum. Fusarium equiseti*
	Orange	Filamentous	Flat	Eroded	3	*2 unidentified species*
	Red	Circular	Convex	Entire	63	*Trichosporon porosum 1 Unidentified species*
	Black	Punctiform	Flat	Entire	13	*Pichia guilliermondii 1 Unidentified species*
Treatment 5	Blue	Filamentous	Flat	Filamentous	9	*Fusarium oxysporum Fusarium equiseti*
	Orange	Filamentous	Flat	Eroded	5	*2 Unidentified species*
	Red	Circular	Convex	Entire	10	*Trichosporon porosum*

Appendix 3: Anaerobic digestion of biochar by methanogenic consortia

Appendix 3.1: LSD test to compare average COD reduction of treatments at each of the 12 sampling events across all the periods

LSD test; variable DV_1 (ANAEROBIC DATA 20110825)
Probabilities for Post Hoc Tests
Error: Between; Within; Pooled MS = 51229., df = 53.226

Cell No.	pH#	TIME	{1} 19.533	{2} 376.90	{3} 21.600	{4} 102.30	{5} 283.80	{6} 245.40	{7} 510.53	{8} 32.200	{9} 315.00	{10} 150.83	{11} 698.40	{12} 346.80	{13} 95.233	{14} 303.70	{15} 339.30	{16} 299.70	{17} 711.60	{18} 198.07	{19} 802.80	{20} 164.20	{21} 967.80	{22} 718.20	{23} 818.40	{24} 439.20	{25} 23.450	{26} 552.10	{27} 278.10	{28} 353.70	{29} 847.80	{30} 127.80	{31} 721.20	{32} 438.43	{33} 503.40	{34} 479.40	{35} 540.00	{36} 1129.2
1	pH1	COD REDUCTION1		0.037	0.990	0.623	0.119	0.182	0.005	0.940	0.082	0.436	0.000	0.055	0.684	0.130	0.089	0.135	0.000	0.338	0.000	0.437	0.000	0.000	0.000	0.027	0.983	0.006	0.168	0.076	0.000	0.560	0.000	0.027	0.011	0.016	0.007	0.000
2	pH1	COD REDUCTION2	0.037		0.038	0.106	0.580	0.435	0.428	0.043	0.713	0.182	0.059	0.858	0.133	0.694	0.840	0.678	0.076	0.338	0.025	0.255	0.002	0.070	0.020	0.737	0.061	0.347	0.595	0.901	0.014	0.183	0.068	0.740	0.497	0.581	0.381	0.000
3	pH1	COD REDUCTION3	0.990	0.038		0.631	0.122	0.186	0.005	0.950	0.084	0.443	0.000	0.056	0.692	0.133	0.091	0.138	0.000	0.344	0.000	0.444	0.000	0.000	0.000	0.028	0.992	0.006	0.171	0.078	0.000	0.568	0.000	0.028	0.012	0.016	0.007	0.000
4	pH1	COD REDUCTION4	0.623	0.106	0.631		0.282	0.396	0.017	0.677	0.208	0.773	0.001	0.149	0.970	0.281	0.205	0.290	0.002	0.606	0.000	0.739	0.000	0.002	0.000	0.074	0.671	0.018	0.346	0.179	0.000	0.891	0.001	0.075	0.034	0.046	0.022	0.000
5	pH1	COD REDUCTION5	0.119	0.580	0.122	0.282		0.819	0.180	0.138	0.853	0.430	0.016	0.708	0.312	0.915	0.765	0.932	0.025	0.645	0.007	0.520	0.001	0.022	0.006	0.404	0.165	0.152	0.976	0.707	0.004	0.402	0.022	0.406	0.240	0.295	0.171	0.000
6	pH1	COD REDUCTION6	0.182	0.435	0.186	0.396	0.819		0.118	0.207	0.679	0.574	0.009	0.547	0.420	0.754	0.613	0.770	0.015	0.799	0.004	0.662	0.000	0.013	0.003	0.299	0.235	0.103	0.860	0.560	0.002	0.527	0.013	0.301	0.168	0.211	0.117	0.000
7	pH1	COD REDUCTION7	0.005	0.428	0.005	0.017	0.180	0.118		0.006	0.247	0.035	0.266	0.332	0.029	0.268	0.358	0.259	0.281	0.097	0.120	0.066	0.017	0.266	0.102	0.701	0.011	0.823	0.214	0.400	0.074	0.043	0.259	0.698	0.969	0.867	0.874	0.002
8	pH1	COD REDUCTION8	0.940	0.043	0.950	0.677	0.138	0.207	0.006		0.096	0.481	0.000	0.065	0.734	0.148	0.102	0.154	0.001	0.373	0.000	0.478	0.000	0.000	0.000	0.032	0.962	0.007	0.189	0.088	0.000	0.607	0.000	0.032	0.014	0.019	0.008	0.000
9	pH1	COD REDUCTION9	0.082	0.713	0.084	0.208	0.853	0.679	0.247	0.096		0.330	0.025	0.850	0.240	0.951	0.896	0.934	0.036	0.530	0.011	0.418	0.001	0.034	0.009	0.504	0.121	0.205	0.842	0.835	0.006	0.316	0.032	0.507	0.313	0.378	0.229	0.000
