

**MICROBIAL BIOMASS IN LIMED AND PERTURBED AREAS IN
SANDY GRASSLAND SOILS IN SWEDEN /1**

BY

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SCIENCES, IN PARTIAL FULFILMENT FOR THE DEGREE OF
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DECLARATION

Declaration by the student

I, Mutonga Mumbi, hereby declare that this is my original work and has not been presented for a degree in any other university to the best of my knowledge.

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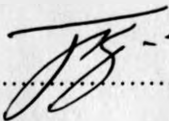
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Dedication

This research is dedicated to year 2010 and my family.

Acknowledgements

First, I would like to thank God for His guidance, protection and strength.

I'm indebted to my supervisors; for their guidance, and supervision throughout the project; I would not have gone this far without their help.

My family, for their support, patience and understanding and not forgetting my friends for their encouragement throughout the study period and Prof. Kinyamario for some financial support.

Finally, Lund University, staff, especially Linda-Maria in Department of Plant Ecology & Systematics, Microbiology Department and Linnaeus- Palme Exchange Program for sponsorship making this project possible.

God bless you all.

ABSTRACT

This study was done in sandy grasslands in eastern Skåne, Sweden, where three experiments were carried out to investigate the effect of changed soil conditions brought about by liming and soil perturbation on different microbial functional groups and how this change affects phosphorous in restoration of sand steppe vegetation in sandy grasslands. In the first experiment, liming was done in three strips of about 0.5 by 3m in June 2007 in order to elevate the soil pH in Rinkaby in a military training area. Sampling was carried out within and outside the strips (controls). In the second experiment, also in Rinkaby area, eight plots of 8 x 8m were delineated; perturbation by soil mixing was done in four of the plots and liming and soil mixing was carried out in the other four. In the third experiment in Lyngsjön area four replicates were carried out along a pH gradient and in each replicate 4 samples were collected. From each experiment, roots and soils samples were collected and amount of mycorrhizal fungi, saprophytic fungi and bacteria tissues were analyzed. Analysis was done using fatty acids and soil chemistry techniques. Biomass estimation was primarily done using the phospholipids fatty acids (PLFAs) technique. Soil chemistry analysis was done to determine the nutrients especially phosphorus (P) and the pH of the soil

Although there were changes in the microbial community, there was no significant changes in limed and non-limed soils in Experiment 1, except for the arbuscular mycorrhizal roots ($t(11) = 2.283, p=0.035$). In Experiment 2 there was also no significant changes in microbial biomass in the limed and perturbed plots and the perturbed plots alone, except in arbuscular mycorrhizal; AM roots ($t(14) = 2.253, p= 0.014$); bacteria ($t(14) = 1.090, p = 0.346$); Saprophytic fungi ($t(14) = 0.708, p= 0.506$). In Experiment 3 a regression line of $[0.08, 2.43 \times (\text{value of pH and P respectively}) - 0.485; r = 0.771]$ was observed showing correlation between arbuscular mycorrhizal in roots and pH and consequently phosphorous. For the saprophytic and bacteria the correlation was very weak, $r = 0.344$ and 0.396 respectively. There was higher microbial biomass of the functional groups in the limed and perturbed plots in comparison to perturbed plots only, having ratios of 14:1, 32:1 and 6:1 for bacteria, AM fungi and saprophytic fungi respectively. The arbuscular mycorrhizal fungal community changed upon pH shift in all of the three experiments. A weak tendency relationship between pH and P was observed in Experiment 2 and 3.

Due to the weak tendencies relationship observed between pH and microbial biomass, the reason for the changes in the microbial community cannot be explained by pH alone, it's therefore important to know how pH affects other factors such as mineralization and solubilisation of nutrients, substrate quality and soil properties.

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List of Acronyms

AM	-	Arbuscular Mycorrhizae
P	-	Phosphorous
NLFA	-	Neutrolipids Fatty Acid
PLFA	-	Phospholipids Fatty Acid
SF	-	Saprotrophic Fungi
C	-	Carbon
N	-	Nitrogen
NPP	-	Net Primary Production

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Grasslands are one of the most widespread vegetation types worldwide, covering nearly one-fifth of the world's land surface ($24 \times 10^6 \text{ km}^2$) and containing more than 10% of global soil carbon stocks (Eswaran *et al.*, 1993). Grassland soils represent a significant carbon pool of the order of 200 - 300pg. Grassland ecosystems store most of their carbon in soils, where turnover times are relatively long (100 – 10,000 years) and so changes, though they may occur slowly, will be of significant duration.

Processes that appear critical to soil carbon dynamics but warrant further research before incorporation into ecosystem models include: below-ground carbon flux and its partitioning among roots, mycorrhizas and exudates; microbial community effects on C sequestration and the effects of temperature and labile C on decomposition. The controls over and consequences of these processes are still unclear at the ecosystem scale (Scurlock and Hall, 1998). These processes include plant and rhizosphere effects on soil respiration, the effect of microbial communities (e.g. mycorrhizas) on productivity and soil-C turnover and interaction among element cycles (e.g. the role of organic nitrogen [N] as a C and N source to microbes). However, current models of the global C cycle seldom explicitly include these processes (Scurlock and Hall, 1997).

Global change should include soil microbiological processes operating over square millimetres of root-mycorrhiza-soil interfaces below ground. This allocates considerable amounts of additional carbon to roots and soil organic matters.

Consistent and complete information is required on the net primary productivity (NPP) of all biomass, including above and below ground detrital inputs to 50m, since transient or long term soil carbon turnover may provide a route for CO₂ sequestration (Trumbore *et al.*, 1996; Bird *et al.*, 1996). Understanding the fate of stored carbon and its potential for anthropological manipulation is critically important if we are to manage the foreseen global change (Tan and Bakwin, 1995; Mellilo *et al.*, 1996).

Changes in processes like land cover, species composition and/or Nitrogen deposition have significant and long lived effects on global Carbon cycles and on the soil-carbon pool. Inclusion of these processes in climate-carbon cycle models would improve their capacity to stimulate current and future climatic changes.

Most grasslands plants form arbuscular mycorrhizal (AM) associations. According to Read (1991) AM is the dominant mycorrhizal type in grasslands because such areas have a high pH where limestone material immobilizes P and makes it very limiting to plant growth. Physiological experiments have shown that the AM fungi are particularly good at taking up inorganic forms of phosphorous (P). However, many grasslands and agricultural soils are highly acidic and it is questionable if AM fungi are as important in these acidic soils since P is less limiting here.

The microbial community is likely to differ between alkaline and acid soils because of nutrient availability in these soils is different, and the abundance of different soil organism groups was therefore investigated. The arbuscular mycorrhizal (AM) fungal community is also expected to change upon a drastic pH shift, and was thus investigated.

Sandy grasslands in eastern Skåne, Sweden, have developed upon glaciofluvial sand, wave-washed sand and glaciolacustrine sediments. In most places the original material contains limestone. The sand-steppe is one of the most rare and threatened plant communities in Sweden, restricted to calcareous sandy soils in eastern Skåne and Öland (Mårtensson and Olsson, 2010).

The most typical plant species of the community is the grass *Koeleria glauca*. This ecosystem type is characterized by broken vegetation and is dependent on regular disturbances of the surface soil layer. On slopes this may occur through the trampling of animals while the biotope on plane areas depends on regular cultivation of the soil. Rabbit burrowing activities are important in some nature reserves (Mårtensson and Olsson, 2010). When disturbance ceases the pH of the upper soil layer decreases, a process enhanced during last decades through the overall soil acidification (Olsson *et al.*, 2009). A general study done in 2004 showed that the pH shifts are dramatic (Olsson *et al.*, 2009) and the vegetation is changing with the changes in soil pH and availability of phosphorus (Mårtensson and Olsson, 2010). Where lime still remains the pH is between 8 and 9, while on most sandy grasslands the pH has dropped to around pH 5. On a horizontal scale these drastic pH shifts may occur within a few meters.

The sandy soils in eastern Skåne, provide an excellent natural field site to investigate pH effects on mycorrhizal colonization since there is little influence of other factors (such as organic matter and various buffering agents) than that of lime. In this general area of Skåne, only small fragments of alkaline sandy soil remain (less than 50 ha) while large areas are naturally (mainly) acidified and have a pH several units below the original material, leading to changed vegetation structure from sand steppe to sand heath.

