Swedish University of Agricultural Sciences
Department of Soil and Environment

Nutrient effects on microorganism communities in nutrient poor soils

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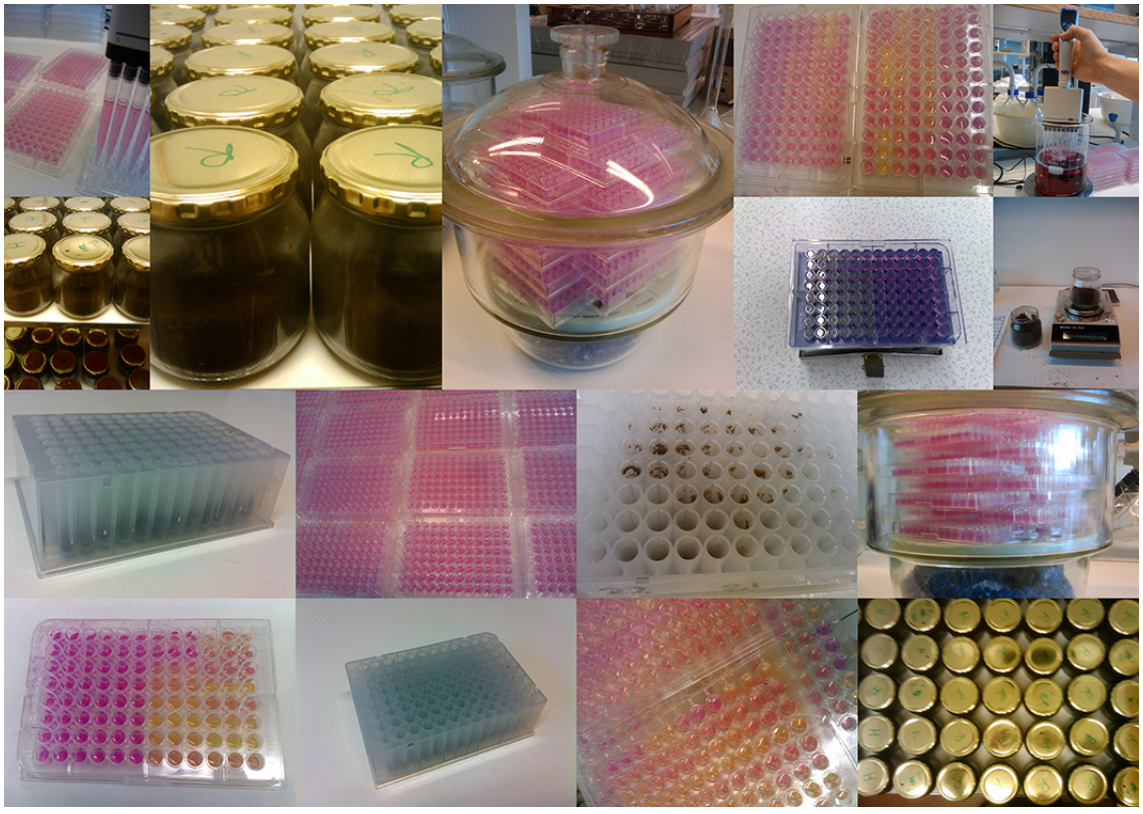

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SLU, Swedish University of Agricultural Sciences
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Abstract

Better, more efficient fertilizers with great productivity and economic returns are needed, but it is important today to find fertilizers that are also sustainable. It is also important to carefully monitor their impact on the environment, including potential side-effects. In this context, the microbial communities that carry out numerous essential functions contributing to a functional ecosystem are of particular interest.