10	pH1	COD REDUCTION10	0.436	0.182	0.443	0.773	0.430	0.574	0.035	0.481	0.330		0.002	0.246	0.765	0.412	0.312	0.424	0.004	0.799	0.001	0.943	0.000	0.003	0.001	0.125	0.494	0.034	0.494	0.277	0.000	0.901	0.003	0.126	0.062	0.081	0.040	0.000
11	pH1	COD REDUCTION11	0.000	0.059	0.000	0.001	0.016	0.009	0.266	0.000	0.025	0.002		0.040	0.002	0.037	0.057	0.036	0.943	0.009	0.574	0.006	0.151	0.915	0.519	0.167	0.001	0.432	0.027	0.068	0.422	0.003	0.902	0.165	0.296	0.241	0.395	0.024
12	pH1	COD REDUCTION12	0.055	0.858	0.056	0.149	0.708	0.547	0.332	0.065	0.850	0.246	0.040		0.179	0.816	0.968	0.800	0.054	0.425	0.017	0.328	0.001	0.050	0.014	0.619	0.086	0.272	0.712	0.970	0.009	0.241	0.048	0.622	0.401	0.476	0.301	0.000
13	pH2	COD REDUCTION1	0.684	0.133	0.692	0.970	0.312	0.420	0.029	0.734	0.240	0.765	0.002	0.179		0.218	0.150	0.226	0.000	0.541	0.000	0.682	0.000	0.000	0.000	0.044	0.699	0.017	0.327	0.168	0.000	0.861	0.001	0.069	0.032	0.042	0.020	0.000
14	pH2	COD REDUCTION2	0.130	0.694	0.133	0.281	0.915	0.754	0.268	0.148	0.951	0.412	0.037	0.816	0.218		0.832	0.981	0.018	0.530	0.004	0.408	0.000	0.016	0.003	0.421	0.135	0.185	0.890	0.788	0.005	0.345	0.028	0.469	0.285	0.346	0.207	0.000
15	pH2	COD REDUCTION3	0.089	0.840	0.091	0.205	0.765	0.613	0.358	0.102	0.896	0.312	0.057	0.968	0.150	0.832		0.814	0.030	0.402	0.007	0.299	0.000	0.027	0.006	0.553	0.093	0.255	0.742	0.938	0.008	0.258	0.044	0.594	0.379	0.452	0.282	0.000
16	pH2	COD REDUCTION4	0.135	0.678	0.138	0.290	0.932	0.770	0.259	0.154	0.934	0.424	0.036	0.800	0.226	0.981	0.814		0.017	0.546	0.004	0.421	0.000	0.015	0.003	0.408	0.141	0.178	0.907	0.771	0.005	0.356	0.027	0.456	0.275	0.335	0.199	0.000
17	pH2	COD REDUCTION5	0.000	0.076	0.000	0.002	0.025	0.015	0.281	0.001	0.036	0.004	0.943	0.054	0.000	0.018	0.030	0.017		0.003	0.588	0.002	0.131	0.969	0.526	0.109	0.000	0.392	0.023	0.058	0.464	0.003	0.959	0.145	0.265	0.214	0.357	0.028
18	pH2	COD REDUCTION6	0.338	0.338	0.344	0.606	0.645	0.799	0.097	0.373	0.530	0.799	0.009	0.425	0.541	0.530	0.402	0.546	0.003		0.001	0.840	0.000	0.003	0.000	0.155	0.349	0.061	0.667	0.403	0.001	0.705	0.007	0.199	0.104	0.134	0.070	0.000
19	pH2	COD REDUCTION7	0.000	0.025	0.000	0.000	0.007	0.004	0.120	0.000	0.011	0.001	0.574	0.017	0.000	0.004	0.007	0.004	0.588	0.001		0.000	0.328	0.615	0.926	0.033	0.000	0.181	0.006	0.018	0.809	0.001	0.661	0.054	0.111	0.086	0.161	0.083
20	pH2	COD REDUCTION8	0.437	0.255	0.444	0.739	0.520	0.662	0.066	0.478	0.418	0.943	0.006	0.328	0.682	0.408	0.299	0.421	0.002	0.840	0.000		0.000	0.002	0.000	0.105	0.450	0.041	0.540	0.310	0.001	0.845	0.004	0.144	0.072	0.094	0.047	0.000
21	pH2	COD REDUCTION9	0.000	0.002	0.000	0.000	0.001	0.000	0.017	0.000	0.001	0.000	0.151	0.001	0.000	0.000	0.000	0.000	0.131	0.000	0.328	0.000		0.141	0.375	0.002	0.000	0.029	0.000	0.002	0.519	0.000	0.188	0.006	0.015	0.011	0.025	0.386
22	pH2	COD REDUCTION10	0.000	0.070	0.000	0.002	0.022	0.013	0.266	0.000	0.034	0.003	0.915	0.050	0.000	0.016	0.027	0.015	0.969	0.003	0.615	0.002	0.141		0.552	0.100	0.000	0.373	0.021	0.054	0.486	0.002	0.987	0.136	0.250	0.202	0.339	0.030