Nitrogen deposition, which have effect on bacteria and saprophytic fungi, and acidification are the major processes that are affecting species composition and land cover in eastern Skåne (Olsson *et al.*, 2009; Hobbie and Hobbie, 2006)). In the restoration of sand steppe vegetation liming and disturbance have been recommended (Olsson *et al.*, 2009). Since *K. glauca* is an arbuscular plant thus relies on arbuscular mycorrhizae for phosphorous absorption, the effect of these processes on microbial community was investigated. Changes in microbial community may led to changes in NPP of the below ground and consequently long term soil carbon turnover which will provide route for CO₂ sequestration. Studies on CO₂ sequestration are on going on this site.

1.2 *Koeleria glauca*

Koeleria glauca is a high pH, low nutrient-tolerant, highly tufted, perennial grass (Ellenberg, 1974), which spreads by vegetative intravaginal tillering (Klimesova and Klimes, 2006). It is more or less restricted to sand steppes and is nationally protected through the Swedish red list. *K. glauca* has preference for dry and acidified areas, nitrogen deposition, and disturbance are important factors in sandy grassland (Mårtensson and Olsson, 2010). Both acidification and N deposition threaten the remaining sand steppes, and these factors were also shown to induce community changes at a distance smaller than 10 meters (Mårtensson and Olsson, 2010).

K. glauca is probably favoured in the soils containing high amounts of lime and/or low amounts of plant-available P. This indicates either tolerance to, or preference for, high lime content and/or low P availability of *K. glauca*. The fact that *K. glauca* also grows in soils with pH below 7, and that the grass community seems to be mainly stress-regulated, indicates that the it tolerates high lime content, and/or low P availability since phosphorous often limit plant growth in calcareous soils (Tyler, 1992).



Plate 1: *Koeleria glauca*

1.3 Effects of perturbation on soil

Soil disturbance leads to decrease in above ground biomass and increase the cover of bare ground (Gendron and Wilson, 2007). Soil disturbances in Skåne often results in increasing lime content and soil pH at the soil surface, which influences the succession from sand steppe vegetation to heath. The intensity of disturbance which favours *K. glauca* growth needs further investigation (Mårtensson and Olsson, 2010).

Disturbance-related shifts in the proportion of ectomycorrhizal to AM plant species may impact soil glomalin concentration, which in turn may be regulated somehow by arbuscular mycorrhizal fungi species composition and abundance (Violi *et al.*, 2007).

1.4 Effects of liming on phosphorous

Liming improves considerably the indices of soil fertility including the pH value, cation exchange capacity, buffer properties, exchangeable calcium ions and magnesium ions percentages, the humus content and contents of phosphates and humates of calcium. The after

effect of liming may last up to 11 years and ensures high levels of soil fertility if the soil is not treated with nitrogen and potassium fertilizers during this period (Mineev and Gomonovan, 2001). The increased content of phosphates and humates of calcium in limed soils is due to a considerable decrease in intensity of leaching (Mineev and Gomonovan, 2001)

Many forage crops are known to be intolerant to soil acidity, and it is for this reason that liming materials have a long history of use (Isabella and Hopkins, 1994). Liming increases phosphate availability in the short term due to an increase in pH resulting in the hydrolysis of strengite and variscite with the release of phosphate ions into soil solution. Liming also reduces the concentration of soluble and exchangeable Fe and Al ions which otherwise could react with added fertilizer phosphate to form sparingly soluble Fe and Al phosphates (Haynes, 1982). Thus liming increases P uptake of plants by decreasing Al toxicity rather than effect on phosphate availability per se (Vickers and Zak, 1978). Furthermore, liming may affect phosphate availability by a third mechanism through influencing microbial activities in soils and thereby the net mineralization or immobilization of soil organic P.

In southern Sweden, sandy soils grasslands show a particularly high variation in soil pH over short distances owing to limited buffering in the silicate and cation exchange (Olsson *et al.*, 2000). Sandy grassland occurs on dunes and glaciofluvial deposits, often near the coast (Olsson, 2000). In certain areas, the sand contains calcium carbonate, in which case an alkaline pH prevails, while other sandy soils are strongly acidic. Sandy soils with intermediate pH appear to be rare (Tyler, 2005), which is probably due to the low base cation buffering capacity of sand once the original lime has been depleted. Cation exchange capacity is very low as a result of the low content of clay and organic matter in sandy soils (Olsson *et*

al., 2009). Lime content has a very strong influence on exchangeable P, and when lime is absent, the P availability switches from being available to unavailable to plant. There is a negative correlation between P availability and pH (Olsson *et al.*, 2009).

1.5 Factors that affect microbial community

Leaching, common in sandy soils, resulting in the loss of base cations and associated reduction of pH all contribute to the production of a syndrome in which mineralization of nutrients is inhibited so that organic matter accumulates as raw humus. This lead to formation of organic acids occurring in their most toxic undissociated form and metallic cations show maximum solubility. The final product is a heath land or tundra ecosystem in which a small number of the most stress tolerant species of plants and microorganisms become dominant.

It is assumed that the initial pH of litter substrate also influences microbial community structure (Blagodatskaya and Anderson, 1998). Prevailing soil pH has a significant influence on total microbial biomass build-up. Initial microbial mineralization processes, and particularly lignin degradation, are dependent on carbon availability which decreases under low pH (Pearson *et. al.*, 1989). In summary, fungi can sustain low pH better than bacteria; they are also known to use the available carbon substrate more efficiently (Blagodatskaya and Anderson, 1998).

1.6 Interactions between pH, microbial community and soil nutrients

Soil pH is a property that affects species distribution and success of plants and soil microorganism (Persson *et al.*, 1991). Soil microbial biomass is a small but labile component of the soil organic matter. It is thought to exert a key controlling influence on the rate at which N, C, P and other nutrients cycle through the ecosystem (Lovell *et al.*, 1995). It has been proposed that liming results in increased microbial biomass and immobilization of N through microbial assimilation (Adams and Cornforth, 1973). Microbial biomass is the chief

biotic regulator in the decomposition of acid organic soils (Swift *et al.*, 1979). Increases in microbial biomass by liming may favour immobilization of N and account for depressed net N mineralization after liming acid peat soils, more humus or raw humus (De Boer *et al.*, 1993).

Varying soil pH may affect the development and functioning of arbuscular mycorrhizal fungi (Abbot and Robson, 1985). Soil pH also changes the concentration of many nutrients in the soil solution as well as leading to higher concentrations of hydrogen ions making the soil toxic (Russell, 1973). In acidic soils, there is often higher concentrations of aluminium and manganese and lower concentration of calcium, magnesium and molybdenum in soil solutions than in alkaline soils (Porter *et al.*, 1987). Again according to Porter *et al.* (1987) it is unlikely that the germination of fungi and hyphal outgrowth caused by acidifying the soil solution, results from an induced deficiency of a factor such as phosphorous or molybdenum. According to Read (1991), the major role of the distinctive mycorrhizal symbioses in the various terrestrial biomes is to provide plants with access to key growth limiting nutrients at crucial stages in their development. On a global scale, the differentiation between biomes dominated by plants species with arbuscular-, ecto- or ericoid- mycorrhizal occurs along a gradient of increasing accumulation of organic matter which essentially involves major changes in the quality rather than quantity of nutrient resources (Read, 1991). The associated increases in the ratios of C: N and C: P inevitably leads to greater recalcitrance of the mineral nutrients (Read, 1991) and to the development of selection pressures favouring mutualistic association between plants and specialized heterotrophic partners which have access to the key resources.

According to Anderson and Domsch (1994), fungi respiratory activity increases under acidic conditions while that of bacteria decreases. Soil pH is one of the major independent driving

variables which strongly influence abiotic factors such as C availability and thus the composition of the microbial community. The additive effect of pH and substrate on fungi to bacteria ratio is but small, nevertheless ecologically significant.