The present study is a complement to a pot experiment that investigated the potential of different waste products for use as fertilizers and how they affect the microbial community. In the pot experiment, the clearest treatment change in microorganism community function was found in the fully fertilized treatment used as a positive control. The question was whether this was indirect, i.e. due to changes to plant growth, or due to a particular nutrient in the fully fertilized treatment. This complementary study sought to identify whether any of the individual nutrient(s) in the fully fertilized treatment affected the soil-microorganism community. This was done through two different incubation experiments (a main experiment and a secondary experiment) in which 12 different nutrients were tested one by one on two soils with low concentrations of trace elements, potassium (K) and magnesium (Mg). Changes in microbial community composition in the soils were examined by measuring the Community Level Physiological Profiles (CLPP) using the MicroResp method.
In the main experiment the incubation lasted for 13 weeks, with assays after 2 and 13 weeks. In the secondary experiment the incubation lasted for 2 weeks, with 4 assays. The results showed a change in physiological soil microbial profile after addition of nitrogen (N), K, Mg and the fully fertilized treatment. These four treatments also gave significantly higher soil electric conductivity than the other treatments, as did the fully fertilized treatment in the original pot experiment. Therefore, it is possible that electric conductivity is the factor causing the change in microbial community composition. However, there is also a possibility of a direct effect of K and Mg in the nutrient-poor soils studied.

Sammanfattning

I jakten på bättre, effektivare och mer ekonomiska gödningsmedel är det av stor vikt att dessa även är hållbara ur ett miljöperspektiv. Det är då extra viktigt med kontroll av vad de betyder för miljön, och däribland hur de påverkar mikroorganismsamhället i jorden där många viktiga och nödvändiga funktioner finns för att bibehålla ett välfungerande ekosystem.
Den här studien är en kompletteringsstudie till ett kärlförsök där man undersökte på vilket sätt olika restprodukter fungerar som gödselmedel och hur de påverkar mikroorganismsamhället i marken, men där kontrollen i form av en fullgödslad näringslösning var den behandling som gav tydligast effekt. Frågan var om effekten i kärlförsöket var indirekt via en effekt på växterna eller om något näringsämne i näringslösningen gav en direkt effekt på mikroorganismerna och i så fall vilket eller vilka näringsämnen?

Detta försök gick ut på att vidare undersöka de i den fullgödslade näringslösningen ingående enskilda näringsämnenas effekt på mikroorganismsamhällets struktur och funktion i marken. Tolv olika näringsämnen testades ett och ett på två svenska jordar med låg halt av spårämnen, kalium och magnesium i två olika inkubationsförsök; ett huvudförsök och ett kompletterande försök. Förändringar i mikroorganismsamhällets metaboliska diverstitet undersöktes med hjälp av metoden Community Level Physiological Profiles (CLPP) i form av MicroResp (en metod som bygger på multipel substrat-inducerad respiration).

I huvudförsöket pågick inkubationen i 13 veckor med provtagning efter 2 och 13 veckor. I det kompletterande försöket pågick inkubationen endast i 2 veckor med 4 provtagningar under perioden. Resultaten visar på en viss förändring i de båda jordarna efter 13 veckor, dessa förändringar är knutna till tillförsel av kväve, kalium, magnesium och den fullgödslade näringslösningen. Dessa fyra näringslösningar har efter 16 veckor signifikant högre elektrisk konduktivitet, vilket även var fallet med den fullgödslade jorden i kärlförsöket, och det är troligt att elektriska konduktiviteten kan vara den faktor som leder till en förändring i mikroorganismsamhället. Dock är det inte möjligt att utesluta en eventuell direkt effekt av K och Mg på jorden från Hollsby.

Populärvetenskaplig sammanfattning

Mikroorganismer som lever i jorden ansvarar för många av markens funktioner, såsom nedbrytning av organiskt material och cirkulation av näringsämnen vilket är viktigt för välfungerande ekosystem. Det är därför viktigt att mikroorganismerna inte tar skada av de gödningsmedel som åkrarna gödslas med. Mikroorganismerna behöver olika näringsämnen men kan påverkas negativt ifall koncentrationen av dessa ämnen blir för hög. För vissa ämnen är spannet mellan eftersträvad koncentration och en giftig koncentration litet.

I ett tidigare försök undersöktes olika gödningsmedels påverkan på mikroorganismerna i marken och man kom fram till att en viss näringslösning ledde till en förändring i mikroorganismsamhället. En kvarstående fråga efter detta försök var varför denna förändring skedde. En tänkbar orsak var att något specifikt näringsämne i lösningen ledde till förändringen genom direkt påverkan på mikroorganismerna, en annan att växterna i försöket först blev påverkade av gödningen och sedan i sin tur påverkade mikroorganismerna.

