Effect of biochar on microbial biomass and biological nitrogen fixation

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University of Helsinki
Department of Forest Sciences
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Christine Ribeiro Moreira de Assumpção

HELSINGIN YLIOPISTO – HELSINGFORS UNIVERSITET – UNIVERSITY OF HELSINKI

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Tiivistelmä – Abstrakt – Abstract
Biochar is a product from the pyrolysis of plant derived-biomass and it is intended to be applied to soil given its potential of carbon sequestration and soil fertility improvement. Some studies also suggest that increasing application rate of biochar has a positive feedback on biological nitrogen fixation (BNF) and on soil microbial biomass. However, these effects are not well known for boreal forests. The purpose of this study was to evaluate the effects of different biochar application rates: 0 t ha^{-1}, 5 t ha^{-1} and 10 t ha^{-1} on BNF, on microbial biomass carbon and nitrogen (MBC and MBN), and on moss biomass. The field experiment was established in Juupajoki, Southern Finland in young Scots pine stands. The stands were amended with biochar one year before the measurements took place. BNF was determined using acetylene reduction assay (ARA), and microbial biomass was estimated using chloroform fumigation-direct extraction (CFDE). The microbial biomass samples were incubated at the temperatures: 10 °C, 15 °C and 20 °C. Biochar amendment raised soil pH, whereas no differences were verified for BNF, MBC, MBN, nor for moss biomass. There was, however, variation in the response of N fixation to incubation temperature, and variation in the response of MBC and MBN to the time of measurement. Observed changes in pH are often likely to justify variations in the rates of BNF and MB, however in this study they were not shown to be of significance. It is possible, however that biochar will have a positive effect on soil vegetation as it is incorporated into the soil in the long-term. Although this study focuses on BNF and MB, the findings may well have a bearing on the use of biochar as a tool for C sequestration, since amendment with biochar was demonstrated as neither beneficial nor harmful to the soil biota.

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1. Introduction

1.1 Biochar overview

Biochar is a carbon-rich product yielded from the thermal conversion (pyrolysis) of organic material in partial or total absence of oxygen (Bruckman et al. 2015). Pyrolysis of biochar concentrates on increasing feedstock recovered as char, which is often seen as more energetically efficient than traditional manufacture of charcoal (Sohi et al. 2010). Moreover, biochar is intended to be applied to soil as it is relatively stable against microbial decomposition under varying environmental conditions due to its stable aromatic forms of organic carbon (Sohi et al. 2010; Bruckman & Klinglmüller 2014).

There are at least three manufacturing related factors essential to the properties of biochar: furnace residence time, heating rate and temperature. Slow pyrolysis tends to last from seconds to hours while fast pyrolysis has faster transfer of heat, lasting from milliseconds to seconds. The faster the heating rate, the faster the temperature peaks. Usually, the peak temperature, or highest treatment temperature (HTT), for slow pyrolysis ranges from 450 to 650 °C and for fast pyrolysis around 500 °C (Sohi et al. 2009; Bruckman & Klinglmüller 2014). Heating rate also affects the fractions of products derived from thermal decomposition (e.g. oil, vapor, and char). Traditionally, pyrolysis engineers have prioritized fast pyrolysis exploration, since it generates larger shares of oil and gas (Sohi et al. 2010).

The main property behind the ability of biochar to store carbon on the soil for long periods of time is recalcitrance. The higher the recalcitrance of an organic compound, the higher its stability is. Sollins et al. (1996) defined recalcitrance as: characteristics of organic substances at the molecular level that influence their degradation by microorganisms. Nevertheless, abiotic processes may facilitate microbial metabolization of recalcitrant structures in biochar (Lehmann & Joseph 2009). Recent observations suggest that microorganism can use biochar to some extent owing to its labile fraction. This comes from a conceptual model that describes biochar mineralization under a two-phased process: a rapid

mineralization followed by a slow mineralization. Biochar is mostly stable, with an amorphous or graphite-like structure. Still, rapid mineralization may occur on particle surfaces within months of exposure to soil, leaving a more abundant fraction (75-95 %) characterized by lower mineralization rates (Lehmann & Joseph 2009; Bruckman & Klinglmüller 2014).

Kloss et al. (2012) suggested that high-temperature biochar, mostly wood-derived, is more resistant to decomposition. In a similar case, Zimmerman (2010), when testing oxidation of biochar, identified a more abundant labile fraction in low-temperature biochar. Furthermore, Bruckman et al. (2015), found priming effect up to one month of amendment (10 t biochar ha^{-1}) on a spruce forest, inferring acceleration of soil organic carbon (SOC) turnover upon input of a new source of C into the soil (Verheijen et al. 2010). After the one-month period, mineralization rates were comparable to those of control, which suggests that microorganisms utilized the labile fraction of biochar immediately after the addition. This is evident in the study of Bruun et al. (2011), that estimated that 90 % of the total CO_2 evolved from biochar treatments occurred within 20 days of measurements. The authors stressed that biochar derived from fast pyrolysis mineralized more rapidly due to fast heat-transfer preventing biomass from being totally converted to char. The same trend was observed by Smith et al. (2010) and Wang et al. (2015). The latter researchers estimated C mean residence time to be 100 days and 550 years for labile and recalcitrant fractions, respectively.

The use of renewable biomass for carbon-neutral energy production as an alternative to fossil fuel sources has been vividly discussed among academia (Johnson 2009; Bruckman & Klinglmüller 2014; Väisänen 2014). There is a concern over how dedicated energy crops produced at large scale might affect natural ecosystems and whether the use of biomass as a fuel is considered carbon neutral. Schwaiger & Schlamadinger (1998) when estimating increases in fuelwood use compared to fossil fuels, found achievable reductions of greenhouse gas (GHG) emissions between 5.0 and 10.5 Mt of CO_2 equivalents in Finland. However, when accounting for forest biomass harvesting and losses

in soil C stocks, Mäkipää et al. (2015) have reported a net increase in CO_2 emissions in Finland when using logging residues and stumps for energy production. In fact, it was estimated that replacing fossil fuel oil by biofuel can produce 40 % higher CO_2 emissions regarding the amount of biomass harvested from 1 ha of Norway spruce grown over an 80-year rotation period.

These trends must be considered when deciding upon biomass feedstocks used to biochar and bioenergy production since both systems have potential for climate change mitigation, and they might as well compete for feedstock. Lehmann (2007a) claims that biochar already produced (as a by-product) by bioenergy companies when added to the soil instead of being pyrolysed, carries an emission reduction potential of 12 to 84 %. The author goes further and suggests that this could render bioenergy a carbon-negative industry.

Until 2004, the most common feedstock of biochar at the commercial and research levels comprised wood chip and pellets, tree bark; crop residues such as straw, nut shells and rice hulls; switch grass; organic wastes including paper sludge, sugarcane bagasse, distillers grain, olive waste; chicken litter, and dairy manure (Yaman 2004 cited in Sohi et al. 2010). Other possible sources of pyrolysis feedstock include municipal green and mixed waste (from gardens and parks), composted urban waste, and digested sewage sludge. An indirect benefit of using off-farm feedstock is the reduction in emission of greenhouse gases compared to typical disposal methods (Sohi et al. 2010). Demirbas et al. (2006) showed that the yield of biochar increases when the feedstock contains lignin. Besides, Wang et al. (2015) showed that wood-derived biochars have slower decomposition rates when compared to grass and crop derived biochar. By contrast, higher nutrient content is found in biochars originated form nutrient rich feedstocks (Gul & Whalen 2016).

A key factor determining the utilization of feedstock for biochar production is the value of biochar in soil. In addition to its use for carbon sequestration and storage, the likelihood of biochar enhancing soil productivity counterbalances the opportunity costs associated with bioenergy (Sohi et al. 2010). In other words, the

benefit of using feedstock for biochar production should be comparable to that of employing it in bioenergy. This is mostly the case for the tropical and subtropical regions, where there is a wide range of feedstock and heavily weathered soils (Bruckman & Klinglmüller 2014). Woolf et al. (2010) projected that the establishment of a sustainable biochar initiative could offset about 12 % of the current anthropogenic CO_2 equivalent emissions. Despite low application frequency, due to its longevity, a cost-benefit-ratio of applying biochar should also be considered.

Assuming that the carbon in biochar is stable, it can be speculated that diversion of biomass, from for instance managed forests, to pyrolysis contributes to a negative feedback to global warming. Had the biomass been left on the forest floor to decompose, the CO_2 emissions would have been larger than if it was pyrolysed into a more stable form (Woolf et al. 2010).

A positive feedback is possible when incorporating biochar to soils on a large spatial scale (Verheijen et al. 2010). Following basic physics principles and suggested by Bowers & Hanks (1965), darker colour surfaces absorb more solar energy and, when it comes to soils, may display higher temperatures depending on water content and plant cover. This could naturally benefit the vegetation by accelerating nutrient cycling and in addition contribute to the productivity of the site. However, simultaneously application of biochar reduces the reflectivity of the Earth's surface, since it is among the darkest substances, hence presenting one of the lowest albedos. Because albedo and GHG effect are the primary mechanisms controlling the Earth's surface temperature, decrease in albedo on a planet scale incites a temperature rise. This could possibly lead to faster decomposition of soil organic matter (SOM) and increased GHG emissions. This leaves room for a scenario where biochar is applied mostly in forests, since the canopy diminishes the effect that biochar may have on albedo.

Different feedbacks are also possible in a scenario where biochar production relies mostly on bioenergy crops. On one side, GHG emissions from land-use changes related to biofuel production are known to increase

(Searchinger et al. 2008). In contrast, Georgescu et al. (2011) have shown that conversion of annual to perennial bioenergy crops across the central United States increased albedo, which contributed to local cooling. These two outcomes reflect the need of evaluation of potential impacts on surface energy and on changes in carbon emissions from land-use change.

As part of the long-term removal of C from the atmosphere, integration of biochar on soil management may influence soil biota, as well as its physicochemical properties. For this reason, when assessing the effects of applying biochar on soil, a range of factors must be considered, including: a) the properties inherent to biochar production (e.g. furnace residence time, peak temperature, feedstock); b) management, which includes application rate and frequency; c) site characteristics (where the experiment took place) and d) duration of the experiment. The importance of biotic and abiotic factors driving biochar decay seem to vary greatly among experiments, depending mostly on the interaction between environment and biochar properties.

The section below further explores the effects of the physical and chemical properties of biochar on soil biological community and nutrient cycles.

1.1.1 Nutrient dynamics

One of the main properties enabling essential soil functions, such as water holding capacity, aeration, nutrient cycling and microbial activity, is specific surface area (Lehmann & Joseph 2009). Optimal proportion of the fine earth fraction (clay, silt, and sand) provides the balance between aeration and water holding-capacity to achieve the best properties for plant growth. As also discussed by Lukac & Godbold (2011), soil structure is not a stable property, therefore it can be altered. A constant supply of organic matter content attenuates the effects of deficiency or excess of water. Due to its large surface area, adding biochar to soil has been found to contribute to soil structure, mostly in sandy and medium-textured soils (Chan et al. 2007; Sohi et al. 2010). This is exemplified in the work undertaken by

Haider et al. (2017), whose research found moisture content to increase (in the topsoil) with increasing rate of biochar application to a temperate sandy soil.