Frostegård *et al.* (1993) suggested that increasing pH levels affects the soil microbial community structure. Liming has been shown to affect soil microbial biomass and activity (Persson *et al.*, 1989; Fritze *et al.*, 1993; Bååth and Arnebrant, 1994). These changes could be related to increases in the pH of soil due to liming treatments. Moreover, pH related changes in response to pattern of thymidine incorporation at different pH values were found for bacterial community (Bååth and Arnebrant, 1994). The influence of soil pH was further implicated for simultaneous changes in the phospholipid fatty-acids and pH response patterns in an area where pH had increased due to alkaline dust deposition (Bååth *et al.*, 1993). Bååth *et al.* (1994) stated that fungi are affected by increased pH due to ash application or liming. In their paper, Rousk *et al.* (2009) suggested that pH strongly influences abiotic factors such as C availability and that in turn affects the fungal and bacteria growth. Microbial biomass, especially for fungi and bacteria, is affected by many factors, for example soil management e.g. fertilization, liming and animal presence, has potential to directly affect the bacterial and fungal composition, with consequent impacts on soil function (Rousk *et al.*, 2009).

1.7 Arbuscular mycorrhizal (AM) fungi and Phosphorus (P) uptake

The fungus appears to be able to absorb P at lower concentrations than does uninfected plants roots (Anderson and Domsch, 1994; Aarle *et al.*, 2003) AM fungi have active phosphatase activity. In their experiment Anderson and Domsch (1994), have shown that phytate, an organic P source, is decomposed by AM fungi, and that the phytate P is taken up.

Polyphosphate make up a significant portion of the P of mycelial strands, and it could be translocated via stream both in the AM hyphae and in the sheath of ectomycorrhizal (EM).

Low insolubility of phosphorous results in low internal concentrations thus allowing for its continued uptake from the soil solution, translocation is proportional to root P concentration. The measurement of the precise amount of soil solubilized P by either roots or soil organisms is complicated by the concomitant mineralization of organic P (Bardgett, 2005).

1.8 Factors affecting P availability

More than 170 Phosphorous containing compounds have been identified in soils (Holford, 1997). The fraction dominating a certain soil is determined by factors such as primary soil properties, soil acidity and rate of organic matter decomposition affects P but, available P to plants is primarily governed by those soil factors influencing P solubility, such as pH, soil moisture and texture, as well as the specific and differing capacities of plants to render P fraction available for uptake (Tyler, 1992). Plants may also alter the equilibrium between different P compounds in the rhizosphere by exuding P-solubilising compounds (Maschner, 1995). Many plants growing in acidic soil exude oxalic acids and this also may be the mechanism for solubilising P in calcareous soil (Ström, 1997; Tyler and Ström, 1995).

The organic P fraction represents a major pool of P in many soils. For example, up to 57% of the total P in acid grasslands soils in Sweden was found to be in organic forms (Tyler and Olsson, 1993). Association between plants and micro-organism may influence P translocation in the rhizosphere and thereby alter the availability of P to plants. AM can increase the influx of P in roots (Filter, 1995; Jakobsen, 1986; Sanders and Tinkers, 1971; Smith and Read, 1997) although a few studies have shown that the AM fungi may have different access to pools of organic P, so that the availability of P could be higher for mycorrhizal than for non-mycorrhizal plants (Jayachandran *et al.*, 1992; Tarafdar and Marschner, 1994). However, AM fungi are usually considered to take up P from similar fractions of soil as do plants (Bolan, 1991; Sanders and Tinker, 1971).

1.9 Research problem

Swedish sand steppe vegetation characterized by *Koeleria glauca*, a rare and threatened species in Sweden, is being replaced by heath vegetation due to decrease in pH which changes microbial biomass. Microbial biomass is highly involved in the carbon-cycling of grasslands, and with global change in mind, knowledge about the microbial communities in restoration areas is of vital importance.

1.9.1 Main objective

To investigate the effect of changed soil conditions brought up by liming and soil perturbation on different microbial functional groups, that is, arbuscular mycorrhizal fungi, bacteria and saprophytic fungi and how this change affects soil nutrients especially phosphorus in temperate sandy grasslands.

1.9.2 Specific objective

The specific objectives were:

- To determine the amount of AM fungi biomass in soil and roots
- To determine the amount of saprophytic fungi and bacteria in soil
- To determine amount of phosphorous in the soil.

CHAPTER 2

MATERIALS AND METHODS

3.1 Study area description

The study sites were sandy grassland in south-eastern Sweden in Rinkaby (55° 58' 0" N, 14° 16' 0" E) and Lyngsjön (55° 56' 36" N, 14° 11' 09" E) (Fig. 1). The topography is characterized by long, narrow primary rock horst-ridges. In most places the original material consists of limestone sand. The Skåne province has maritime climate, especially the south and east coasts, where the study areas were located. The average temperatures are around 16.8 °C in summer and winter is -0.2 °C with an annual precipitation of between 500 and 800 mm. The sand-steppe is one of the most rare and threatened plant communities in Sweden, restricted to calcareous sandy soils in eastern Skåne and Öland.

The ecosystem type is characterized by broken vegetation and is dependent on regular disturbances of the surface soil layer (Plate 2). On slopes, these disturbances may occur through trampling by animals while the biotope on plane areas depends on regular cultivation of the soil. Rabbit activities are important source of disturbance in some nature reserves of the biome. It has been observed that when disturbance ceases, the pH of the upper soil layer decreases; a process enhanced during the last decades through the overall soil acidification. Preliminary survey during 2004 showed that shifts in soil pH are dramatic. Where lime still remains, the pH ranges 8 and 9 while on most sandy grasslands the pH has dropped to around pH 5. On a horizontal scale these drastic pH shifts may occur within a few meters (Mårtensson and Olsson, 2010).