För att besvara detta har ett nytt försök gjorts för att undersöka samma näringslösning i detalj. Näringslösningen bestod av 12 grundämnen: kväve(N), fosfor(P), svavel(S), magnesium(Mg), kalium(K), kalcium(Ca), mangan(Mn), koppar(Cu), järn(Fe), zink(Zn), bor(B) och molybden(Mo). De 12 ämnena tillsattes dels vart och ett för sig, dels alla tillsammans, såsom den ursprungliga näringslösningen var sammansatt, till två olika jordar – Hollsby och Rådde. Dessutom blandades ett prov från vardera jord med enbart vatten för att användas som kontroll. En metod att mäta aktiviteten hos mikroorganismerna är att mäta den koldioxid de avger. I försöket mättes därför mängden koldioxid som avgick från jordproven under 6h. Den elektriska konduktiviteten (som är ett mått på hur väl jorden kan transportera elektrisk laddning) och pH mättes också. Båda jordarna i försöket var från början näringsfattiga och särskilt Hollsby hade låga halter av näringsämnena kalium och magnesium.

Resultat

Förutom näringslösningen i sin helhet gav även enbart kväve, magnesium (endast Hollsby) respektive kalium (endast Hollsby) en förändrad aktivitet bland mikroorganismerna och resulterade även i högre elektrisk konduktivitet än övriga näringstillsatser. Detta resultat pekar på att det skulle kunna vara den elektriska konduktiviteten orsakad av nämnda näringsämnen som leder till förändring i mikroorganismsamhället. I fallet Hollsby kan även jordens ursprungliga brist på kalium och magnesium ha spelat in, genom att förändringen då skulle ha orsakats av tillförseln av dessa ämnen till en näringsfattig jord. Det tyder på en direkt effekt på mikroorganismerna även om en indirekt effekt inte helt går att utesluta.

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1 Introduction

1.1 Background

Microbial activities are important in the soil ecosystem. Microorganisms (MO) are responsible for many functions in the soil, such as decomposition and nutrient cycling, including immobilization and mineralization of nutrients and biological nitrogen fixation (Marshall et al., 2011; Bünemann et al., 2006). They thus constitute a vital link between plants and their environment. Microorganisms require a number of nutrients, but can also be negatively affected if the concentrations of nutrients or non-nutrient elements are too high (e.g. Giller et al., 1998). This is especially true for some of the trace elements (which are essential elements for biological systems but are needed in very small amounts), where the span between deficiency concentrations and toxic concentrations is relatively narrow.

It is important to economize on nutrients and recycle nutrient sources, both for the environment and for the financial cost. There is an on-going search for better, more effective fertilizers with high productivity and economic returns that are also environmentally sustainable and possible to recycle in an efficient way (Bünemann et al., 2006). Waste products from agriculture and industry are currently being promoted as fertilizers. However, because of the vital role of microorganisms in soil functioning, it is essential to ensure that the fertilizers used in agriculture have minimal negative effects on the environment and soil microbial communities. It is important that the nutrients supplied in fertilizer can be used by the crops to a high extent, and lead to minimal gaseous losses or nutrient leakage that can contribute to increased global warming or result in eutrophication (Ju et al., 2009). Furthermore, waste products that negatively affect the soil MO or the crop (quantity and/or quality) in the long run should be avoided, even if they work well as fertilizers in the short term.

1.2 Laboratory methods to study microbial community function

The most common state for soil microorganisms is (more or less) starvation. However, given an easily degradable substrate they quickly respond by increasing their metabolic activity. The maximum respiration rate is roughly positively correlated to the size of the living, non-resting biomass, which makes it possible to measure the quantity of microorganisms in a soil sample by giving them access to an easily degradable substrate at a constant temperature, (Anderson & Domsch, 1978). This method to measure the living, non-resting biomass in soil is called the Substrate-Induced Respiration (SIR) method.

With SIR, it is also possible to determine the functional profile of the microbial community by using a number of different carbon sources added one by one, thus producing Multiple Substrate-Induced Respiration (MSIR). The Community Level Physiological Profile (CLPP) derived from the pattern of the combined MSIRs acts as a measure of the catabolic diversity of microbial communities in soil (Degens & Harris, 1997). With the CLPP, it is possible to characterize and identify trends and changes in microbial communities and activities in soils.