On the question of nutrient value, Lehmann & Joseph (2009) argue that biochar can directly and indirectly influence site productivity. A variety of macro- and micro-nutrients, pointed as valuable resources in the soil food web (Lehmann et al. 2011), can be found in biochar. Most of the studies reviewed by Lehmann & Joseph (2009) credited enhanced nutrient dynamics to indirect effects triggered by biochar amendment. Overall, the cases support the view that applying biochar increases or maintains the pH of the soils. pH is a negative logarithm of the concentration of H+, and express the degree of acidity of a solution. It is a widely-held view that the lower the pH of the soil, the lower the availability of nutrients. In some podzolic soils of boreal forest, pH can be as low as 3 (Lukac & Godbold 2011).

Cation exchange capacity (CEC) is the inherent soil mechanism acting against acidification. CEC is defined as the total capacity of a soil to hold exchangeable cations. These cations are divided into base cations, that are necessary to plant growth, and acid cations. At low pH, H^+ ions displace base cations from the cation “bank” into the soil solution (Lukac & Godbold 2011). Base cations adhere to negatively-charged sites of biochar surface as they do to clay particles and organic matter (Verheijen et al. 2010). Therefore, applying biochar on soil can also lead to an increase in CEC, especially aged biochar (pyrolyzed at or below 600-700) in nutrient-poor sandy soils (Glaser et al. 2002; Lehmann 2007b; Kookana et al. 2011). According to Clough & Condron (2010), this increase in CEC can indicate the ability of biochar to retain cations such as NH_4^+.

The literature on applying biochar to soils has highlighted several benefits related to N drainage flow and gaseous emissions. Asada et al. (2002) have demonstrated the potential of biochar to adsorb NH_3 on biochar manufactured from bamboo. Rondon (2005) cited in Clough & Condron 2010 reported a reduction in N_2O emission of at least 50 % post biochar amendment, and DeLuca & Sala (2006) verified that the addition of charcoal from a recent fire site to an

unburned site increased nitrification potential. The authors claim the importance of charcoal for N fluxes to fire-dependent ecosystems in the long term.

1.1.2 Soil biota

The effects of biochar on soil biota is a topic of growing interest, although still poorly understood. This is partially due to the complexity surrounding biochar properties and its behaviour upon contact with soil. The understanding of the impacts biochar amendment may have on the soil biota is crucial to safeguard soil functions and ecosystem services, as they are susceptible to the health and diversity of the biological community (Brussaard 1997 cited in Lehmann et al. 2011). In that regard, Moore et al. (2004) have stressed the importance of quality, quantity, and distribution of detritus (defined as any form of non-living organic matter) for the structure and functioning of food web. The heterogeneous nature of biochar (i.e. labile and recalcitrant fractions) will affect its decay when added on soil.

Lehmann et al. (2011) suggest that pH changes proceeding biochar amendment can influence microbial abundance. The degree of change would depend on the native pH of the soil as well as on the magnitude of change in pH. Relative microbial abundance is expected to increase with pH, as verified by Aciego Pietri & Brookes (2008). Therefore, applying biochar with high pH might contribute microbial biomass growth. Additionally, there can be an increase in the microbial biomass simply because of increase in colonisable surface. This is evident in the case of marine sediments, in which Yamamoto & Lopez (1985) found a positive relationship between bacterial abundance and specific surface area of sediment. Because of the porous structure and water-holding capacity of biochar, microorganisms might benefit from moist pore spaces during periods of drought in sandy soils (Lehmann et al. 2011; Bruckman & Klinglmüller 2014). Lehmann et al. (2011) discuss the possibility of biochar reducing the tensile strength of the soil and how it makes nutrient mining more effective by roots and mycorrhizas.

1.2 Nitrogen cycle and boreal forest

Nitrogen is a plant macronutrient essential to the survival of ecosystems. Yet, in most cases the amount of N available to plants is low (Robertson & Groffman 2007), which limits the gross primary productivity (GPP) of the site (Tamm 1991 cited in Gundale et al. 2011). This is mostly the case in pristine and northern sites, where natural N deposition is low, averaging approximately 0.21 kg N ha^{-1} yr^{-1} (Brenner et al. 2005). Boreal forests are relatively protected from large airborne deposition (Nordin et al. 2005). However, when closer to urban areas, N deposition can be as high as 7.4 kg ha^{-1} yr^{-1} (Korhonen et al. 2013).

In boreal ecosystems, large part of the N pool is found in undecomposed biomass (Robertson & Groffman 2007), where N bounds to complex recalcitrant C-compounds, making mineralization and N recycling a lot slower and costly compared to other elements (Vitousek et al. 2002). For this reason, inputs of N to the system, such as from BNF, are substantial to the natural productivity of the vegetation (DeLuca et al. 2008; Korhonen et al. 2013). N input from BNF has been estimated to be 0.1–4 kg N ha^{-1} yr^{-1} (Cleveland et al. 1999; DeLuca et al. 2002; Zackrisson et al. 2004; DeLuca et al. 2008; Korhonen et al. 2013). In northern sites, BNF may even exceed atmospheric N deposition. This is exemplified in the work undertaken by Gundale et al. (2011), in which a contrasting variation of deposition across Sweden, ranging from 10–15 kg N ha^{-1} yr^{-1} in the south to 1–3 kg N ha^{-1} yr^{-1} in the north, was reported.

Nitrogen is most abundant in the biosphere as unreactive dinitrogen gas (N_2) (Robertson & Groffman 2007). Since this form is not usable for most organisms, N_2 should be fixed into reduced nitrogen forms (e.g. ammonia, ammonium, and amines) that are essential in food production, ecology and in the environment (Erisman et al. 2007). N_2 can be fixed biologically by certain groups of microorganisms (Archaea and Bacteria). Moreover, fixed N is naturally produced by lightening (Galloway et al. 2004), and in minor scale by emissions from volcanoes (Ward 2012) and natural fires (Levy et al. 1991). Nonetheless, during the last century anthropogenic activity has heavily affected the N cycle due

to increased emissions of fixed N. This increases the amount of reactive nitrogen (Nr) in the atmosphere. Nr includes inorganic reduced forms of N (e.g. ammonia, ammonium), inorganic oxidized forms (e.g., NO_x, HNO_3, N_2O, NO_3), and organic compounds (e.g., urea, amines), which will be deposited to forests mostly around urbanized areas (Galloway et al. 2004).

Mineralization is the source of the most common soluble forms of N that plants can uptake (Robertson & Groffman 2007). Nevertheless, it has been suggested that certain tree species such as Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) can also uptake organic forms of nitrogen (Schimel & Bennett 2004; Näsholm et al. 2009), as well as crop plants (Gioseffi et al. 2012; Paungfoo-Lonhienne et al. 2012). The process of mineralization includes: a) the depolymerisation of organic macromolecules to dissolved organic N, and b) conversion of these molecules to ammonium. Whereas, immobilization represents the allocation of soluble forms of N available in the soil solution to the tissues of living organisms. Microorganisms realize this conversion and the quality of the plant detritus that is targeted by the microbe regulates whether mineralization or immobilization occurs. As any other living organism, microbes need energy, C, N, and several other nutrients to support their growth. These microbes vary widely, including aerobes, anaerobes, fungi, and bacteria. Soil fauna, such as termites, also contribute to this process, by for example macerating wood (Robertson & Groffman 2007; Lukac & Godbold 2011).

Whenever a microbe consumes a plant detritus, there can be either release of nutrients (mineralization) or immobilization. The path depends on the detritus quality: if microbial needs are met, mineralization takes place and any extra nitrogen is to be released to the soil solution; whereas if the detritus is nitrogen poor, microbes must find extra supply of nutrients from the vicinity (soil solution) to proceed consumption, while the nitrogen from the detritus is immobilized in the microbe (Robertson & Groffman 2007).

One way of determining the quality of the detritus is verifying the C:N-ratio, which is “the availability of C in the material relative to its available N” (Robertson

& Groffman 2007, p. 346). In general, a high C:N-ratio stimulates immobilization, whereas a low C:N-ratio stimulates mineralization (Read 1991; Robertson & Groffman 2007). For instance, alfalfa residues have C:N-ratio 16:1 and pine litter is 300:1 (Robertson & Groffman 2007). Microbes have more difficulty in decomposing pine litter than alfalfa residues, and should immobilize nitrogen from their surroundings to proceed with degradation. Another factor controlling mineralization versus immobilization rate is specific to the organism-specific growth efficiency: different organisms have different cell walls structures. For example, fungi are known to have a wider C:N-ratio in their tissues compared to other microorganisms. Therefore, they are less demanding for N-rich detritus (Robertson & Groffman 2007). It is very likely that mineralization and immobilization occur at the same time within small volumes of soil. Therefore, gross mineralization, immobilization and net mineralization can be quantifiable. If gross mineralization exceeds gross immobilization, net mineralization is positive, and soil inorganic nitrogen is increasing.

Besides mineralization, nitrification is also known to increase plant available N. This mechanism represents the oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) and nitrate (NO_3^-), ions that are known to be mostly important to crop nutrition (Andrews et al. 2013). Because NO_3^- has higher mobility than NH_4^+, it is more prone to leaching (Smolander et al. 2012). Nitrogen may also flow through the denitrification pathway, when NO_3^- is converted to gaseous forms of N, such as nitrous oxide (N_2O) and N_2 (Smolander et al. 2012). It is known that prior to human intervention, natural N fixation and denitrification were processes in equilibrium (Galloway et al. 2004). Currently, in undisturbed boreal forests, a relatively small N-leakage is found (Smolander et al. 2012). Korhonen et al. (2013) reported rather small N losses in Southern Finland. About 5 % of N inputs (atmospheric N deposition, BNF, and fertilization) are lost via leaching of NH_4^+, NO_3^-, and via emissions of N_2O and NO_x. However, in cases where above 30 % of the catchment area is clear-cut, the losses of total N, total organic N and NO_3^- can be higher (Palviainen et al. 2014). In this context, utilization of biochar as soil

amendment can potentially reduce NO_3^- leaching (Haider et al. 2017), thus indirectly contributing to mitigation of N_2O emission.

1.3 Moss-cyanobacteria associations

The symbiotic association between moss and nitrogen-fixing cyanobacteria is one of the mechanisms of biological N input to many natural systems, including boreal forest ecosystems. In this section, both groups of organisms are described, and key ecosystem controls on nitrogen fixation in boreal feather moss communities are discussed.

1.3.1 Nitrogen-fixing cyanobacteria

Cyanobacteria are a highly diverse group of bacteria found both in terrestrial and aquatic environments. They are free-living organisms, but a range of them can form symbiotic relationships, such as cyanobacterial-associates in feather moss carpets (Bergman et al. 1996; DeLuca et al. 2008). They are also known to colonize lichens, animals and higher plants (Cleveland et al. 1999; Rai et al. 2000). Cyanobacterium *Nostoc* is the most common to habit terrestrial environments, owing to its versatility (Stal 2015). *Nostoc sp.* has been found to colonize *P. schreberi* and *H. splendens*, but not *D. polysetum* (Bay et al. 2013). In fact, literature in *D. polysetum* associated with cyanobacteria appears to be inexistent. As discussed above, there are cyanobacteria that conduct BNF in a free-living state (Gentili et al. 2005). Those are often found in decaying wood, where fungi would depolymerize sugars to supply bacteria with energy (Sylvester & Musgrave 1991 cited in Bottomley & Myrold 2007), in soil crusts of arid areas (Belnap 2003), and in high arctic habitats, where Solheim et al. (1996) described them as one of the most important sources of BNF in a Norwegian soil.