3.1 The Study Sites

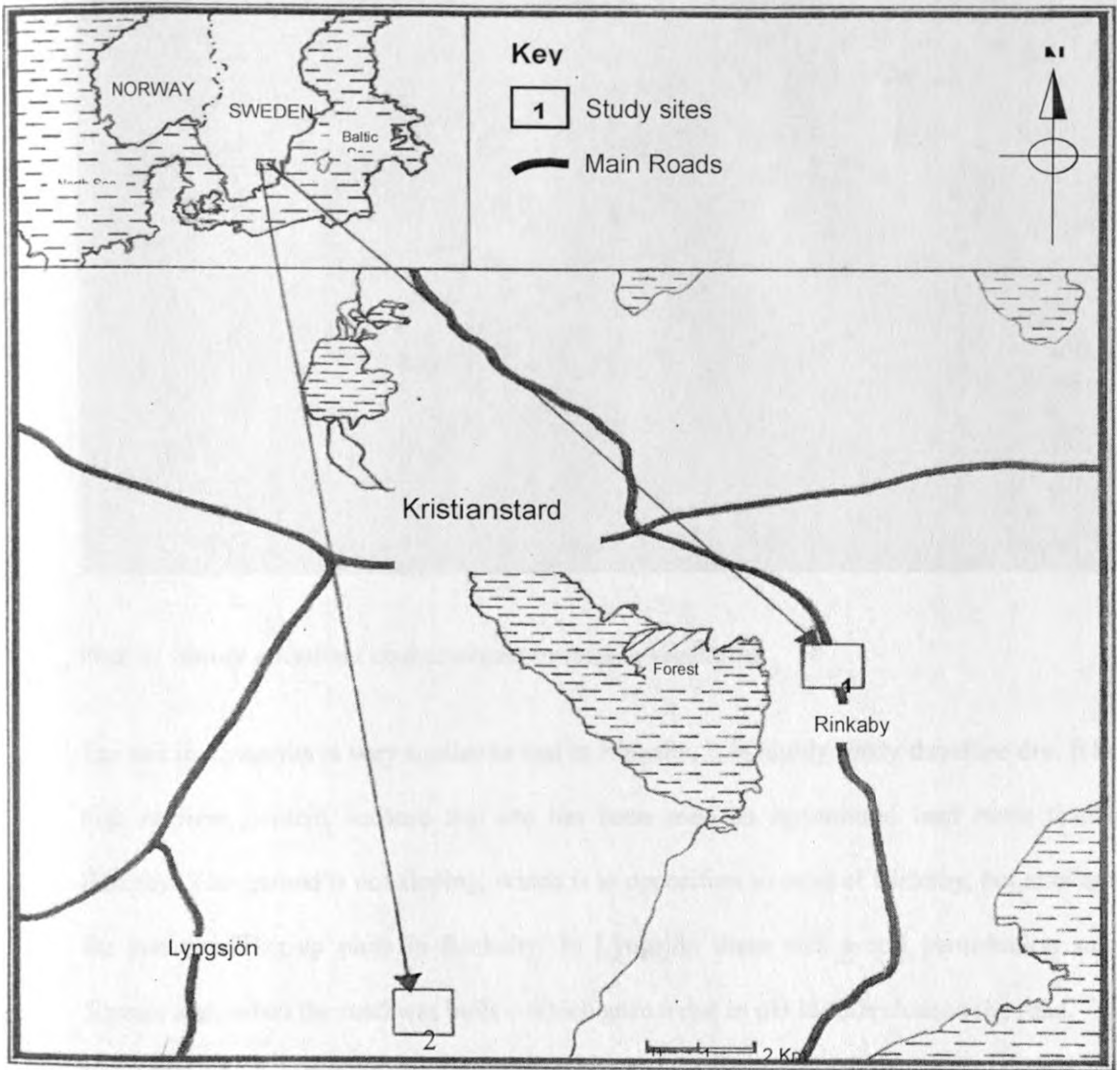


Figure 1: A map showing the study sites 1. Rinkaby 2. Lyngsjön

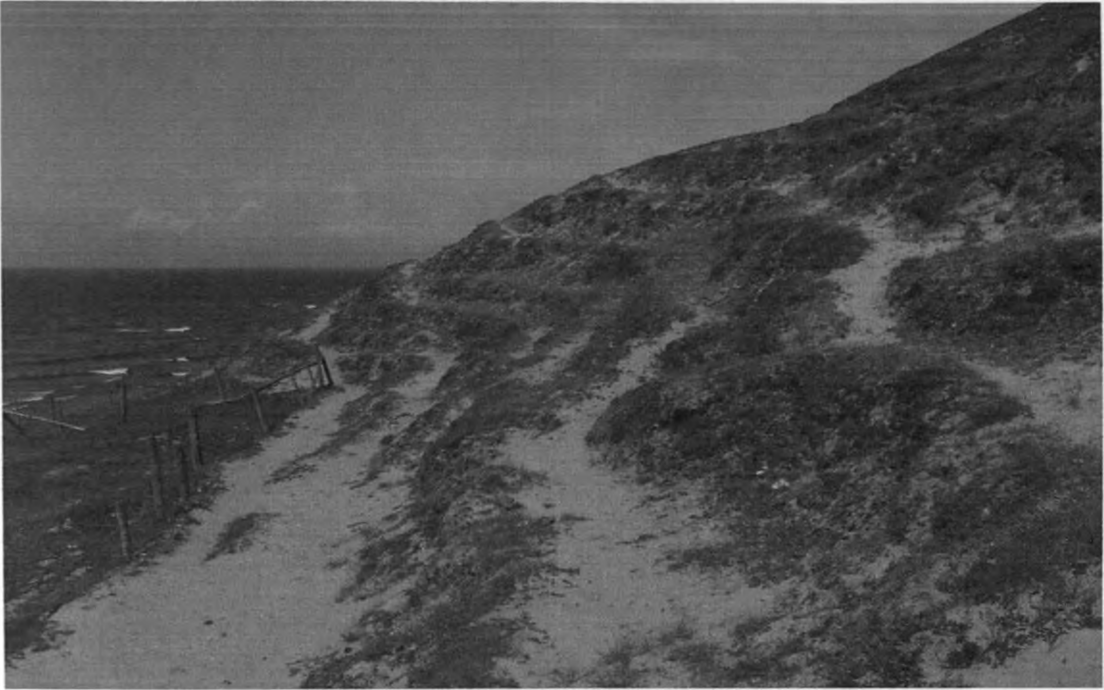


Plate 2: *Sandy grassland characterized by broken vegetation*

The soil in Lyngsjön is very similar to that in Rinkaby. It is highly sandy therefore dry. It has high nutrient content because the site has been used as agricultural land more than in Rinkaby. The ground is not sloping, which is in opposition to most of Rinkaby, but similar to the perturbed\dug-up plots in Rinkaby. In Lyngsjön there was a soil perturbation some 50years ago, when the road was built – which gave a rise in pH in soils close to the road. The Lyngsjön site is rather close to Rinkaby site about 15 km, so the climate is similar. The species under study in this site was *Pilosella officinarum* (Plate 3). The plant is from the Compositae family and requires well-drained soils and can grow in nutritionally poor soil. The plant also prefers any pH levels (acid, neutral and basic soils). The plant cannot grow in the shade and requires dry or moist soils.



Plate 3: *Pilosella officinarum*

2.2 Experimental design

In Rinkaby, a liming experiment had been done earlier. In June 2007, three strips of about 0.5 m by 3 m were limed in order to elevate soil pH (Olsson 1999; Olsson *et al.*, 1995) (Fig. 1). The vegetation within and outside the strips (controls) were sampled to analyze the amount of mycorrhizal tissues and bacteria in the soil by taking three subsamples from each strip and outside the strip.

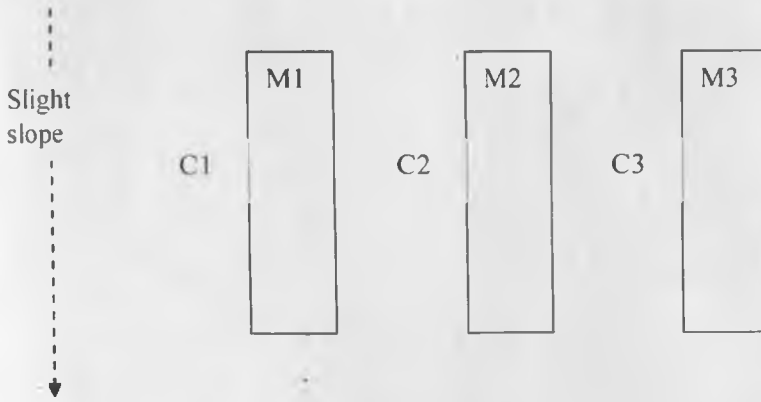


Figure 2: Experimental layout of limed plots (M) and non-limed plots (C).

Eight plots of 8 x 8 m were delineated, separated by 4 m corridor in Rinkaby. In four plots liming and soil disturbance were applied, and in the other four soils disturbance only was applied. The two treatments were systematically assigned among the eight plots (Fig. 2). Disturbance was through mechanical means created by tilling (Plate 4). This was done to find out the effect of disturbance and liming and disturbance only on the microbial biomass and vegetation. Soil and root samples from each plot were collected at random locations, but 1m away from the edges of the plots, from the upper 10 cm of soil and five samples of soil and roots were collected from each plot to estimate microbial biomass and phosphorous.



Plate 4: *creation of 8 x 8 m plots and soil mixing*

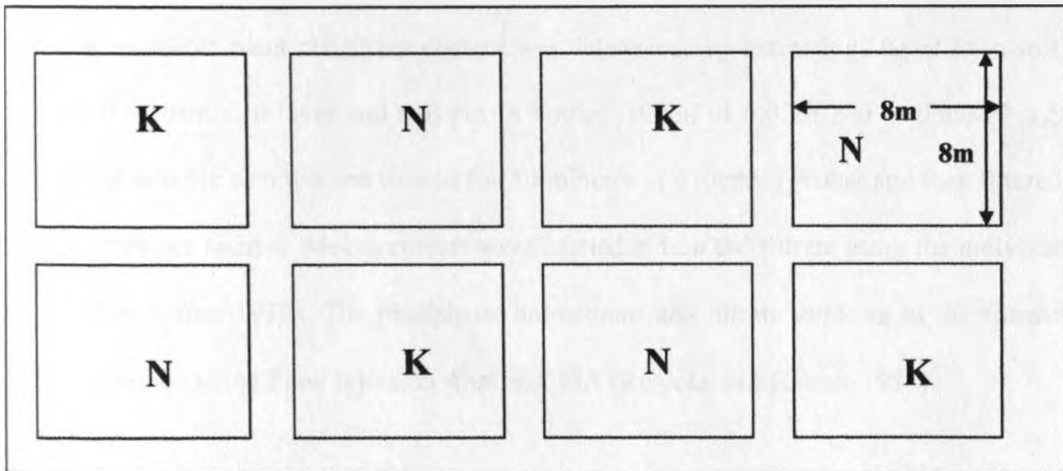


Figure 3: Experimental layout for liming and soil mixing (K) and soil mixing only (N) treatments.