23	pH2	COD REDUCTION11	0.000	0.020	0.000	0.000	0.006	0.003	0.102	0.000	0.009	0.001	0.519	0.014	0.000	0.003	0.006	0.003	0.526	0.000	0.926	0.000	0.375	0.552		0.027	0.000	0.155	0.005	0.015	0.874	0.000	0.601	0.045	0.094	0.072	0.138	0.098
24	pH2	COD REDUCTION12	0.027	0.737	0.028	0.074	0.404	0.299	0.701	0.032	0.504	0.125	0.167	0.619	0.044	0.421	0.553	0.408	0.109	0.155	0.033	0.105	0.002	0.100	0.027		0.029	0.544	0.387	0.645	0.031	0.098	0.133	0.997	0.730	0.829	0.588	0.000
25	pH3	COD REDUCTION1	0.983	0.061	0.992	0.671	0.165	0.235	0.011	0.962	0.121	0.494	0.001	0.086	0.699	0.135	0.093	0.141	0.000	0.349	0.000	0.450	0.000	0.000	0.000	0.029		0.002	0.133	0.053	0.000	0.535	0.000	0.016	0.006	0.008	0.003	0.000
26	pH3	COD REDUCTION2	0.006	0.347	0.006	0.018	0.152	0.103	0.823	0.007	0.205	0.034	0.432	0.272	0.017	0.185	0.255	0.178	0.392	0.061	0.181	0.041	0.029	0.373	0.155	0.544	0.002		0.107	0.240	0.082	0.014	0.316	0.500	0.772	0.666	0.943	0.001
27	pH3	COD REDUCTION3	0.168	0.595	0.171	0.346	0.976	0.860	0.214	0.189	0.842	0.494	0.027	0.712	0.327	0.890	0.742	0.907	0.023	0.667	0.006	0.540	0.000	0.021	0.005	0.387	0.133	0.107		0.653	0.001	0.373	0.010	0.342	0.183	0.234	0.123	0.000
28	pH3	COD REDUCTION4	0.076	0.901	0.078	0.179	0.707	0.560	0.400	0.088	0.835	0.277	0.068	0.970	0.168	0.788	0.938	0.771	0.058	0.403	0.018	0.310	0.002	0.054	0.015	0.645	0.053	0.240	0.653		0.004	0.182	0.032	0.615	0.375	0.455	0.270	0.000
29	pH3	COD REDUCTION5	0.000	0.014	0.000	0.000	0.004	0.002	0.074	0.000	0.006	0.000	0.422	0.009	0.000	0.005	0.008	0.005	0.464	0.001	0.809	0.001	0.519	0.486	0.874	0.031	0.000	0.082	0.001	0.004		0.000	0.452	0.017	0.044	0.031	0.071	0.098
30	pH3	COD REDUCTION6	0.560	0.183	0.568	0.891	0.402	0.527	0.043	0.607	0.316	0.901	0.003	0.241	0.861	0.345	0.258	0.356	0.003	0.705	0.001	0.845	0.000	0.002	0.000	0.098	0.535	0.014	0.373	0.182	0.000		0.001	0.068	0.028	0.040	0.016	0.000
31	pH3	COD REDUCTION7	0.000	0.068	0.000	0.001	0.022	0.013	0.259	0.000	0.032	0.003	0.902	0.048	0.001	0.028	0.044	0.027	0.959	0.007	0.661	0.004	0.188	0.987	0.601	0.133	0.000	0.316	0.010	0.032	0.452	0.001		0.096	0.198	0.153	0.283	0.018
32	pH3	COD REDUCTION8	0.027	0.740	0.028	0.075	0.406	0.301	0.698	0.032	0.507	0.126	0.165	0.622	0.069	0.469	0.594	0.456	0.145	0.199	0.054	0.144	0.006	0.136	0.045	0.997	0.016	0.500	0.342	0.615	0.017	0.068	0.096		0.699	0.807	0.546	0.000
33	pH3	COD REDUCTION9	0.011	0.497	0.012	0.034	0.240	0.168	0.969	0.014	0.313	0.062	0.296	0.401	0.032	0.285	0.379	0.275	0.265	0.104	0.111	0.072	0.015	0.250	0.094	0.730	0.006	0.772	0.183	0.375	0.044	0.028	0.198	0.699		0.886	0.828	0.000
34	pH3	COD REDUCTION10	0.016	0.581	0.016	0.046	0.295	0.211	0.867	0.019	0.378	0.081	0.241	0.476	0.042	0.346	0.452	0.335	0.214	0.134	0.086	0.094	0.011	0.202	0.072	0.829	0.008	0.666	0.234	0.455	0.031	0.040	0.153	0.807	0.886		0.719	0.000
35	pH3	COD REDUCTION11	0.007	0.381	0.007	0.022	0.171	0.117	0.874	0.008	0.229	0.040	0.395	0.301	0.020	0.207	0.282	0.199	0.357	0.070	0.161	0.047	0.025	0.339	0.138	0.588	0.003	0.943	0.123	0.270	0.071	0.016	0.283	0.546	0.828	0.719		0.001
36	pH3	COD REDUCTION12	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.024	0.000	0.000	0.000	0.000	0.000	0.028	0.000	0.083	0.000	0.386	0.030	0.098	0.000	0.000	0.001	0.000	0.000	0.098	0.000	0.018	0.000	0.000	0.000	0.001	