Even though these bacterial microorganisms mostly obtain energy through oxygenic photosynthesis and CO_2 fixation, they can also assimilate amino acids. Furthermore, several cyanobacteria, known as diazotrophs, are capable of fixing N_2 (Stewart 1980). However, the reduction of N_2 to NH_3 is still an energy-

expensive process, costing 16 ATP (adenosine triphosphate), which makes other sources of N preferable when available (Rees & Howard 2000).

Another factor to N-fixation is that nitrogenase, the enzyme responsible to reduce N_2, is inactivated in the presence of O_2. Since cyanobacteria are photosynthetic organisms, they had to develop strategies to protect nitrogenase from O_2 inactivation (Gallon 1992). For instance, they might have spatial and/or temporal separation of N_2 fixation from photosynthesis (Stal 2015). The high-energy cost associated with BNF makes photosynthesis and BNF intertwined processes because of dependency on light (Rousk et al. 2013).

In the absence of atmospheric N deposition, cyanobacterial-associates in feather moss carpets contribute significantly to N input into the boreal forest floor, ranging from 1.5 to 2.0 kg N ha^{-1} yr^{-1} in mid to late successional sites (DeLuca et al. 2002; DeLuca et al. 2007). The relationship between these two organisms is best described as a mutualistic symbiosis: "the plant host receives N in the form of ammonium (NH_4^+) or amino acids and in return provides carbohydrates, shelter and protection" to the symbiont, also called cyanobiont (Rousk et al. 2013). Approximately 80 % of the fixed N is released to the moss (Adams 2002). The fixed C provided to the cyanobiont mitigates the costs of ATP to fix N_2 (Wolk et al. 1994 cited in Rai et al. 2000). Knowing the relationship between the actors involved in the symbiosis is a prerequisite to evaluate responses of N_2 fixation systems to environmental factors, as well as the effects of these factors to BNF (Leppänen 2013).

1.3.2 Boreal mosses

Mosses (Bryophyta) belong to one of the three major taxa of the bryophytes, that comprised the first land plants descendent from green algal-like ancestors (Lewis & McCourt 2004; Goffinet & Shaw 2010). Molecular phylogenies estimate that mosses originated during the Ordovician period, at least 400 million years ago, when most of the area north of the tropics was ocean (Buck & Shaw 2010; University of California Museum of Paleontology 2011). Currently, mosses are

estimated to include about 12 700 species distributed in aquatic and terrestrial biomes from the tropics to high latitudes (Crosby et al. 1999).

In this thesis, the moss species studied were pleurocarpous *Pleurozium schreberi* (Brid.) Mitt, *Hylocomium splendens* (Hedw.) Schimp., and acrocarpous *Dicranum polysetum* Sw. These are the most common bryophyte species in Finland, while *P. schreberi* and *H. splendens* are one of the most dominant and widespread feather mosses in boreal forest (Reinikainen et al. 2000; Ininbergs et al. 2011). Together, feather mosses can account for over 80 % of ground cover in boreal forests (Dickson 2000 cited in Ackermann et al. 2012).

Mosses have been regarded as opportunistic in terms of CO_2 assimilation even during low irradiance and temperature: their leaves are usually arranged in a way to maximize light interception (Harley et al. 1989). Despite having rudimentary control over desiccation, they might also develop mechanisms to hold excess water prolonging periods suitable for photosynthesis. In Bryopsida, leucocysts, modified cells to store water temporarily, are the most common mechanism (Buck & Shaw 2010; Lindo & Gonzalez 2010). In boreal zones, during hot midsummer period, it is common for mosses to become dormant prior desiccation and rehydrate when water becomes available. This is known specially for the species P. *schreberi* and H. *splendens* (Proctor 2001; Carleton & Dunham 2003), when in the understorey of evergreen boreal forests. Sexual reproduction (via spores) also relies on water availability, but it is not such a limiting factor since asexual reproduction (vegetative propagation) is known from most families of mosses (Buck & Shaw 2010).

In a classic study of growth and nutrition of *H. splendens* (Tamm 1953 cited in Bates 2010), it was shown that the most important obstacle to the productivity of this species in Norwegian forests was nutrient limitation. It was concluded that *H. splendens* receives mineral nutrients as wet deposition. In fact, atmospheric deposition seems to be the main source of N for Bryophita (Turetsky 2003, Ackermann et al. 2012). Often tissue N concentration in this vegetation is used as indicator of air pollution (Woolgrove & Woodin 1996). Moreover, N can be

potentially toxic if above plant tolerance levels, as verified by Gunnarsson & Rydin (2000) when testing N influx on *Sphagnum* growth.

The factors mentioned earlier in this section, such as leaf or plant morphology, light, water and nutrient availability are considered as controls on bryophyte growth (Turetsky 2003). On the other hand, mosses influence the ecosystem in many ways since they have a high CEC (contributing to N-interception), their tissues decompose at much slower rates than vascular plants, and some species host N-fixing cyanobacteria, facilitating BNF (Bates 2010; Turetsky 2003). Their association with symbiotic cyanobacteria contributes significantly to boreal C and N budgets (Turetsky 2003). Lindo & Gonzalez (2010) referred to the bryosphere as an important C and N sink, but often excluded from C stocks and fluxes models (Hagemann et al. 2010). Feather moss net primary productivity (NPP) in boreal forests can reach 80 g C m^{-2} yr^{-1} (Swanson & Flanagan 2001). They also serve as habitat to a diversity of microflora, microfauna and mesofauna that integrate the detrital food web (Lindo & Gonzalez 2010), and a nutrient reservoir to microbes under moss carpets (Biasi et al. 2005).

1.3.3 Environmental controls on nitrogen fixation

The main environmental factors controlling BNF in the Arctic and Subarctic ecosystems regarding *H. splendens*-cyanobacteria associations are the availability of N (Sorensen et al. 2012), light and temperature (Gundale et al. 2012a). For *P. schreberi*-cyanobacteria association, they are moisture (Smith 1984; Gundale et al. 2012b), temperature, light (Gentili et al. 2005; Gundale et al. 2012a), and nutrient conditions (Zackrisson et al. 2004; Gundale et al. 2011).

DeLuca et al. (2007) found a higher number of cyanobacteria on the leaves of *P. schreberi* growing in areas with lower N-deposition, resulting in higher fixation rates. Gundale et al. (2011) verified decrease in BNF rates with artificial fertilization of as little as 3 kg N ha^{-1} yr^{-1}. However, in laboratory conditions, the thresholder for BNF inhibition has been at least three times higher (Ackermann

2013). This indicates that mosses have higher tolerance to N-deposition in the field than expected (Rousk et al. 2013).

There is some evidence to suggest that higher biological nitrogen fixation (BNF) of beans was achieved as a result of reduced availability of N and improved availability of boron (B) and molybdenum (Mo) following addition of biochar (Rondon et al. 2007). The importance of B for bean nodules has been previously suggested by Carpena et al. (2000). A recent study by Rousk et al. (2016) verified that BNF on moss- cyanobacteria associations is also limited by Mo availability.

It is speculated that due to the dormancy period mosses experience during midsummer, their growth as well as the activity of cyanobacterial associates peak in early spring and late summer (Carleton & Dunham 2003, Zackrisson et al. 2004). This behaviour shows how the response of both cyanobacteria and mosses to environmental changes affects BNF (Sorensen et al. 2012). Species variation within mosses also provides different environments to the cyanobacteria. This is supported by Zackrisson et al. (2009), when investigating BNF in *H. splendens* and *P. schreberi* distributed from southern to northern Fennoscandia. It was found that nitrogen-fixation rates varied between the two species at northern latitudes, where *P. schreberi* demonstrated higher fixation rates. In addition, the fixation rates varied between the species when located in sites with different fertility index. In this case, *H. splendens* contributed to higher fixation only in high fertility sites, while total feather moss nitrogen-fixation was significantly higher in sites with low fertility.

Reports on the interactive effect of temperature and light on BNF highlights the complexity found in an epiphytic association part of a forest. On one side, enhanced air temperature increases mineralization rates, which is expected to favour the establishment and growth of fast-growing vascular plants. Finally, higher canopy coverage reduces light available for ground layer mosses, affecting negatively their growth (Van Der Wal et al. 2005 cited in Turetsky et al. 2012). On the other side, Gentili et al. (2005) found that the N-fixation rates peaked at temperatures of 13 °C and 22 °C, and declined from 31.5 °C. Moreover, BNF was

found to also be active at low light intensities, with lower rates, though (Gentili et al. 2005; Gundale et al. 2012a). Gentili et al. (2005) describe it as a possible adaptation mechanism to the higher canopy coverage. Root & McCune (2010) did not find a relationship between bryophyte and canopy cover whatsoever.

1.4 Analysis of biological nitrogen fixation and microbial biomass

Since the work of (Dilworth 1966), it has been known that the same enzyme that reduces acetylene (C_2H_2) to ethylene (C_2H_4) is also responsible for nitrogen (N_2) fixation. As acetylene competes with nitrogen for the active site of nitrogenase, conversion of acetylene to ethylene provides the estimation of nitrogenase activity.

To date, several experiments have ensured the validity of the acetylene-reduction assay (ARA) for nitrogenase activity (Leppänen 2013; Rousk et al. 2016; Stuiver et al. 2016). By comparison, the method is criticized when quantifying BNF from previously unidentified moss-associated cyanobacteria (Darnajoux et al. 2017). For this reason, the $^{15}N_2$ tracer method is recommended for direct calibration of the ARA (Montoya et al. 1996). Still, ARA is a more affordable method for identifying nitrogenase activity, and it is 10^3-fold more sensitive than is possible with $^{15}N_2$ analysis (Hardy et al. 1968). To express the ARA data in N fixation estimates, a conversion ratio of 3 is commonly adopted, as it has already been established for both *P. schreberi* and *H. splendens* (DeLuca et al. 2002; Zackrisson et al. 2004)

As early as in 1907, Darbishire (cited in Jenkinson and Powlson 1976) investigated the effects of partial sterilisation of soil on the action of microorganisms. As a soil is exposed to a volatile fumigant, over a short period post fumigation, the rates of respiration are higher than in an unfumigated control soil. The reason behind this process was first proposed by Störmer (1908 cited in Jenkinson et al. 2004) and further described by Vance et al. (1987) as due to the death of indigenous microorganisms by soil microorganisms that have survived the fumigation and could decompose cell lysates. Based on this

assumption, soil microbial biomass C (MBC) and N (MBN) can be estimated from the difference of the CO_2 evolved (or N mineralized) by a fumigated soil from a CO_2 evolved (or N mineralized) by a non-fumigated soil (Jenkinson & Powlson 1976).