In Lyngsjön, where there was a pH gradient four replicates were carried out and carbon labeling was done using Carbon 13 (^{13}C). The plants of interest were marked and using a clear, plastic containers were hooded and the ^{13}C introduced using injection into hooded area

of the plant in three places, along the four replicates. Roots and soil samples were collected after 3 hours. Using a shovel, the plant was scooped with the surrounding soil and put in labeled plastic bag for transportation to the laboratory for analysis at Lund University, Sweden.

2.2 Soil nutrient analysis

Soil samples were collected from the three experiments. After identification of key plants, the plant and its roots were scooped by using a shovel taking care that the roots were not destroyed and then the soil near the plant roots was collected. The soil samples were frozen until analysis.

2.2.1 Chemical Analyses of the soil

The available plant phosphate content was determined by extracting 10g of fresh soil, in the top 0 - 10cm soil layer and soil put in bottles. 100ml of 0.02M NaF + 0.05M Na₂SO₄ was added into the samples and shaken for 30 minutes in a rotating shaker and then filtered for 10 minutes per sample. Measurements were carried out on the filtrate using the molybdate-blue method (John 1970). The phosphate, ammonium and nitrate contents in the filtrates were determined using Flow Injection Analysis, FIA (Ruzicka and Hansen 1981).

The calculations were done using the formula

$$(mg \div l) \times extraction\ volume \div dry\ weight\ of\ soil$$

2.4 pH analysis

Soil pH was determined by putting 10 g of fresh soil in 50 ml distilled water and shook for 1 hour. It was left to sediment for another 1 hour then the pH was measured using probe and

meter method (ORION Research microprocessor ionalyzer/901, Optilab –Bo Philip instrumentation AB, Stockholm, Sweden).

2.5 Biomass estimation in roots and soils

The roots were collected using a shovel where the whole plant was scooped plus its roots taking care not to destroy the roots from the subsequent species in the three experiments.

The roots samples were carefully washed and then put in clean plastic tubes. Stones and other materials in the soil were removed and the soil placed in clean plastic tubes. The above was done sample by sample. The samples were then freeze dried to remove all moisture.

Estimation of biomass in the soil and roots using phospholipids fatty acids and neutral lipids fatty acids was done following the steps below.

2.5.1 Extraction of lipids in roots material and soil;

A sample of 30 g freeze dried roots were weighed and milled in Eppendorf tubes. The milled roots were placed in 50 ml centrifuge test tubes (Teflon, with screw-caps).the Eppendorf tubes were rinsed with 2 ml of Bligh and Dyer [CHCl_3 : MeOH: citrate buffer (0.15M at pH 4.0. citrate buffer was prepared as follows; 31.25 g of citric acid in 1 litre of millipore water, the pH was adjusted with 40 pellets of NaOH it was mixed using magnetic stirrer without heating and was stored in fridge when not in use)] using a plastic pipette tip and poured into the (Teflon) test tubes. Another 8 ml of Bligh and Dyer was added and then vortexed for 2 minutes. Extraction was done for 2 hours at room temperature.

3 g freeze dried soil was weighed and placed in 50 ml centrifuge test tubes (Teflon, with screw-caps), 15 ml of Bligh and Dyer was added, then vortex for 1 minute and left for 2 hours at room temperature. Soils centrifugation (at 5000 rpm, 10 min) was done (in Labofuge 200

Heraeus Sepatech, Tillquist Analys AB, Stockholm, Sweden) and the supernatant transferred to a large glass test tube and the remaining soil was rinsed with 5 ml of Bligh and Dyer, vortex and centrifugation was done again. The supernatant was added to the earlier obtained supernatant.

Splitting of phases was done by adding 4 ml of chloroform using a pump and 4 ml citrate buffer using a plastic pipette tip, vortexed for 2 minutes and left overnight at room temperature for phases to separate.

The next day, 1.5 ml of lipid extract, which was in the lower phase, was transferred using Pasteur glass tips to small test tubes with screw cap, this was done twice and stored in freezer before evaporation.

Lipid fractionation was carried out and the different lipids separated into classes with increasing polarity. The extracted lipids were fractionated into neutral lipids, glycolipids, and polar lipids (phospho-) using the following steps;

1. Evaporation using a stream of nitrogen gas on a heating block at 40 °C. After evaporation 100 µl of chloroform (CHCL₃) was added to the evaporated lipid extract and vortex for 15 seconds.
2. Using a yellow pipette tip calibrated at 150 µl sucking twice before taking the sample to ensure that the entire sample is taken, the dissolved lipid extract was applied on pre-packed silicic acid (100-200 mesh, Unisil) columns. The test tubes were washed one time with 100 µl of CHCL₃, vortex for 5 seconds and applied to the silica columns.
3. Neutral lipids were eluated with 1.5 ml of CHCL₃ into small, burned (autoclaved) test tubes labelled N, (for neutral lipids). The columns were then moved to a trash

collector and 6 ml of acetone was added to the columns to eluate glycolipids. The columns were then moved to new small, burned (autoclaved) test tubes labeled P (for phospholipids) and sample number and 1.5 ml of methanol was added to the columns to eluate phospholipids. Both test tubes labeled N and P were stored in a freezer before evaporation.

2.5.3 Methanolysis (Transesterification)

Evaporation was done using a stream of nitrogen gas on a heating block at 40°C. In the neutral and polar lipids samples, 100 µl of methyl nonadecanoate (fatty acid methyl 19:0) was added as internal standard. Evaporation of the samples was done under a stream of nitrogen with no heating.

The samples were dissolved in 1 ml toluene: methanol (1:1) and vortexed for 5 seconds. 1 ml of freshly made 0.2 M KOH in methanol was added to the samples and incubated in a water bath at 37°C for 15 minutes and cooled for 20 minutes.

2 ml of hexane: chloroform (4:1, v/v), 0.3 ml 1M Hac and 2 ml water was added to the samples and vortexed for 1 minute. Using a bench centrifuge (Heidolph Instrument, Lab Equipments Sales, 2615 River Rd., Cinnaminson, NJ 08077, USA) the samples were centrifuged at 3000rpm for 5 minutes. The upper phase was transferred to small, burned test tubes and stored in a freezer before evaporation.

2.5.3 GC-analysis for quantification of fatty acids

Evaporation was done under stream of nitrogen without heating and the samples dissolved in 200 µl (for neutral fatty acids) and 150 µl (for phospho-fatty acids) of hexane and vortexed

for a few seconds for both roots and soil samples. Small vials were filled with 200 μ l hexane. The inner assembly was put in and filled with 25 μ l of the dissolved sample and capped.

The vials were analyzed on a gas chromatograph (Hewlett Packard 5890, 50 m HP5 capillary tube, Palo Alto, CA, USA) where the fatty acid methyl esters were separated by gas chromatography (flame ionization detector) using a 50-m HP-5 (phenyl methyl silicone) capillary column. Hydrogen was used as a carrier gas (Frostegård *et al.*, 1993).

Identification of the peak from the standard (19:0) was done, which was the same for both PLFA and NLFA data. The retention time of the standard was 19.458.