Appendix 3.2: Repeated measures ANOVA on the means of COD reductions of each treatment over the three periods

Effect	Repeated Measures Analysis of Variance Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degrees of Freedom	MS	F	p
pH#	332658	2	166329	4.3731	0.067361
Error	228206	6	38034		
PERIOD	589797	2	294899	46.7382	0.000002
PERIOD*pH#	45638	4	11410	1.8083	0.192100
Error	75715	12	6310		

Appendix 3.3: Bonferroni comparisons test between the average COD reductions of each treatment during each period

Variable: COD reduction over periods 1-3
Probabilities for Post Hoc Tests
Error: Between; Within; Pooled MS = 16885., df = 10.087

pH#	COD reductions in periods 1-3	1 130.08	2 267.98	3 377.76	4 259.48	5 469.17	6 735.90	7 301.84	8 533.81	9 663.00
pH=5.5	COD REDUCTION1		1.000000	0.088012	1.000000	0.340397	0.006815	1.000000	0.122495	0.018214
pH=5.5	COD REDUCTION2	1.000000		1.000000	1.000000	1.000000	0.046323	1.000000	1.000000	0.140264
pH=5.5	COD REDUCTION3	0.088012	1.000000		1.000000	1.000000	0.250925	1.000000	1.000000	0.813503
pH= 7	COD REDUCTION1	1.000000	1.000000	1.000000		0.258441	0.000321	1.000000	0.971253	0.122891
pH =7	COD REDUCTION2	0.340397	1.000000	1.000000	0.258441		0.051819	1.000000	1.000000	1.000000
pH =7	COD REDUCTION3	0.006815	0.046323	0.250925	0.000321	0.051819		0.076903	1.000000	1.000000
pH =8	COD REDUCTION1	1.000000	1.000000	1.000000	1.000000	1.000000	0.076903		0.136998	0.004395
pH =8	COD REDUCTION2	0.122495	1.000000	1.000000	0.971253	1.000000	1.000000	0.136998		1.000000
pH =8	COD REDUCTION3	0.018214	0.140264	0.813503	0.122891	1.000000	1.000000	0.004395	1.000000	