1.2.1 MicroResp™

MicroResp is a microplate-based MSIR system that can be used to measure the respired carbon (C) released from soil, sediment and water samples (Campbell et al., 2003; Figure 1). MicroResp shows the activity of the organisms before the onset of growth and can provide large volumes of data quickly. The most common procedure is to use 15 different substrates one by one, in addition to distilled water (as a control). These substrates include easily degradable carbohydrates, carboxylic acid and amino acid, and have been discovered in root exudates, so the microorganisms can be expected to be capable of utilizing them. Because the substrates are added directly to the soil samples, the results do not suffer from bias against organisms that can or cannot be cultured in nutrient solution. MicroResp uses deepwell plates with 96 wells for samples and a silicone rubber seal with an airhole for each well, allowing the carbon dioxide (CO_2) to pass from the deepwell plate to the detection plate on top of the seal. There, the CO_2 evolved from each sample is trapped in an alkaline gel which may be stained with an indicator dye for colorimetric determination (Campbell et al., 2003), as was done in the present study.

1.2.2 Colorimetric method

The colorimetric method uses an indicator dye, cresol red, which changes colour with the change in pH when CO_2 reacts with bicarbonate, as shown in the equation:

$$CO_2 \text{ (gas)} + H_2O + HCO_3^- \leftrightarrow 2CO_3^{2-} + 3H^+$$

The cresol red changes from pink to yellow as the pH decreases (Rowell, 1995) and the change is determined by measuring light transmittance by the detection gel (CO_2 trap) at 570 nm (corresponding to the evolving yellow colouring) before and after substrate addition and sample incubation.

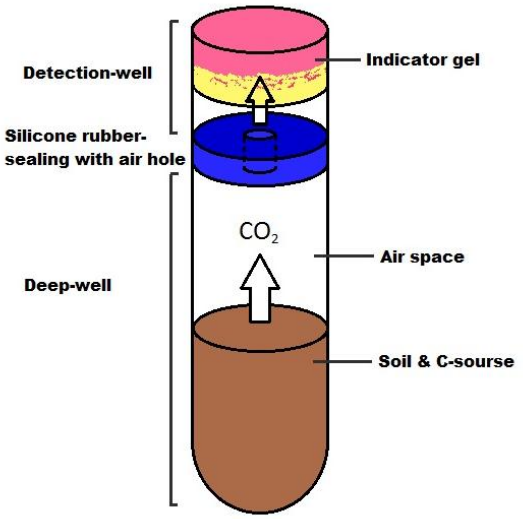

Figure 1. Schematic cross-section of a deepwell unit connected to a detection well with a silicone rubber seal in between.

1.3 Previous studies

The present MSc study was carried out as a complement to a pot experiment that formed part of a PhD project (Ramezanian et al., unpublished). The pot experiment examined the extent to which the microbial community structure in soil is determined by soil mineralogical composition and how it reacts to changes in nutrient availability in the soil brought about by soil amendment. Different waste products (Rock dust, Rock dust + N, Ash (wood), Ash (wood) + N, Distillery waste and Biogas digest) were tested on two soils, with an unfertilized control and a fully fertilized treatment included for reference. The two soils (Hollsby and Rådde) were chosen because they have low concentrations of micronutrients, potassium (K) and magnesium (Mg), and also have distinctly different geological origins. A mix of perennial ryegrass (*Lolium perenne* L.) and red clover (*Trifolium pratense* L.) was used in the pot experiment to simulate a mixed ley, which is a common crop on less fertile soils. The research questions were: How efficient are these waste products as nutrient sources? And how do they affect the microorganisms in the soil?

In the pot experiment, it was surprisingly only one of the treatments that led to a detectable change in microorganism community function: the fully fertilized treatment. The present study therefore sought to investigate which (if any) specific element in the fully fertilized treatment caused the change.

1.4 Objectives

The overall aim of this study was to investigate the causes of the shift in microbial community function observed in the pot experiment. Specific objectives were to:

a) Identify the nutrient or nutrients in the fully fertilized treatment that caused the change in microorganism community function observed in the pot experiment.

b) Investigate the impact of time on the change(s).

c) Investigate whether the changes were caused by a direct effect on the microorganisms or an indirect effect via the plants used in the pot experiment.