These findings were achieved by employing chloroform fumigation-incubation (CFI), a method that has been criticized on three main aspects: it is (1) excessively time consuming and (2) unsuccessful for strongly acid soils and for (3) soils recently amended with substrate (Jenkinson et al. 2004). In addition to those, when measuring MBN, the results could be masked by immobilization and denitrification by the soil population during incubation (Brookes et al. 1985a).

It was not until the early 1980s when estimations of MBC and MBN were tested immediately after fumigation, by employing a chloroform fumigation-direct extraction (CFDE). The innovative method was based on the knowledge that chloroform ($CHCl_3$) fumigation causes an increase in total N extractable by potassium sulfate (K_2SO_4). This is due to $CHCl_3$ lysing living soil organisms while having very little effect on other soil fractions extractable by K_2SO_4 (Brookes et al. 1985b).

The principle to estimate MBC and MBN is the same as for the earlier version of the method. The total C and N extracted by K_2SO_4 from fumigated soil minus the total C and N extracted by K_2SO_4 from non-fumigated soil. A factor 0.45 was proposed by Jenkinson & Ladd (1981 cited in Wu et al. 1990) to represent the fraction of microbial C evolved as CO_2 during the incubation. The widespread use of $CHCl_3$ in this technique owes to its effectiveness on not solubilizing non-microbial soil organic matter and facility to remove from soil after fumigation (Jenkinson & Powlson 1976). Vance et al. (1987) and Wu et al. (1990) compared both CFDE and CFI for measuring MBC, and have proposed the use of CFDE especially in acid soils, which is a common condition among coniferous forest soils (Persson & Wirén 1995).

1.5 Research motivation, goals, and hypotheses

A key aspect of applying biochar on soil is the understanding of its environmental behaviour. As it fulfils its target, e.g. carbon storage or soil fertility, biochar undergoes several changes in soil, some of which will be heavily influenced by its physical-chemical properties derived from feedstock and pyrolysis conditions. Equally, the interaction of biochar to the variety of existent soils will have implications to its use strategy, as in residence time in soil or plant production. The existing body of research on biochar reasserts the demand for empirical evidence on biochar use. This thesis aims to contribute to the current knowledge on biochar systems and support their effective implementation. The specific objectives of this study were to determine:

i. The effect of biochar addition on moss biomass and species composition;
ii. The effect of biochar addition on soil microbial biomass (MB);
iii. Whether biochar addition affects BNF;
iv. Whether possible changes in BNF are explained by changes in the ground vegetation and microbial biomass;
v. To estimate the BNF rate of a boreal forest floor at different temperatures and different biochar rates;
vi. To estimate MB of a boreal forest floor in different months.

Several mechanisms have been proposed regarding the effect of biochar on MB and BNF, including:

i. Mosses tend to become dormant preceding desiccation and rehydrate when water becomes available (Proctor 2001; Carleton & Dunham 2003);
ii. Microbes might benefit from moisture retained in biochar pores (Lehmann et al. 2011; Domene et al. 2014);
iii. Microbial growth is likely to increase with rising pH values (3.7 to 8.3 gradient) (Aciego Pietri & Brookes 2008);

iv. There is a positive relationship between bacterial abundance and specific surface area of sediment (Yamamoto & Lopez 1985);
v. Detritus with C:N-ratio higher than 25:1 is known to stimulate immobilization (Robertson & Groffman 2007);
vi. Biochar is a bi-phased compound, with a labile and a recalcitrant fraction (Lehmann & Joseph 2009; Bruckman & Klinglmüller 2014);
vii. Cyanobacteria are known to have higher affinity to some moss species (Bay et al. 2013), yet they are free-living organisms (Bergman et al. 1996).

The main study hypotheses were that biochar application on soil:

i. Increases moss biomass as a result of better growth conditions;
ii. Increases soil MB due to continued hydration, higher pH, and increased colonisable surfaces, both on moss and on biochar;
iii. Increases MBN over MBC due to higher immobilization;
iv. Increases BNF if there is an increase in MB and/or moss biomass.

2. Materials and Methods

2.1 Study site, experimental design, and measurements

The field experiment was established at the Hyytiälä Forestry Field Station, in Juupajoki, Southern Finland (61^{o} 51' N, 24^{o} 17' E, 181 m above sea level), in four approximately 20-year-old Scots pine (*Pinus sylvestris* L.) stands (Figure 1), in mid-May 2015.

Figure 1. Field experiment. A: Overview of one of the forest stands. B: Forest floor. C: Biochar.

According to Köppen's climate classification, Finland belongs to the boreal coniferous-mixed forest zone with cold and wet winters. The annual mean temperature is 3 °C and precipitation is 700 mm. The soil in the stands was analysed prior to biochar application in 2015 (Table 1).

Table 1. Soil chemical and physical characteristics

Horizon	Organic C	Organic N	C/N	pH	EC	BD	Particle size distribution		
	%			1:5 v/v	µS cm^{-1}	g cm^{-3}	Clay %	Silt %	Sand %
O	31.13	0.94	33.13	3.51	209.45	0.09			
E	2.64	0.1	26.26	4.28	58.35	0.47	0	15.48	84.52
Bs	1.14	0.06	19.13	4.76	29.26	0.58	0	12.67	87.33

Soil organic carbon (C) and nitrogen (N), C/N ratio, pH, electrical conductivity (EC), bulk density (BD) and particle size distribution by soil horizon (organic, eluviated, and illuviated) at the Hyytiälä study site. Soil pH was determined in the lab on field-moist soil (1:5 v/v soil:distilled water).

Biochar was incorporated into the plots (15 m x 15 m) at different rates: 0, 5 and 10 t biochar ha^{-1}, treatment 1, 2 and 3 respectively. Biochar was spread on the humus layer, therefore not mixed with the mineral soil. This experimental design was replicated in four stands (Figure 2).

Stand 1	Stand 2	Stand 3	Stand 4
T1	T1	T1	T1
T2	T2	T2	T2
T3	T3	T3	T3

Figure 2. Experimental design. T1: 0 t biochar ha^{-1}, T2: 5 t biochar ha^{-1}, T3: 10 t biochar ha^{-1}.

The biochar used in this experiment was purchased overseas (Sonnenerde, Riedlingsdorf, Austria), where it was produced under controlled conditions by pyrolyzing Norway spruce chips at 650°C. Chemical analyses were performed on 15 subsamples, except Organic C and N, which used 3 subsamples (Table 2).

Table 2. Chemical characteristics of the added biochar

Biochar characteristics		
Organic N		0.25
Organic C		76.91
LOI	%	90.75
Ash content		15.92
C/N ratio		313.92
pH		8.92
EC	$\mu S\ cm^{-1}$	1719.5
Ca		33.16
K		5.07
Fe		4.00
Al		3.72
Mg		2.91
P		1.84
S	$mg\ g^{-1}$	0.96
Mn		0.66
Na		0.56
Si		0.39
Zn		0.07
Cu		0.04
Ni		0.01

Biochar organic carbon (C) and nitrogen (N), C/N ratio, pH, electrical conductivity (EC), and mean elemental concentrations ($mg\ g^{-1}$).

Two sets of samples per treatment plot were collected in May, June, and July of 2016, repetition 1, 2 and 3, respectively. The sampling spots were selected to avoid disturbance of other experiments ongoing in the area. For the BNF experiment, there were 108 samples, which included 3 samples per treatment, 9 samples per area and 36 samples per repetition. Moreover, the repetitions were further incubated at different temperatures for 24 hours, adding the second factor to the experiment (Table 3).

Table 3. The various treatments in the BNF experiment

Factors	BNF experiment
A. Biochar application rate	0 t ha^{-1} 5 t ha^{-1} 10 t ha^{-1}
B. Incubation temperature	10 °C 15 °C 20 °C

The samples were collected with a soil core cylinder (diameter: 0.058 m) and each sample consisted of moss, litter, biochar (T2 or T3), and organic layer. The samples were moved from the core to glass jars in the field and left at room temperature for the maximum of two days (Figure 3A).

Samples for the MB analysis were collected simultaneously from the same holes to avoid soil disturbance. A different soil core cylinder (diameter: 0.058 m) was used for that purpose and root material was removed with tweezers before the samples were placed into 45 mL plastic tubes. Between each sample, the tweezers were sterilized with alcohol. There were in total 72 samples as only the samples corresponding to June and July were analysed. They were placed in the freezer at -20°C preceding the experiment that happened from November 2016 to March 2017.

Soil temperature at 5 cm depth was measured continuously on all sample plots at two hours intervals with iButton temperature sensors (Maxim Integrated, San Jose, California, USA). Soil pH was measured 15 months after the experiment was established. 10 ml of soil was mixed with 25 ml of deionized water and the suspension pH (H_2O) was measured with a glass electrode (PHM210, Radiometer Analytical, France) on the next day.

2.2 Acetylene Reduction Assay (ARA)

Nitrogen fixation was estimated using acetylene reduction assay (ARA). The experiment was conducted in the Tree laboratory and in the Soil Physics

laboratory at the Department of Forest Sciences of the University of Helsinki. The condition of the samples was converted from field-moist to field capacity, a moisture state where there was enough water in the samples so that the soil particles could not hold onto it. Then, samples were subjected to incubation. Once in this state, 10 % of the volume of the jar was evacuated and replaced with acetylene (Figure 3B). Moss samples were incubated in an environmental chamber (WEISS WK11 340, Weiss Klimatechnik GmbH, Germany) at 10°C (repetition 1), 15°C (repetition 2) and 20°C (repetition 3), at 80 % humidity for 24 hours with artificial light (LED Grow Light Spider 1, Twilight Groups Co, China) applied in all repetitions.

After incubation, a gas sample was taken from each jar by a 50-ml polypropylene syringe (BD Plastipak 60, BOC Ohmeda, Helsingborg, Sweden) and injected into a 12 ml exetainer vial (Labco limited, Lampeter, UK). To have it not over pressurized, the vials were first vented with a needle while 10mL were pumped in with a syringe. Secondly, the needle was removed and, with the syringe, the remaining air was injected up to the maximum capacity of the vial (Figure 3C).

Figure 3. ARA experiment. A: Moss samples. B: Addition of acetylene before incubation. C: Gas transfer to vial after incubation.

The vials were retained in a cold room with temperatures reaching from 3.6°C to 6°C. The acetylene reduction was measured with a gas chromatography (GC), carried out at the Natural Resources Institute Finland (LUKE) by Bartosz Adamczyk in August 2016. The moss samples post incubation were dried at 40°C for about three days. Sample weights in all stages were recorded and ultimately the dry mass of moss, which allowed to express the measured N fixation in terms of moss dry mass.

The GC provided ethylene concentration in $nmol\ cm^{-3}d^{-1}$. To determine the acetylene reduction during the incubation, each estimate was multiplied by the volume of the jar (Equation 1),

$$Ethylene\ (\mu mol\ d^{-1}) = x\ (nmol\ cm^3) \times V\ (cm^3) \div 1000\ (\mu mol\ nmol^{-1}), \quad (1)$$

where x is the concentration of ethylene in $nmol\ cm^3$, V is the volume of jar (500 cm^3) and 1000 is the conversion factor from $nmol$ to μmol.