Appendix 3.4: Newman-Keuls test between the average COD reduction from each treatment during period 2

Probabilities for Post Hoc Tests **Error: Between MS = 10682., df = 6.0000**			
pH#	**Average COD reduction**		
	(pH= 5.5) **267.98**	**(pH= 7)** **469.17**	**(pH= 8)** **533.81**
pH= 5.5		0.054642	0.045359
pH= 7	0.054642		0.472930
pH= 8	0.045359	0.472930	

Appendix 3.5: Repeated measures ANOVA on the means of COD removal efficiencies of each treatment over the three periods

Effect	**Repeated Measures Analysis of Variance** **Sigma-restricted parameterization** **Effective hypothesis decomposition**				
	SS	**Degrees of Freedom**	**MS**	**F**	**p**
pH#	1294.99	2	647.50	3.8449	0.084189
Error	1010.42	6	168.40		
PERIOD	68.25	2	34.12	0.9138	0.427186
PERIOD*pH#	69.50	4	17.38	0.4653	0.760172
Error	448.12	12	37.34		

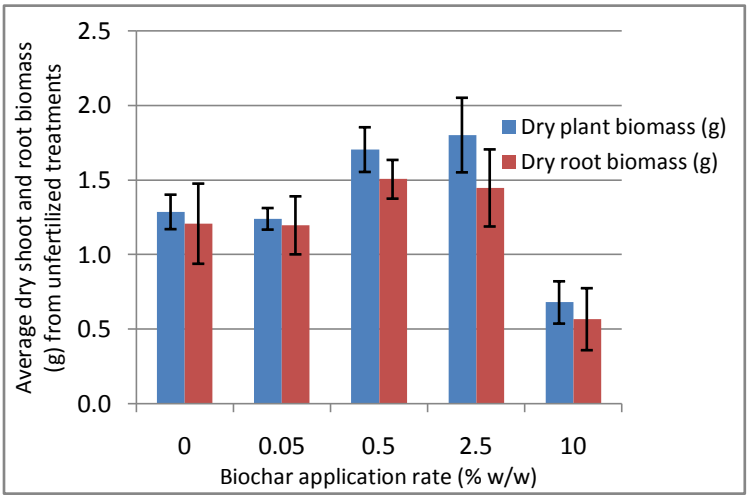
Appendix 3.6: Repeated measures ANOVA on the means of CO_2 produced over three periods

Effect	**Repeated Measures Analysis of Variance** **Sigma-restricted parameterization** **Effective hypothesis decomposition**				
	SS	**Degrees of Freedom**	**MS**	**F**	**p**
pH#	0.000147	2	0.000074	0.2374	0.795747
Error	0.001858	6	0.000310		
Period	0.000135	2	0.000068	6.1399	0.014575
Period*pH#	0.000059	4	0.000015	1.3333	0.313386
Error	0.000132	12	0.000011		

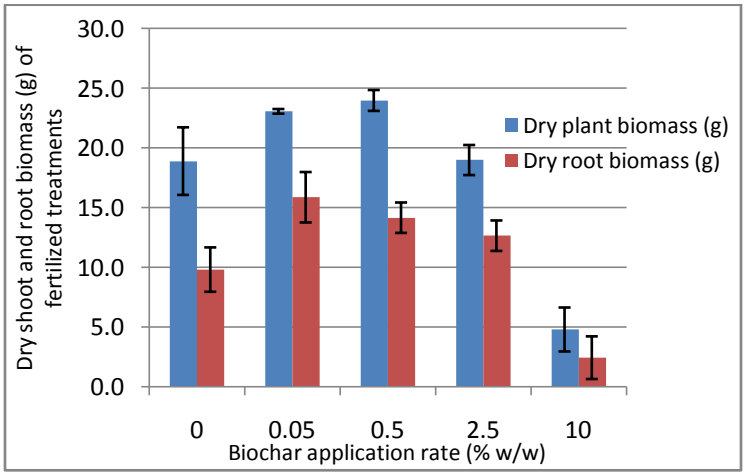
Appendix 3.7: Bonferroni comparisons test between the average CO_2 evolution during each period