The relative retention time, which is the retention time of each fatty acid in relation to the retention time of the peak of 19:0, was used to find the other peaks as follows;

Identification of fatty acids followed the nomenclature of fatty acids that of Tunlid and White (1992). NLFA and PLFA 16:1w5 were analyzed and could be used as indicators of AM fungal biomass in roots (Olsson *et al.*, 1995; Graham *et al.*, 1995; Olsson, 1999) while 16:1w5 in NLFA indicates AM fungal biomass in soils (Olsson, 1999) since this fatty acid is normally not found in other fungi (Muller *et al.*, 1994). Fatty acid 16:1w5 is, however, also present in bacteria and the ratio between NLFA16:1w5 and PLFA16:1w5 was calculated, since a ratio >1 indicates this NLFA originates from AM fungi (Olsson, 1999). PLFA 18:2w6, 9 was used as an indicator of saprotrophic fungal biomass (Federle, 1986; Frostegård and Bååth, 1996; Olsson, 1999) and PLFA 18:1w9 (Bååth, 2003). Bacteria-specific PLFAs (i15:0, a15:0, 15:0, i16:0, 16:1w9, 16:1w7, i17:0, a17:0, cy17:0, 17:0, 18:1w7 and cy 19:0) were used as indicators of bacterial biomass (Frostegård and Bååth, 1996). The amount of PLFA 16:0 was used to estimate the general microbial biomass, while NLFA 16:0 in most cases is indicative of AM fungal biomass (Olsson *et al.*, 1995).

The values of chosen fatty acids were written in excel for recalculations into mol g⁻¹ of roots and soils:

AREA is the amount of a specific fatty acid that was detected by the gas chromatography machine

Microbial biomass was calculated using the below formula:

$$= \frac{\text{AREA for chosen fatty acid}}{\text{AREA for 19:0} \times 2300 \text{mg} \times (\text{X soil or root}) \div \frac{312 \text{ g}}{\text{mol}}}$$

Where:

- X for soils = total volume of chloroform in the extraction inclusive the splitting of phases = $(1 / (1+2+0.8)) * (15 \text{ ml} + 5 \text{ ml Bligh and dyer}) + 4 \text{ ml} = 9.263 \text{ ml}$
- X for roots = total volume of chloroform in the extraction inclusive the splitting of phases = $(1 / (1+2+0.8)) * (10 \text{ ml Bligh and dyer}) + 3 \text{ ml} = 5.632 \text{ ml}$.
- y = volume of samples taken out of the lower phase after splitting = $2 * 1.5 \text{ ml} = 3 \text{ ml}$.
- 2300mg is how much standard (19:0) added to the samples.
- 312 g mol^{-1} was the molar mass of fatty acid 19:0

The bacteria-specific PLFAs area were calculated individually and added into one group after calculations.

2.6 Statistical analysis

All statistical analyses were performed with SPSS version 17.0 To test for differences between means, paired T-test in the limed plots was used and independent T-test in the disturbed plots and one way ANOVA and correlation to test for relationship between microbial organism, P and pH for the experiment in Lyngsjön and Rinkaby.

CHAPTER 3: RESULTS

3.1 Experiment 1: limed strips and control

In the control plots the arbuscular mycorrhiza and bacterial biomass was higher than in limed plots, but the saprophytic fungi biomass was higher in limed plots than in control plots (Fig. 3). The ratio of control plots and limed plots for bacteria were 1:1 as for arbuscular mycorrhiza was 2:1 and for saprophytic fungi was 1:2. The range of pH in the control plots ranged from 6.09 to 6.72 while that of limed was 7.93 to 8.26 (Table 1). The phosphorous concentration in the control plots ranged from 0.347 μg to 0.408 μg while that in limed ranged from 0.339 μg to 0.372 μg (Table 1).

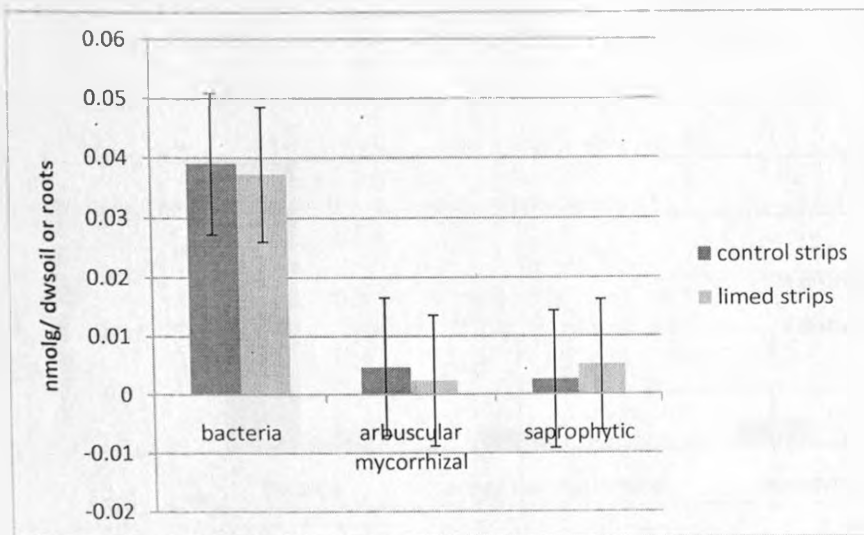


Figure 4: Bar graphs showing effects of liming on microbial community in the limed strips and controls and their SE

3.2 Experiment 2: limed and disturbed and disturbed area only.

The disturbed and limed plots had a higher microbial biomass in comparison to the disturbed plots (Fig. 4), having a ratio of 14:1, 32:1 and 6:1 for bacteria, arbuscular mycorrhiza fungi and saprophytic fungi respectively. In contrast to disturbed plots, a higher variation was observed in the limed and disturbed plots with K having 0.017 while N had 0.005. A narrow range of pH was observed in the disturbed plots ranging from 6.19 to 6.99 as compared to the limed and disturbed plots which ranged from 6.73 to 8.36 (Table 1). The phosphorous in the soil in the disturbed (N) was higher than in plots limed and disturbed plots (K), an average of 0,387 μg in N and 0, 291 μg in K.

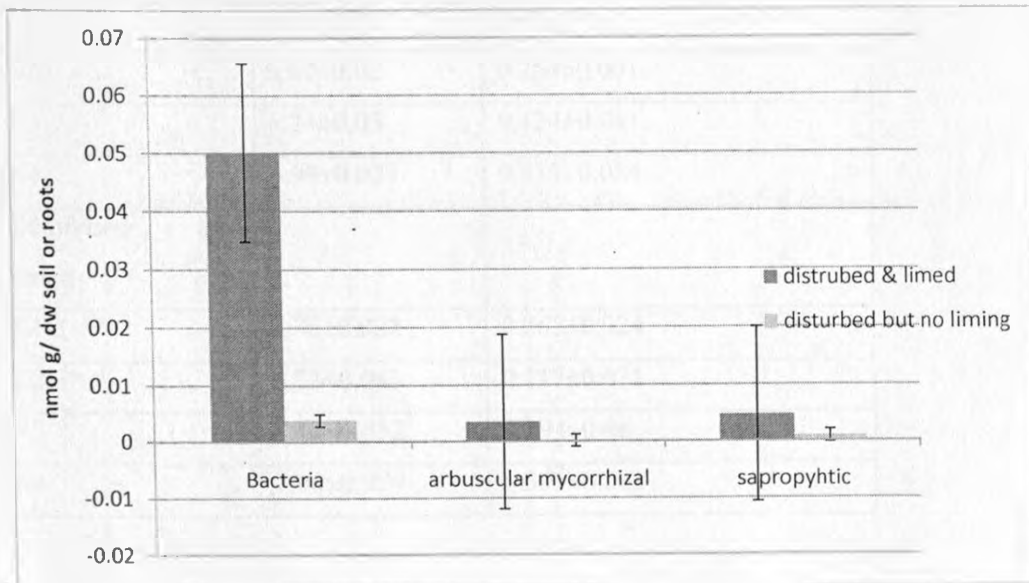


Figure 5: effects of liming and perturbation and perturbation on microbial community and their SE

Table 1 Mean of pH and P in the different treatments; M1 - M3; limed strips. C1 - C3; plots that have not been limed, N1 - N4; disturbed but not limed plots and K1 - K4; limed plots and disturbed plots.