Acetylene reduction was reported in an aerial basis (AB) (Equation 2),

$$Ethyelene_{AB}\ (\mu mol\ m^{-2}\ d^{-1}) = Ethylene\ (\mu mol\ d^{-1}) \div Ab\ (m^2), \quad (2)$$

In this study, a ratio of 3 moles of reduced acetylene per mole of N fixed was used. Thus, by dividing $ethylene\ (\mu mol\ d^{-1})$ by 3, a theoretical mass of fixed nitrogen was calculated (Equation 3),

$$N\ fixation\ (\mu g\ d^{-1}) = Ethylene\ (\mu mol\ d^{-1})\ \div\ F \times M\ (g\ mol^{-1}) \quad (3)$$

where, F is the conversion factor (3) and M is the molecular mass of N_2 (28.014 $g\ mol^{-1}$).

BNF was reported as N-fixation on aerial basis (Equation 4), on annual basis (AnB) (Equation 5), on moss mass basis (MMB) (Equation 6) and on sample mass basis (SMB) (Equation 7):

$$N\ fixation\ (AB)\ (\mu g\ m^{-2}\ d^{-1}) =\ N\ fixation\ (\mu g\ d^{-1})\ \div\ Ab\ (m^2), \quad (4)$$

where Ab is the base area of the core cylinder (.00255 m^2).

$$N\ fixation\ (AnB)\ (kg\ ha^{-1}\ yr^{-1}) = N\ fixation\ (AB)\ (\mu g\ m^{-2}\ d^{-1})\ \div$$
$$1\ 000\ 000\ 000\ (\mu g\ kg^{-1}) \times 10\ 000\ (m^2\ ha^{-1}) \times 180\ (d), \quad (5)$$

$$N\ fixation\ (MMB)\ (\mu g\ g^{-1}\ d^{-1}) = N\ fixation\ (\mu g\ d^{-1})\ \div\ m_m\ (g), \quad (6)$$

$$N\ fixation\ (SMB)\ (\mu g\ g^{-1}\ d^{-1}) = N\ fixation\ (\mu g\ d^{-1})\ \div\ m_{sm}\ (g), \quad (7)$$

where m_m is the mass of the dry moss, m_s is the mass of the dry sample, 180 is the average length of the growing season in Juupajoki, 1 000 000 000 is the conversion from μg to kg and 10 000 is the conversion from m^2 to ha.

Moss biomass was calculated for total moss mass per m^2 (Equation 8),

$$Moss\ biomass\ (g\ m^{-2}) = m_m(g) \div Ab\ (m^2) \quad (8)$$

and for moss species (Equation 9 and 10),

$$P.schreberi\ biomass\ (kg\ ha^{-1}) = [m_{P.schreberi}\ (g) \times 10\ 000\ (m^2 ha^{-1})] \div Ab \div$$
$$1000\ (g\ kg^{-1}) \quad (9)$$

$$D.polysetum\ biomass\ (kg\ ha^{-1}) = [m_{D.polysetum}\ (g) \times 10\ 000\ (m^2 ha^{-1})] \div Ab \div$$
$$1000\ (g\ kg^{-1}) \quad (10)$$

where, $m_{P.schreberi}$ and $m_{D.polysetum}$ are dry mass of each species and 1000 is the conversion factor from g to kg. Fragments of leaves from the moss samples were identified based on their leaf morphology and arrangement, and then weighed.

2.3 Chloroform fumigation-direct extraction (CFDE)

Microbial biomass carbon and nitrogen were estimated using chloroform fumigation-direct extraction (CFDE). The analysis was conducted in the Soil Physics laboratory and in the Analysis Laboratory of the University of Helsinki. Prior to the chloroform extraction, the samples were transferred from the freezer (-20 °C) to the cold room (3.6 °C to 6 °C), where they had an acclimation period of 7 to 10 days. Each sample was homogenized by having as many roots and other material removed as possible, and ground to fine texture by a mill (DeLonghi

KG49). Each sample became 3 subsamples weighing about 2 g: Soil fresh mass (SFM), Fumigated mass (FM) and non-fumigated mass (NFM).

Soil water content was determined by weighing the SFM subsamples before and after drying them for 24 hours at 105 °C (Equation 11):

$$Water\ content = \frac{Weight_{SFM}\ (g)}{Weight_{SFM105C}\ (g)} \quad (11)$$

Dry mass (DM) was calculated for FM and NFM subsamples (Equation 12 and 13):

$$FDM = Weight_{FM}\ (g) \times Water\ content \quad (12)$$

$$NFDM = Weight_{NFM}\ (g) \times Water\ content \quad (13)$$

where, FDM and $NFDM$ represent the dry mass of the FM and NFM subsamples, respectively.

Soil ash content was determined by weighing the oven-dried (105 °C) soil subsamples before and after placing them in a muffle furnace for 3 hours at 550 °C (Equation 14):

$$Ash\ content = Weight_{SFM105C} - Weight_{SFM550C} \quad (14)$$

The FCN samples were incubated with ethanol-free $CHCl_3$ for 24 hours before extraction, whereas the NFCN samples were kept in the dark for the same period. Briefly, the subsamples were placed into a vacuum desiccator, containing wet paper towel on its inner borders and 2 beakers with 30 mL of $CHCl_3$ in the bottom (Figure 4A). The vacuum was locked and evacuated until the $CHCl_3$ boiled. Evacuation was repeated three times with 1 minute interval. The vacuum was covered to prevent the $CHCl_3$ from breaking down.

At the end of the incubation period, the vacuum was released and vented 6 times to remove the excess of $CHCl_3$. Both FCN and NFCN subsamples were extracted with 0.5 K_2SO_4 and shook for 1 hour at 200 RPM. A volume of K_2SO_4 of 20 times the $Weight_{FM105C}$ was used for the extraction, except when the FM weighed about 1 g. Then a volume of K_2SO_4 of 40 times the $Weight_{FM105C}$ was

used, which was the case when there was not enough soil to make up three 2 g subsamples. The subsamples were then filtered using Whatman No. 42 ashless filter paper and stored (-20 °C) (Figure 4B). All materials were handled with gloves.

Before proceeding to the TOC (total organic carbon) analyser (Shimadzu TOC-V_{CPH}) equipped with a Total Nitrogen Measuring Unit (TNM-1) and an Auto Sampler (ASI-V), subsamples were unfrozen, syringe filtered (0.45 µm, Minisart High-Flow, Sartorius Stedim Biotech, Goettigen, Germany) and diluted 8 times (Figure 4C).

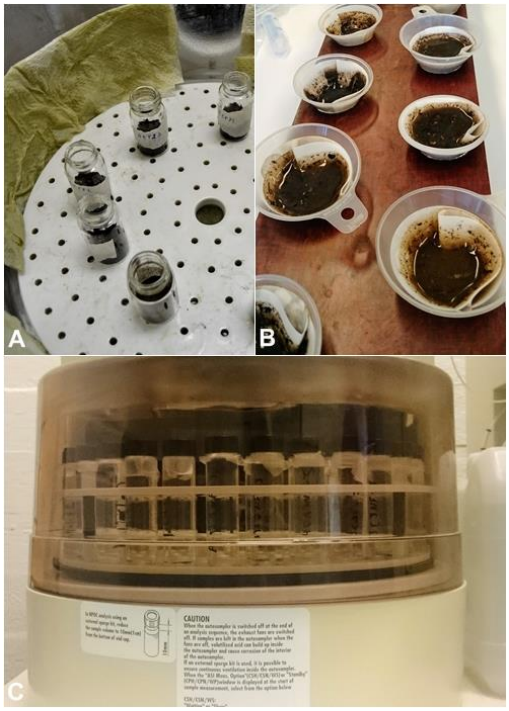


Figure 4. The determination of soil microbial biomass C and N. A: FCN samples in the vacuum desiccator. B: Filtering samples after K_2SO_4 extraction. C: Diluted samples in the TOC analyser.

MBC and MBN were determined by the Equations 15 and 16:

$$MBC\ (mg\ g^{-1}) = [TOC\ FM\ \ (mg\ L^{-1}) \div FDM] - [TOC\ NFM\ (mg\ L^{-1}) \div\ NFDM] \times V\ (L) \times 8 \div 0.45 \quad (15)$$

$$MBN\ (mg\ g^{-1}) = [TN\ FM\ (mg\ L^{-1}) \div FDM] - [TN\ NF\ (mg\ L^{-1}) \div\ NFDM] \times V\ (L) \times 8 \div 0.45 \quad (16)$$

where, $TOC\ FM$ and $TN\ FM$ are the measured total organic carbon and nitrogen from the FM subsamples, $TOC\ NFM$ and $TN\ NFM$ are the measured total organic carbon and nitrogen from the NFM subsamples, V is the added volume of K_2SO_4 at the extraction, 8 is the dilution factor, FDM and $NFDM$ represent the dry mass of the FM and NFM subsamples, and 0.45 is the adjust factor of mineralized microbial biomass during incubation (Jenkinson & Ladd 1981).

The C:N-ratio of the microbial biomass was calculated by dividing MBC values by MBN (Equation 17):

$$C{:}N = MBC\ (mg\ g^{-1}) \div\ MBN\ (mg\ g^{-1}) \quad (17)$$

2.4 Statistical analysis

The datasets were tested for outliers using boxplots from the unstandardized residuals of analysis of variance (ANOVA). Any data point that was more than 1.5 box-lengths from the edge of their box was assumed to be an outlier. There were 5 outliers within the MB dataset, and 2 within the moss biomass dataset. There were 16 outliers in the data within the BNF dataset, approximately 2 for each combination of treatment and temperature. One extreme data point (more than 3 box-lengths away from the edge of their box) believed to be a measurement error was removed. The analyses were then performed with and without the remaining outliers to assess their impact on the results. Since both analyses provided the same results, no other outlier was removed from the datasets.

Variables were log-transformed as required to obtain normality (assessed by Shapiro-Wilk's test) and homogeneity of variance (assessed by Levene's test), which were investigated with residuals from ANOVA. Soil MB data were analysed by two-way ANOVA to compare treatments across time (June and July), and one-way ANOVA to compare treatment effects at any given time. Two-way ANOVA examined the interaction effect between biochar application rate treatments and

incubation temperature, and the main effects of treatments on BNF and on moss biomass. One-way ANOVA also analysed soil MB and BNF in each month.

Tukey's HSD (honestly significant difference) *post hoc* tests were subsequently performed to investigate significant differences among biochar application rates and temperature at $p \leq 0.05$ level. Results are reported using untransformed values, as the conclusions reached were the same. Differences in pH, soil temperature and moss biomass abundance of each species between treatments were analysed using the Bonferroni correction.

Scatterplot of BNF against moss biomass, MBC and pH, and MBC against moss biomass were inspected for linearity. Regression lines were fitted for all relationships between the variables.

All analyses were performed using IBM SPSS Statistics for Windows, version 24 (IBM Corporation, Armonk, NY, USA).