Probabilities for Post Hoc Tests **Error: Within MS = .00001, df = 12.000**			
REPEAT	**1** **.04500**	**2** **.04075**	**3** **.03987**
Period 1		0.056330	0.019882
Period 2	0.056330		1.000000
Period 3	0.019882	1.000000	

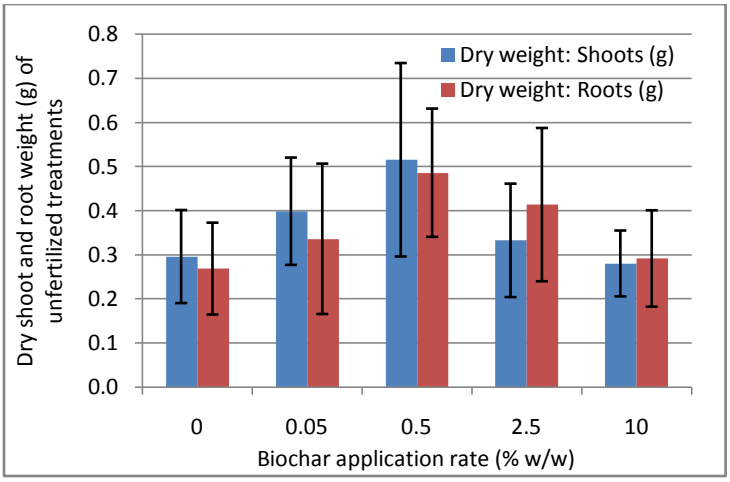
Appendix 4: Pot trials to assess the effects of biochar presence in a virgin and fertilized acidic sandy soil on total microbial populations, plant roots and associated symbionts.



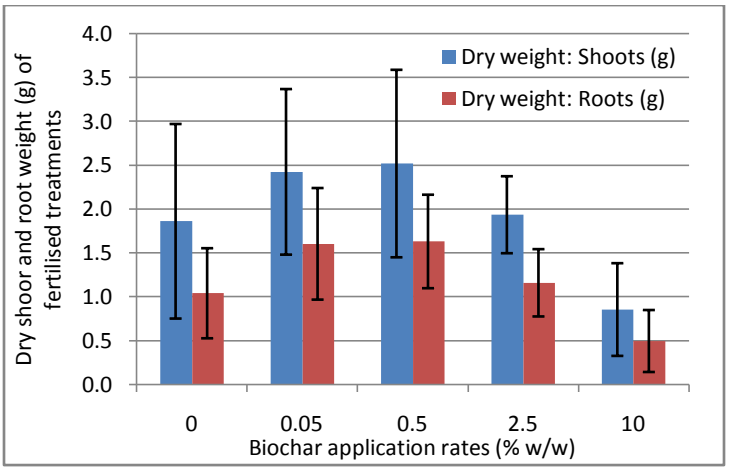
Appendix 4.1: Shoot and root dry weight of the unfertilized wheat treatments



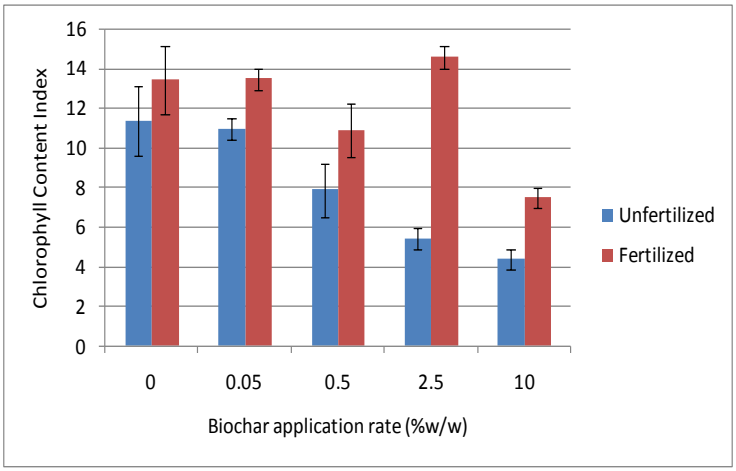
Appendix 4.2: Shoot and root dry weight of the fertilized wheat treatments



Appendix 4.3: Shoot and root dry weight of the unfertilized bean treatments



Appendix 4.4: Shoot and root dry weight of the fertilized bean treatments



Appendix 4.5: Chlorophyll content index of bean leaves with increasing biochar application rate (Zeelie, 2011)