Limed strips and controls	pH	P (μg)
M1	8.26 \pm 0.102	0.372 \pm 0.018
M2	8.22 \pm 0.1	0.339 \pm 0.025
M3	7.93 \pm 0.05	0.372 \pm 0.003
C1	6.27 \pm 0.188	0.367 \pm 0.009
C2	6.09 \pm 0.234	0.347 \pm 0.026
C3	6.72 \pm 0.2	0.389 \pm 0.07
Disturbed but no liming		
N1	6.19 \pm 0.04	0.452 \pm 0.01
N2	6.60 \pm 0.02	0.254 \pm 0.001
N3	6.24 \pm 0.03	0.424 \pm 0.041
N4	6.99 \pm 0.023	0.415 \pm 0.054
Disturbed and limed		
K1	8.36 \pm 0.028	0.262 \pm 0.024
K2	6.73 \pm 0.043	0.117 \pm 0.071
K3	7.77 \pm 0.032	0.294 \pm 0.06
K4	7.66 \pm 0.079	0.391 \pm 0.011

3.3 Experiment 3: Lyngsjön pH Gradient

The saprophytic fungi and bacteria decreased with increase in pH was not significant (Fig. 5).

The arbuscular mycorrhiza fungi increased with increase in pH (Fig. 6). There was negative tendencies between P concentration and pH although not so significant $p = 0.135$. The correlation between bacteria, arbuscular mycorrhiza fungi and saprophytic fungi with P

concentration was neither significant ($p = 0.35$ bacteria and 0.42 saprotrophic fungi) nor strong except in AM roots which had $r = 0.47$; $p = 0.12$ and also their correlation with pH was not significant. There is a positive tendency between arbuscular mycorrhiza and pH. No correlation was found among the microbial communities. The AM fungi showed an increase as pH increased while the saprophytic fungi and bacteria showed a slight decrease, almost constant; as pH increased although the pH range was narrow ranging from 5.42 to 6.35 (Table 2). Treatment 1 which was the closest to the road had the highest pH of an average of 6.27 and the lowest arbuscular mycorrhiza and the highest bacteria biomass among the four treatments (Table 2)

Table 2: Mean of pH (± 0.01), P (± 0.021) and microbial biomass over a pH gradient and after long time disturbance.

I	pH (P Conc. (μg).	PLFA soil (nmol g^{-1} dw soil)			NLFA (mg g^{-1} dw soil)	
			Bacteria	AM	SAPROPHYTIC	AM- roots	AM- soil
1	6.35	0.114	0.041	0.001348	0	0	0
2	5.69	0.112	0.020	0	0.007	0	0.019
3	5.68	0.165	0.048	0	0.009	0	0.018
4	5.70	0.435	0.032	0	0.005	0.102	0
1	6.37	0.064	0.029	0	0.006	0.068	0.027
2	6.18	0.092	0.035	0	0.009	0.029	0.028
3	5.42	0.265	0.068	0	0.015	0.004	0
4	5.90	0.413	0.005	0	0.008	0.086	0.047
1	6.09	0.059	0.060	0	0	0.064	0.055
2	5.67	0.174	0.036	0	0.001	0.017	0.021
3	5.73	0.169	0.020	0	0.003	0	0
4	5.42	0.197	0.035	0	0.003	0	0.017

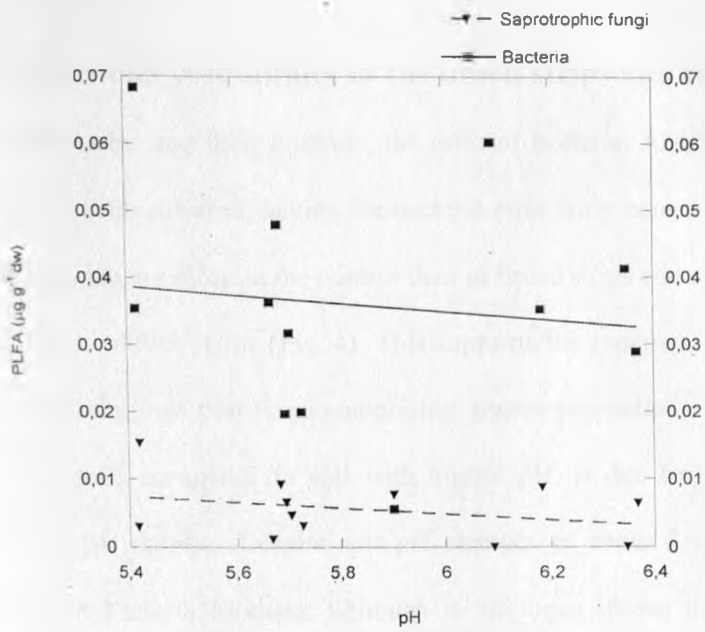


Figure 6: Effects of pH on saprotrophic fungi and bacteria

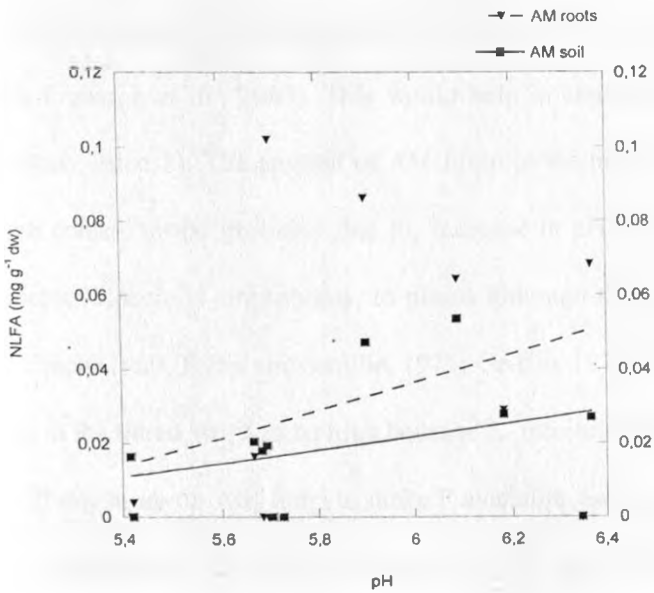


Figure 7: Behaviour of AM fungi biomass over a pH gradient

CHAPTER 4: DISCUSSION

4.1 Microbial community in the limed strips and their controls

In limed strips and their controls, the ratio of bacteria, AM fungi and saprophytic fungi in both treatments differed, having the bacteria ratio fairly constant in both treatments, while the AM fungi having more in the control than in limed strips and saprophytic fungi being more in limed than control strips (Fig. 4). This supports the statement by Read, (1991); Pennanen *et al.* (1998) findings that fungi comprising greater proportion of the soil microbial biomass in low pH soils compared to soil with higher pH, is due to soil fungi and bacteria having different pH optima. Despite soil pH changes of about 2 units, there was no significant change in bacteria biomass, although it has been shown that neutral or slightly alkaline conditions (6-8) favours bacterial growth (Rousk *et al.*, 2009).

Mycorrhizae formation in limed soil could be associated with the response to the level of soil phosphate available for plant growth as well as pH *per se* (Thomson *et al.*, 1986; Sano *et al.*, 2002; Fransson *et al.*, 2003). This would help in explaining the differences in AM fungi in the plots (Table 1). The amount of AM fungi in the limed strips was lower compared to that in the control strips probably due to, increase in pH that can decrease the availability of nutrients, especially phosphorus, to plants although this is not always the case (Murrmann and Peech, 1969; Ryan and Smillie, 1975; Griffin, 1971). Thus we would have expected AM fungi in the limed strips to be high because *K. glauca* does not have root exudates and thus, it would rely more on AM fungi to make P available. Sano *et al.* (2002) helps to explain this by demonstrating that the innate competitiveness or aggressive characteristics of AM fungi may be overshadowed if the quantity of the inoculum present or the soil conditions allow increased growth of hyphae in soil prior to root colonization. Increases in either the AM inoculum or lime alters the relative amounts of infective hyphae of each fungus at the root

surface, thus decreasing AM fungi hyphae competitive ability in mycorrhizal formation and also there is inhibition of the growth of mycorrhizas hyphae in alkaline soil (Abbott and Robson, 1984; 1985)

The low AM biomass in limed plots could be due to changes in soil pH. This affects the competitive ability of various AM fungi during mycorrhizal formation primarily by affecting hyphae growth in soil and the relative abundance of hyphae growth in soil at the root surface and subsequently inside the roots (Sano *et al.*, 2002). Little is known of the interaction between species during mycorrhizal formation (Juniper *et al.*, 1998). The quantity of mycorrhizae formed by species of AM fungi in field soils will depend on both the amount of infective hyphae and their relative competitive ability (Abbott and Robson, 1984), which is based on antagonism (Hepper *et al.*, 1984), and depletion of nutrients within roots (Wilson and Trinick, 1983).