3. Results

Biochar comprised 76.91 % C and 0.25 % N with a C:N-ratio of 314:1 (Table 2). This was the equivalent to an addition of 12.5 and 25 kg N ha^{-1} in the 5 and 10 t biochar ha^{-1} application rates, respectively. The biochar had a pH of 8.92 and an EC of 1719.5 µS cm^{-1} (Table 2). Biochar amendment increased soil pH from 3.68 to 4.07 with the addition of 10 t ha^{-1}, whereas the mean soil temperature was statistically equal in all treatment areas (Table 4).

Table 4. The mean soil temperature (May, June, and July) and pH in biochar application rate treatments

Soil properties	0 t ha^{-1}	5 t ha^{-1}	10 t ha^{-1}
Temperature (°C)	11.52 $(0.53)_a$	11.52 $(0.55)_a$	10.8 $(0.91)_a$
pH	3.68 $(0.05)_a$	3.84 $(0.06)_{a,b}$	4.07 $(0.14)_b$

Values in the same row not sharing the same subscript are significantly different at $p<0.05$. SE in parenthesis.

The ash content was 15.92 %. The major elements available in biochar were Ca (33.16 mg g^{-1}), K (5.07 mg g^{-1}), Fe (4.00 mg g^{-1}), Al (3.72 mg g^{-1}), Mg (2.91 mg g^{-1}) and P (1.84 mg g^{-1}) (Table 2).

Table 5 provides an overview of MBC, MBN, and MB C:N-ratio in June and July, and in each biochar application rate within each month. As it can be seen, MBC ($p<0.001$), MBN ($p=0.006$) and MB C:N-ratio ($p=0.028$) were higher in July. However, biochar amendment had no significant effect in any of the variables within each month ($p=0.337$, $p=0.490$ and $p=0.572$, MBC, MBN and MB C:N-ratio, respectively).

Table 5. The mean soil microbial biomass C N and C:N-ratio in each month, and in each biochar application rate within each month

	June	July	June			July		
Response variables			0 t ha^{-1}	5 t ha^{-1}	10 t ha^{-1}	0 t ha^{-1}	5 t ha^{-1}	10 t ha^{-1}
MBC (mg g^{-1})	2.12_a	3.52_b	2.01_a	2.36_a	2.04_a	4.07_a	3.09_a	3.36_a
MBN (mg g^{-1})	0.21_a	0.32_b	0.21_a	0.21_a	0.23_a	0.37_a	0.27_a	0.31_a
MB C:N-ratio	10_a	12_b	10_a	11_a	10_a	12_a	12_a	11_a

Values in the same row and subtable not sharing the same subscript are significantly different at $p<0.05$.

Biochar amendment had no significant effect on average MBC ($p=0.866$), nor on MBN ($p=0.628$) (Figure 5). MBC and MBN were 2.94 mg C g^{-1} and 0.28 mg N g^{-1} without biochar addition and 2.76 mg C g^{-1} and 0.27 mg N g^{-1} at 10 t biochar ha^{-1}, respectively. Microbial biomass C:N-ratios were 11, 12 and 10 at 0, 5 and 10 t biochar ha^{-1}, respectively, showing no difference between treatments ($p=0.438$).

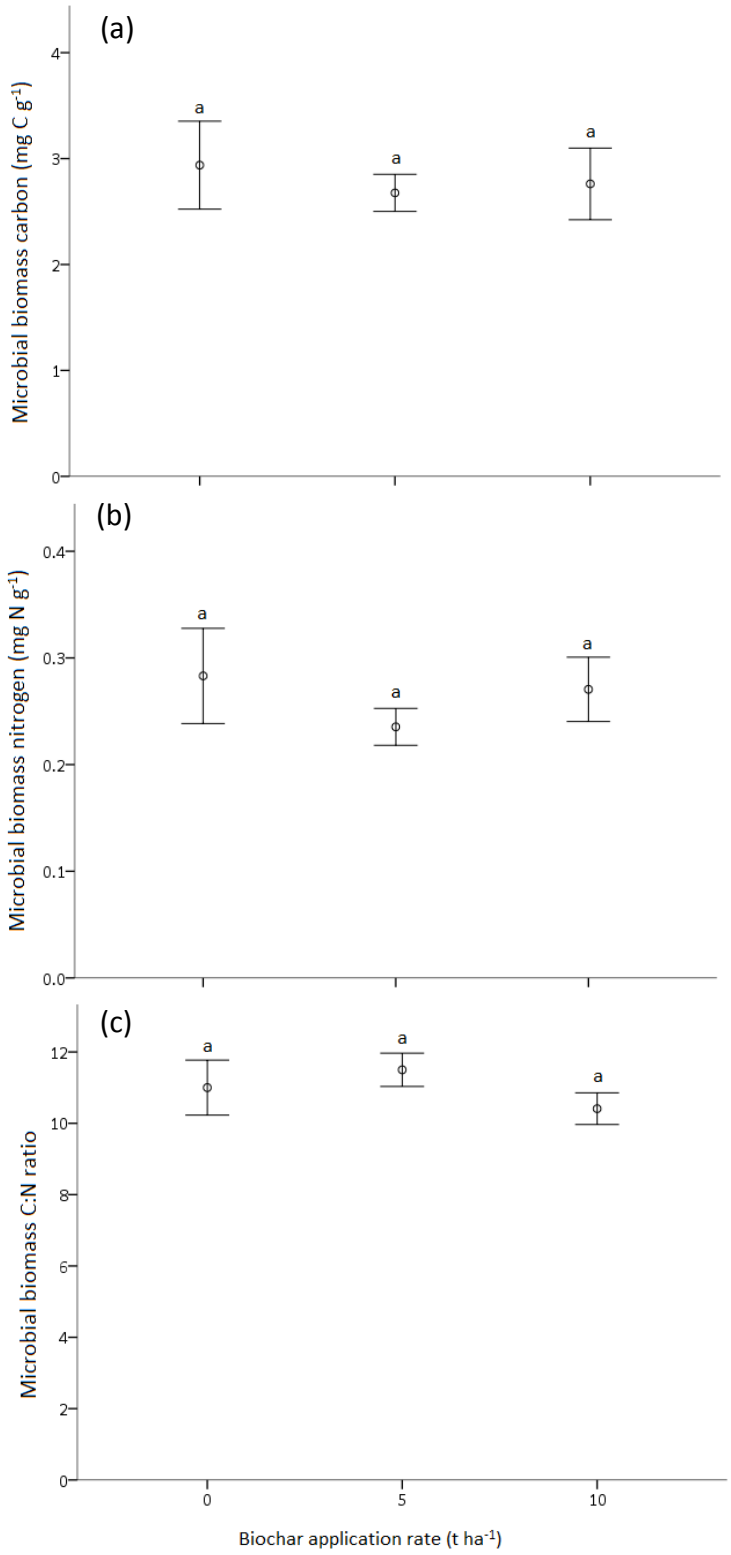


Figure 5. The mean (+/- SE) (a) microbial biomass carbon (mg C g^{-1}), (b) microbial biomass nitrogen (mg N g^{-1}) and (c) microbial biomass C:N-ratio. Different letters indicate significant differences between treatments.

The dominant moss species on the studied site were *P. schreberi* and *D. polysetum*. *H. splendens* was only found in very few samples, therefore not reported. Alone, *P. schreberi* formed approximately 64 % of the total biomass of mosses. There was higher abundance of *P. schreberi* in plots amended with 5 t biochar ha^{-1} compared to plots amended with 10 t biochar ha^{-1} (p=0.007). Despite low p-value (p=0.056), plots with 0 and 5 t biochar ha^{-1} had the same abundance of *P. schreberi* statistically. *D. polysetum* was equally abundant in all treatments (p=0.592) (Figure 6).

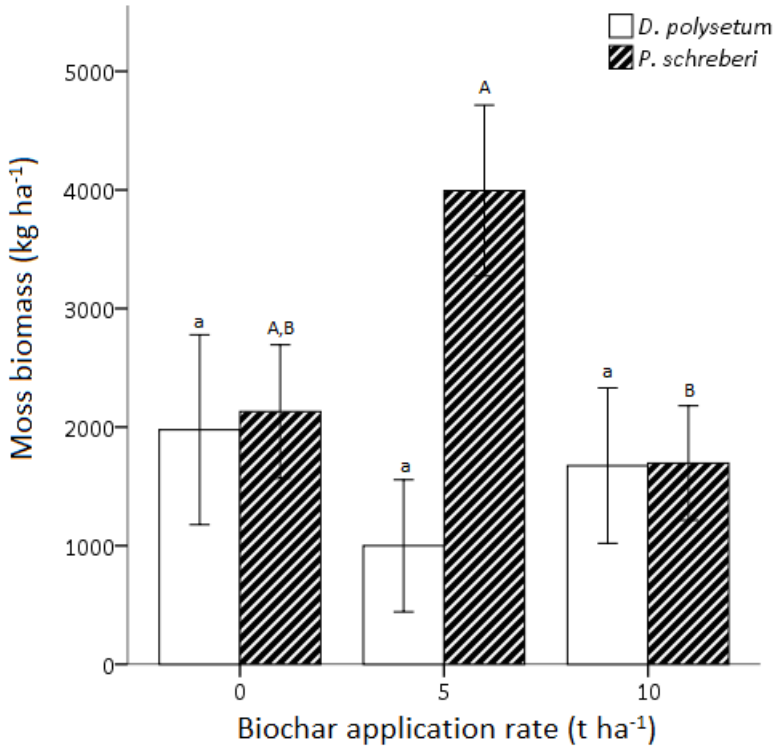


Figure 6. The mean *D. polysetum* and *P. schreberi* biomass per unit area (kg ha^{-1}). Error bars +/- SE. Different letters indicate statistically significant differences between treatments.

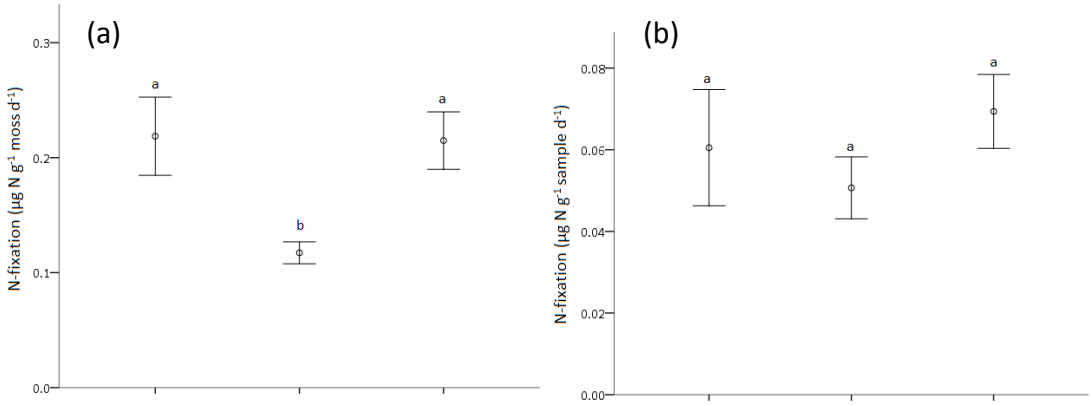
There was no significant interaction effect between biochar application rate and temperature of incubation on none of the BNF categories nor on moss biomass (Table 6). The mean N-fixation on a moss mass basis was lowest (0.12 µg g^{-1} d^{-1}) at biochar application rate of 5 t ha^{-1}, with a significant difference from application rates of 0 and 10 t ha^{-1} (Figure 7). At biochar application rate of 5 t ha^{-1}, the mean N-fixation on an aerial basis was 238 µg m^{-2} d^{-1}, and on a sample

mass basis it was 0.05 µg g^{-1} d^{-1}. However, at biochar application rate of 5 t ha^{-1}, the mean moss biomass found (2352 g m^{-2}) did not statistically differ from the other treatments at p=0.060. Moss biomass per unit area (Table 6) was about five times higher than the mean *D. polysetum* and *P. schreberi* biomass per unit area (Table 6) because there was approximately four times higher unidentified moss material than identified.

Table 6. The interaction effects between biochar application rate and incubation temperature, and the main effects of biochar treatments on BNF on an aerial and a mass basis, and on dry mass of moss, and their mean values

Response variable	Treatment x Temperature	Treatments			
	p-value §	p-value†	0 t ha^{-1}	5 t ha^{-1}	10 t ha^{-1}
N-fixation					
Aerial basis (μg m^{-2} d^{-1})	0.458	0.166	309.16_a	238.13_a	327.02_a
Mass basis (μg g^{-1} d^{-1})					
Moss	0.345	0.006	0.22_a	0.12_b	0.22_a
Sample	0.135	0.556	0.06_a	0.05_a	0.07_a
Moss biomass (g m^{-2})	0.584	0.060	1866.33_a	2352.39_a	1782.06_a

Values are § p-values from interaction effects between biochar treatments and temperature, † p-values from main effects of biochar treatments, and average of the treatments. Different letters within each row indicate significant *post hoc* differences.



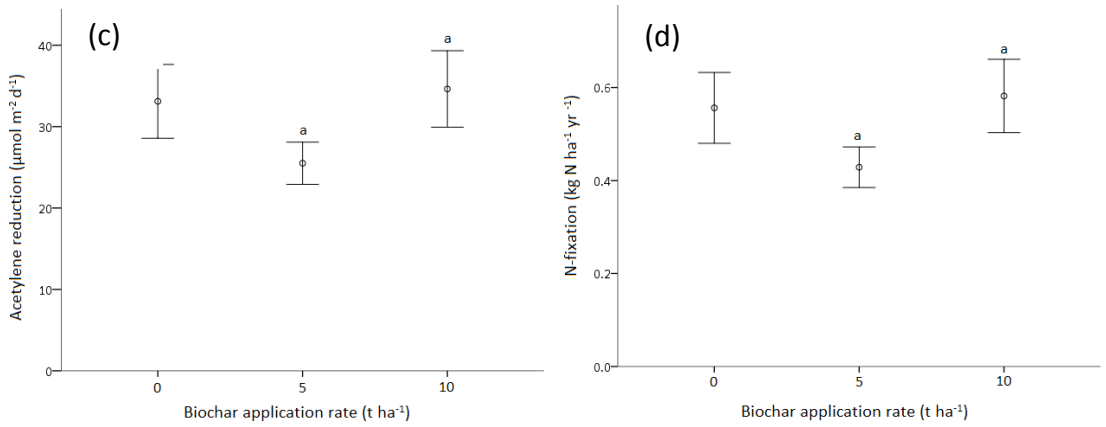


Figure 7. The mean (+/- SE) (a) N-fixation per unit of moss mass (µg N g^{-1} moss d^{-1}), (b) N-fixation per unit of sample mass (µg N g^{-1} sample d^{-1}), (c) acetylene reduction per unit area (µmol m^{-2} d^{-1}), (d) N-fixation per unit area in annual basis (kg N ha^{-1} yr^{-1}). Different letters indicate significant differences between treatments.

The mean acetylene reduction rates were 21 µmol m^{-2} d^{-1}, 25 µmol m^{-2} d^{-1} and 47 µmol m^{-2} d^{-1}, at 10 °C, 15°C and 20 °C respectively (Table 7). Fixation rates were higher at an incubation temperature of 20 °C ($p<0.001$) and did not differ between 10 °C and 15°C ($p=0.762$) (Figure 8).

Table 7. The mean acetylene reduction rates per unit area (µmol m^{-2} d^{-1}) in each incubation temperature (10 °C, 15°C and 20°C) per treatment

Temperature	Biochar application rate	Acetylene reduction
°C	t ha^{-1}	µmol m^{-2} d^{-1}
	0	19.8
10	5	19.9
	10	24.5
	0	23.0
15	5	20.1
	10	31.7
	0	56.6
20	5	36.5
	10	48.0

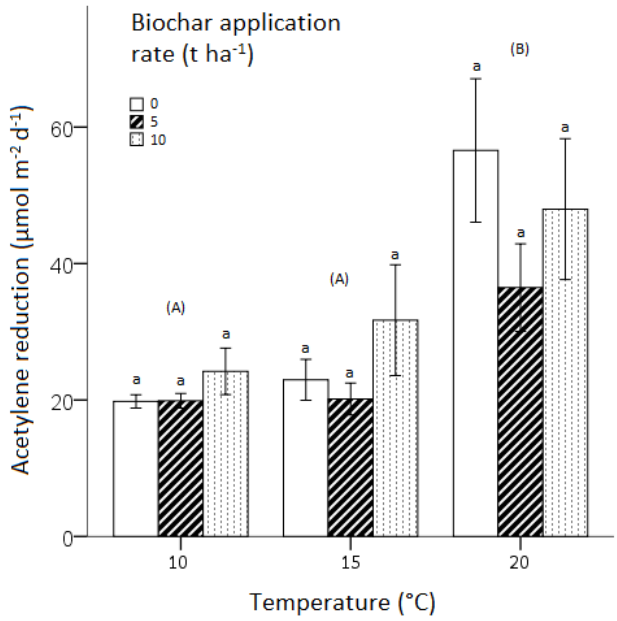


Figure 8. The mean (+/- SE) acetylene reduction rates per unit area (µmol m^{-2} d^{-1}) in each incubation temperature (10 °C, 15°C and 20°C) per treatment. Significant differences between treatments in each temperature group are indicated by different lower-case letters, whereas significant differences between temperatures are indicated by upper-case letters in parenthesis.

No relationship was found for the variables analysed (Figure 9). MBC, moss biomass and pH accounted for 6.5 %, 2.9 % and 1.1 %, respectively, in the variation in acetylene reduction. Moss biomass accounted for 0.4 % in the variation of MBC.

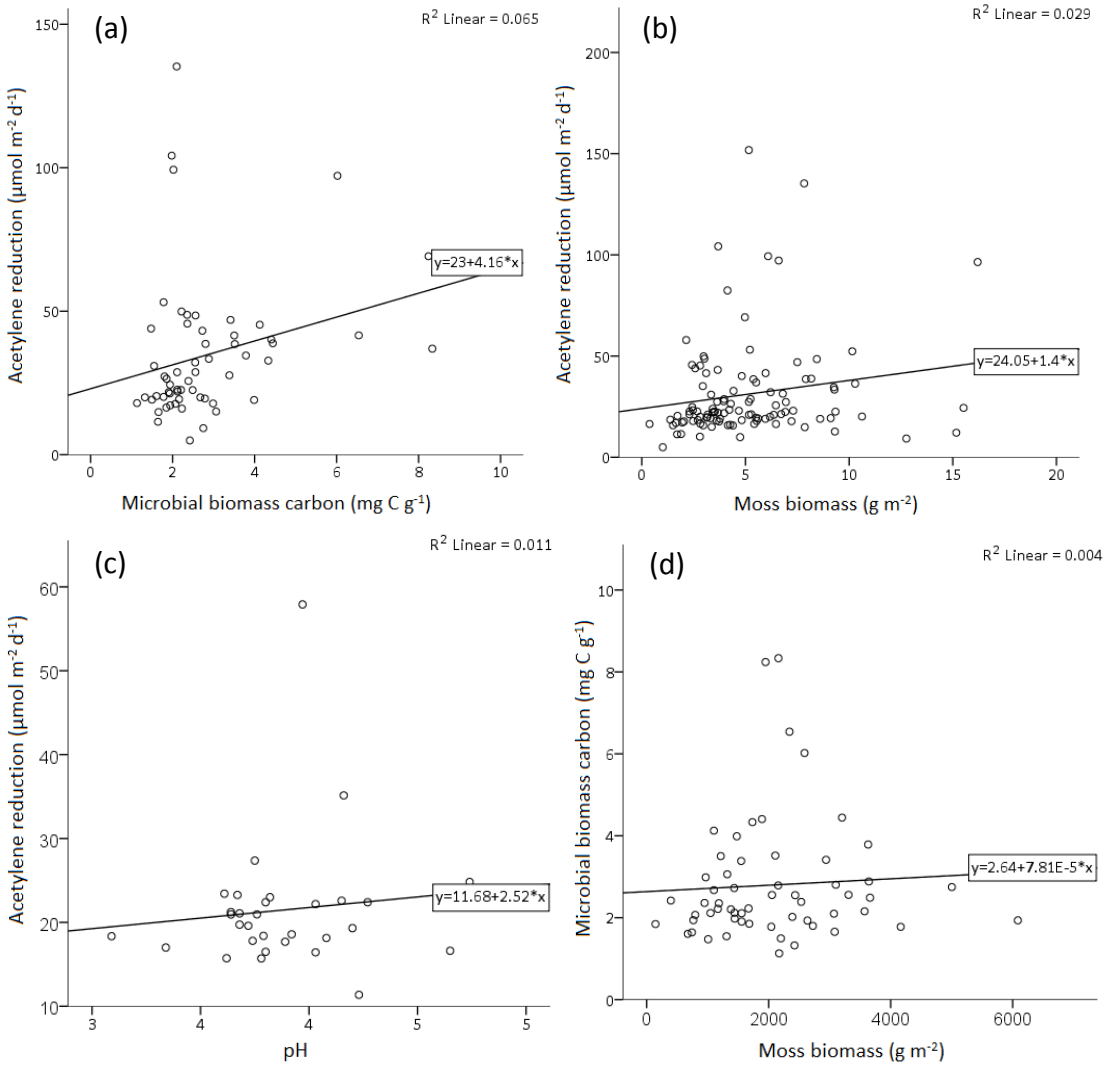


Figure 9. Fitted regression lines for (a) acetylene reduction and microbial biomass carbon, (b) acetylene reduction and moss biomass, (c) acetylene reduction and pH, and (d) microbial biomass carbon and moss biomass.

4. Discussion

The current study found that biochar amendment on the soil of a boreal forest led to an increase in soil pH, whereas no differences were verified for soil temperature, microbial biomass, moss biomass and biological nitrogen fixation. There was, however, variation in the response of nitrogen fixation to incubation temperature, and variation in the response of microbial biomass C and N (and C:N-ratio) to the time of measurement.

The first question in this study sought to determine the effect of biochar addition on species composition and moss biomass. On the question of species composition, this study found that *P. schreberi* and *D. polysetum* were the dominant moss species in the bottom layer of control plots. The same dominant moss species composition was described by Tonteri et al. (2013) in young less fertile forests in Finland. One interesting finding was that *P. schreberi* was more dominant in plots amended with 5 t biochar ha^{-1} than in plots with 10 t biochar ha^{-1}, whereas both treatments showed statistically equal moss biomass of control plots. These results demonstrate that connections between biochar amendment and moss growth is likely to exist. Nevertheless, further studies with more focus on the effects of biochar application rates on moss growth and species composition is suggested.