Some fungus like, *Acaulospora laevis*, cannot infect the roots above pH 7.3 unless in very high inoculum and also addition of lime may alter the relationship between mycorrhiza formation and sporulation of AM fungi (Davis *et al.*, 1985; Porter *et al.*, 1987a). A variation in relative numbers of propagules of fungi with changes in soil pH will perpetuates shifts in the population of AM fungi in limed soils (Sano *et al.*, 2002).

However, it could be argued that soil pH was not uniform in the soil matrix, and the pH measured might therefore not be indicative of the pH in microhabitats (Båath *et al.*, 1995) especially in the limed and control strips where seepage was feared to have happened.

4.2 Microbial communities in limed and perturbed plots and perturbed plots

Changes in PLFA analysis (biomass) pattern after liming were due to pH, since a good correlation between these 2 measurements was found. This was also true for the present study (Bååth and Arnebrant, 1993; Bååth *et al.*, 1995; Neale *et al.*, 1997). However, by including the disturbance treatment, the effect of pH became less evident, this is so because the pH was almost similar to that of control strips (Table 1) but P concentration was different. This difference was due to mixing of soil in the perturbed treatment (Table 1). Even though they had different P concentration, in the perturbed plots having higher P than control strips, the microbial biomass of the three groups studied, bacteria, AM fungi and saprophytic fungi were almost the same (Table 3). Assuming that the same mechanism is behind these similar changes in microbial biomass in the different treatments, an underlying explanation other than the altered pH is necessary (Bååth and Arnebrant, 1993, 1994; Frostegård *et al.*, 1993; Bååth *et al.*, 1995).

Disturbance coupled with liming seems to significantly affect the classes of microbial community under study at all the four plots, unlike liming without disturbance (figure 4). Changes in microbial biomass might be due to an altered substrate quality as also an altered substrate quantity, that is, availability of soil organic matter might change due to the treatments. It is well known that the availability of soil organic matter increases after liming or other pH raising treatments (Persson *et al.* 1989). This was reflected in an increased specific respiration rate expressed as CO₂ evolution per biomass unit (qCO₂) in a study done by Bååth and Arnebrant (1994). Perturbed-treated plots would have an increased input in decomposable substances due to the mixing of soil, which increases available substrate to the microflora, as well as nutrients needed by the microflora. This is reflected by the high numbers of microflora in the perturbed and limed plots especially saprophytic fungi and

bacteria. An increase in the bacteria could be explained by the increase in materials that have a low C: N ratio and as for the saprophytic fungi it was availability of new substrate material from the surface.

Roots exudates play an important role in solubilisation of soil nutrients, e.g. P (Bar-Yosef, 1991; Camerford, 1992; Kafkafi *et al.*, 1998). Acidic soils and limestone soils have no general differences in total inorganic P or total P content. The main difference is that the concentration of soil solution and easily exchangeable phosphate is much lower in limestone soils (Tyler and Olsson, 1993). To develop successfully in limestone soil plants must be able to mobilise considerable amounts of phosphate from other than the minute soil pools. Unlike most of the acidifuges, plants that grow in calcareous soil, *K. glauca* does not produce oxalate which increases solubility of Ca-apatite. It's thought to rely on AM fungi to make P available.

The quantitative effect of the pH changes on microbial biomass differed between the sites, which was also the case with P concentration. One explanation for these sites differences could be that, it was not pH *per se* that was the reason for altered PLFA pattern (microbial biomass) since no correlation was found between bacteria and saprophytic fungi and pH. Maybe pH changes altered some other property of soil that in turn affected the microbial composition (Bååth and Arnebrant, 1993; 1994).

At pH 8, which was present in the limed strips and the limed and perturbed plots, calcium phosphate is highly insoluble and the minute share of phosphate present in the soil solution is mainly HPO_4^{2-} . This form is taken up less readily by plants than the H_2PO_4^- species predominant at a lower pH. In addition, the OH^- and HCO_3^- ions present in solution at pH 8 may act antagonistically on P uptake (Lin, 1979).

Calcareous soils are consistently low in soluble and easily exchangeable phosphate (Zohlen and Tyler, 2004). The inorganic P of such soil is mainly present as insoluble apatite or apatite-like Ca compounds (Haynes, 1989; Tyler, 1992) which plants or plants-microbial system need to solubilize to render the P constituent available for uptake. Reason for no increase in microbial biomass after decrease in P. calcicoles species (which grow on calcareous soils) exude much oxalic acid/ oxalate (powerful solubilizer of mineral Ca phosphate due to the very low solubility of Ca oxalate) (Ström *et al.*, 1995; Tyler and Ström, 1995; Ström, 1997).

4.3: Effect of pH gradient and long-term soil disturbance on microbial community

The low amount of AM fungi in the Lyngsjön (Table 2) could be explained by the presence of chelating acids in root exudates produced by *Pilosella officinarum*, important in phosphate solubilisation (Nye, 1979). However, due to rapid nitrification, nitrate is known to be major source in calcareous soils (Ao *et al.*, 1987; Tyler, 1992). Release of OH⁻ or HCO₃⁻ ions as an exchange for NO₃⁻ ions will neutralize the excess acidity generated in nitrification and keep the pH at a high level also at the root soil solution interface. This might prevent acid dissolution of Ca phosphate from the soil phase. Increased root exudates due to root growth may result in seasonal changes in microbial biomass, carbon and nitrogen (Bristow and Jarvis, 1991). The high bacteria biomass can be due to enhanced root growth and increased root exudates which are thought to stimulate the growth of bacteria (Lovell *et al.*, 1995). The perturbation that occurred in Lyngsjön 50 years ago due to the building of the road didn't have much effect on the microbial biomass and P concentration in the soil.

The P-uptake rate by roots is directly proportional to P concentration at the root-soil interface (Nye, 1979). This concentration is largely controlled by the amount of labile (adsorbed) P as related to the P buffering capacity (Holford and Mattingly, 1975; Kuo, 1990). P adsorption in

calcareous soils correlates with dithionite-soluble Fe and surface area of the lime. Westermann (1992) suggests that the surface area of the lime is the major factor affecting P availability on calcareous soil; however, neither surface area nor dithionite-soluble Fe was estimated in these soils. The effects of lime on P availability was not clear, since in the limed plots and control plots there was very little difference in P (Table 1) even after having a 2 unit difference in pH. In the plots where liming and perturbation was carried out, the P in the soil was very low while in the plots where perturbation only was done, the P was the high than that of the other treatments. This could be because of the nutrients made available by mixing the soil and also increase in decomposable material. As for the plots where liming and perturbation was done, mixing of the soil increased the pH and decomposable material. Due to liming P adsorption is reduced and this leads to increase in the AM fungi. Increase in decomposable materials leads to increase in the bacteria and saprophytic fungi. This cannot be stated with finality as what happened but it can be assumed that a combination of disturbance and liming caused the changes in these plots.

CONCLUSION AND RECOMMENDATIONS

Soil microbial community is defined by soil composition as well as by the plants community, especially in the rhizosphere in close contact with the fine roots. It plays a key role for plants nutrition and its response to changes thus is a key to any prediction of effects of, e.g. liming, perturbation and CO₂ elevations. The emerging evidence points to perturbation and liming as the way forward for the conservation of *K. glauca* since it increases the pH but P concentration decreases there is also a marked increase in AM fungi. This is so because *K. glauca* is a high-pH, low -nutrient-tolerant perennial grass (Mårtensson and Olsson 2004). The high variation was brought about by *K. glauca* being a rare species and this lead to a low sample size. Liming and perturbation has effect on the microbial biomass, by changes in pH, substrate quality and quantity, P and other nutrients availability. Acidification, nitrogen deposition and disturbance are important factors in sandy grasslands, thus how nitrogen affects the microbial biomass should be studied especially saprophytic fungi and bacteria.

This study is particularly relevant to global environmental changes as grasslands hold a considerable stock of carbon and have, in the past, been considered as net sinks of atmospheric carbon (Staddon *et al.*, 2003). Any environmental change that affects soil microbial communities and alters mycorrhizal abundance is likely, therefore, to have a significant effect on the net ecosystem carbon flux.

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