It is somewhat surprising that no differences regarding moss biomass between 5 t biochar ha^{-1} and the other biochar application rates were verified. There is currently a lack of studies that especially consider the differences in moss growth in relation to biochar addition. In any case, Gundale et al. (2015) reported no differences in total cover of the ground layer vegetation (vascular plants and mosses) in response to biochar addition (10 t ha^{-1}). Conversely, Güereña et al. (2015) verified increases in bean shoot, root, and nodule biomass in response to biochar amendment. The latter authors suggested that biochar application improved mycorrhizal colonization, contributing to phosphorus (P) uptake. Associations between bryophytes and mycorrhizae have been described as beneficial regarding nutrient acquisition (Glime 2008). It is possible, therefore, that biochar amendment increases moss biomass. However, it was not possible to identify differences with the application rates tested over the time scale of this experiment.

The changes in nutrient dynamics promoted by the addition of biochar has been shown to increase the growth of crops and trees (Robertson et al. 2012; Biederman & Harpole 2013; Thomas & Gale 2015). This is particularly linked to the cycling of P and K. Once these nutrients are made available through the

organic labile compounds from biochar, they are either used by plants or leached (Biederman & Harpole 2013). Whether mosses can benefit from such short lived nutrient availability, and whether the potential of biochar in reducing leaching losses affects this dynamic should be investigated.

Gundale et al. (2015) suggest that vegetation is more responsive to disturbances than to nutrient availability, at least over a two-year period after biochar amendment. Because the effect of biochar on soil water holding capacity, porosity and CEC is probably more significant in mineral soil than in the humus layer, in long-term studies when biochar becomes incorporated into the soil, its effects on vegetation variables may be greater. An implication of this is the possibility that effects on the biochar-soil system in the long-term (above 3 years) will be different than those found in the short-term (Gul et al. 2015).

In this study, an average soil pH of 3.51 was measured for the site before the experiment was established, which stands within the pH range determined by Ackermann et al. (2012) for forests near busy roads in northern Sweden. This experiment detected that application of 10 t biochar ha^{-1} increased soil pH from 3.68 to 4.07. These results are consistent with data obtained in similar environmental conditions by Pietikainen et al. (2000), who found a pH increase in a forest humus layer under biochar. In addition, Güereña et al. (2015) found pH to increase with biochar additions in a pot experiment using Acrisol, DeLuca & Sala (2006) reported higher pH across frequently burned stands in a temperate forest, and Van Zwieten et al. (2010) found application of 10 t biochar ha^{-1} to increase soil pH of a Ferrosol from 4.2 to 5.4. Whereas, Chan et al. (2007) did not find differences in pH when applying biochar at the same rate in an Alfisol.

As mentioned in the literature review, several mechanisms have been proposed to justify changes in microbial biomass in response to biochar amendment. These mechanisms include for instance pH changes (Steiner et al. 2004) and increased colonizable surfaces, that is linked to microorganisms protection against leaching (Pietikainen et al. 2000) and desiccation (Lehmann et al. 2011). Surprisingly, the treatments with biochar application showed soil MBC,

MBN and microbial biomass C:N-ratio almost identical to the control soil. Additionally, no relationship was observed between MBC and moss biomass (Figure 9D). This outcome is contrary to the second hypothesis, and to what has been shown in earlier studies (Chan et al. 2007; Steiner et al. 2008; Jin 2010; Liang et al. 2010), especially to that of Kolb et al. (2009), who found microbial biomass to increase with increasing biochar application in four different soils tested, one of them being a Spodosol. Dempster et al. (2012) found MBC to be higher without biochar addition, whereas MBN remained unaltered, when applying 5 and 25 t biochar ha^{-1} in a Grey Orthic Tenosol.

Some previous studies have also found that biochar additions did not have significant effect on microbial biomass in forest soils (Zhang et al. 2014; Gundale et al. 2015; Noyce et al. 2015; Sackett et al. 2015). Moreover, Bruun et al. (2012) found no differences concerning soil MBC and MBN between a sandy loam soil amended with slow pyrolysis biochar and control. In agricultural soils, it has been observed that microbial biomass increases only with biochar additions of 30 t ha^{-1} (Domene et al. 2014), in which moisture is the main driver for increased MBC. As mentioned previously, it may be that the effect of biochar on mineral layer is more evident than on humus layer. In regards to microbial responses, this study agrees to what has been proposed by Noyce et al. (2015), that such responses are delayed and occur mainly in the mineral soil. Another possible explanation for the negligible change in microbial biomass is associated with the abundance of the labile fraction in the biochar. In other words, most of the biochar could have been to be recalcitrant and relatively non-bioavailable.

Based on the results of measurements carried out in May, August, and September of 2015 in the same plots of the present study (unpublished data), the mean MBC on plots amended with biochar was the same than on control plots (4.5 mg g^{-1}). The mean MBN mirrored MBC means, remaining approximately 0.5 mg g^{-1} in all treatments. These results are likely to be related to the abundance of the labile fraction in the biochar used in this study. Bruun et al. (2011) demonstrated that soils amended with slow pyrolysis biochar developed similarly

to unamended soils regarding MBC. Zhang et al. (2014) observed the same trend when using high temperature (700 °C) biochar. High temperature (Zimmerman 2010; Kloss et al. 2012) and slow pyrolysis biochars (Smith et al. 2010; Wang et al. 2015) have been previously described as more stable material. Even though only one type of biochar was tested, it can be assumed that it was composed of a rather recalcitrant C form, as it considered to be true for wood biochars (Kloss et al. 2012). As a result of high C:N-ratio of biochar, no evidence was found for the third hypothesis that MBN would increase with biochar additions.

The measured BNF in both moss mass basis (0.12–0.22 µg N g^{-1} moss d^{-1}) and sample mass basis (0.05–0.07 µg N g^{-1} sample d^{-1}) was lower compared to other studies, 1 ~ 4 µg N g^{-1} moss d^{-1} (Stuiver et al. 2016), ~40 µg N g^{-1} moss d^{-1} (Bay et al. 2013). This discrepancy could be attributed to the BNF measurements. In most studies a defined number of moss shoots is sampled and incubated (DeLuca et al. 2002; Ackermann et al. 2012; Leppänen et al. 2013; Stuiver et al. 2016), whereas in this study ground vegetation and organic layer were sampled and incubated altogether, as possible BNF performed in the forest floor (soil and vegetation together) was of interest. Even though the methodology of this study accounted for fixation on moss mass basis, the moss dry mass included all three moss species (*P. schreberi*, *H. splendens* and *D. polysetum*), of which *D. polysetum* is not known to be colonizable by Nostoc (Bay et al. 2013).

The measured BNF rates in aerial (9.57–11.06 µmol m^{-2} d^{-1}) and annual basis (0.43–0.59 kg N ha^{-1} yr^{-1}) were at the lower end of the range of those of previous studies in boreal forests, 0.74 ~ 495.0 µmol m^{-2} d^{-1} (Ackermann et al. 2012; Leppänen et al. 2013), and 0.3 ~ 1.0 kg N ha^{-1} yr^{-1} (Stuiver et al. 2016). However, one of the most comprehensive studies regarding BNF for the Fennoscandia Peninsula reported higher annual average rate, 2.1 kg N ha^{-1} yr^{-1}, than the one verified in this study (DeLuca et al. 2002). This result might be explained by the fact that moss carpets are less abundant in early successional forests, as showed by DeLuca et al. (2007) and Zackrisson et al. (2004), where the average annual BNF is estimated to be below 0.5 kg ha^{-1} yr^{-1}.

Contrary to expectations, this study did not find a significant difference between the application rates of biochar, 0, 5 and 10 t ha^{-1}, on BNF. Enhancement of BNF have been commonly reported in the agriculture field (Rondon et al. 2007; Oka & Rungrattanakasin 1993 cited in Ogawa & Okimori 2010; Mia et al. 2014; Van Zwieten et al. 2015). There are some mechanisms proposed to explain increased N fixation in leguminous plants (Mia et al. 2014). Two of the ones that can be extended to moss-cyanobacteria associations are covered in this study: increasing soil pH, and input of nutrients through biochar. It was hypothesised that better growth conditions for mosses and microbes would lead to higher BNF. Even though there was a pH increase in plots amended with biochar, no relationship between pH and BNF was found. According to the fourth hypothesis, increases in BNF were expected to follow increases in MB and moss biomass. Even though no significant changes were observed in those variables, which could justify the lack of changes in BNF, no relationships were found between the variables and BNF (Figure 9).

According to Vitousek et al. (2002) and Rondon et al. (2007), higher rates in BNF were related to greater availability of P, K, Fe, Ca, and Mo, which are important nutrients for rhizobia. In this study, biochar amendment promoted the addition of several nutrients (Table 2) to the forest floor, including P, K, and Fe. In tropical rainforests, both Mo and P availability have been shown to be limiting to free-living N fixation (Reed et al. 2013). On the other hand, it has been previously shown for boreal forest that bioavailability of N might affect BNF negatively, even to the extent of 4.5 kg N ha^{-1} yr^{-1} (Zackrisson et al. 2004). Based on the chemical characteristics of the biochar used in this experiment (Table 2), it included addition of 12.5 and 25 kg N ha^{-1} to the soil in T2 and T3, respectively. The rate at which N becomes bioavailable may or may not affect BNF negatively. Further studies, which take this variable into account, will need to be undertaken.

Biological nitrogen fixation rates and microbial biomass were found to increase with temperature. These results support previous findings that BNF is temperature limited (Gentili et al. 2005; Gundale et al. 2012a). Nonetheless, it

should be highlighted that the level of light intensity at moss surface is an important factor to guarantee the N fixation yields (Sorensen et al. 2012).

5. Conclusions

The present study was designed to determine the effect of biochar on biological nitrogen fixation and on soil microbial biomass in a boreal forest in Finland. These effects were further analysed under the light of changes in soil pH, moss biomass, as well as the chemical characteristics of the added biochar.

The medium-term effects of biochar in the studied soil were restricted to higher soil pH in plots amended with 10 t biochar ha^{-1}, and higher dominance of *P. schreberi* in plots amended with 5 t biochar ha^{-1}, whereas no differences were verified for moss biomass, microbial biomass, and biological nitrogen fixation. Observed changes in pH are often likely to justify variations in the rates of BNF and MB, however in this study they were not shown to be of significance. It is possible, however that biochar will have a positive effect on soil vegetation as it is incorporated into the soil in the long-term. Further research could usefully explore how these changes are to affect soil biota in the future.

Notwithstanding, this research extends the knowledge of BNF and MB rates in boreal forests, and will serve as a base for future studies concerning applying biochar on forests. For instance, it would be interesting to compare biochars with different furnace residence times in future researches.

Although this study focuses on BNF and MB, the findings may well have a bearing on the use of biochar as a tool for C sequestration, since amendment with biochar was demonstrated as neither beneficial nor harmful to the soil biota.

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