2 Materials and Methods

Two different experiments were carried out. In the main experiment, the two test soils were mixed with the respective nutrients and incubated in glass jars, from which subsamples were taken for analysis. In a secondary experiment, the main experiment was repeated but on micro-scale, carried out in its entirety in two deepwell plates, where the nutrients were added directly to the soil in the plate wells. This experiment was carried out in order to study how the soil MO reacted to nutrient addition on a short-time basis.

2.1 Experimental design

The experiments were set up in a two-factorial block design with soil (Hollsby and Rådde) and fertilizer treatment as main factors. The treatments included twelve nutrients (nitrogen (N), K, Mg, manganese (Mn), sulphur (S), copper (Cu), phosphorus (P), calcium (Ca), iron (Fe), boron (B), zinc (Zn) and molybdenum (Mo), added singly or as a fully fertilized treatment (a positive control), and a negative control (H_2O only). All combinations of soil and treatment were replicated three times.

2.1.1 Soils

Topsoil from the 0-20 cm layer of a permanent semi-natural pasture (Hollsby) and a second year ley (Rådde) was used for this experiment. At Hollsby (59˚48'27.69N'', 13˚31'15.96''E) (Figure 2), the soil is a silt developed from river sediments originating from an area dominated by granitic and sandstone bedrock. The soil at Rådde (57˚36'25.99''N, 13˚15'42.89''E) (Figure 2) is a till with sandy loam texture, developed from granitic or gneissic parent material.

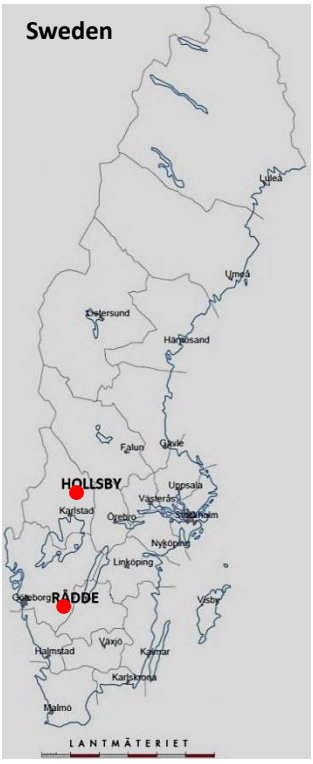

Figure 2. Location of the Hollsby and Rådde sites (Lantmäteriet).

Soil total N and C concentrations in the original soils were determined by high temperature induction furnace combustion using LECO CN2000 (LECO Corporation, St Joseph, MI, USA). Total elemental analysis of other elements was done at ALS, Scandinavia AB lab, Luleå, Sweden. For analysis of Cu, Mo, S and Zn, the soils were digested in concentrated HNO3, HCl and HF in closed teflon containers in a microwave digestion system and measured on ICP-SFMS, and for analysis of Ca, Fe, K, Mg and Mn fused with lithium metaborate, then dissolved in HNO3 and measured on ICP-AES (Table 1). Both soils have low to very low concentrations of a number of nutrients. In particular, the content of Cu, Mg and Zn are very low. The S and Ca content are closer to the mean value for Swedish soils, but still low. Nitrogen and Mo differ most between the two soils, with quite ordinary values for Rådde but low values for Hollsby. pH is quite low for both soils (<6.31, which is the mean value for Swedish arable land (Eriksson et al., 2010, Swedish environmental monitoring program on arable soils 2012).

Table 1. pH and total concentrations of 12 different nutrients in the Hollsby and Rådde soils

			Total analysis(mg/kg dw)											Org matter (% dw)	
Soil	pH(H2O)	pH(CaCl2)	K	Mg	Mn	S	Cu	P	Ca	Fe	B	Zn	Mo	N	C
Rådde	5.53	5.15	20 600	3 510	431	481	6.5	1 190	11 400	18 000	Missing	30	0.85	0.27	3.5
Hollsby	5.43	4.81	25 000	3 010	531	334	6.9	782	9 980	16 000	Missing	46	0.4	0.15	1.9

The soils were homogenized by sieving through a 2-mm plastic sieve. During sieving, plant roots and other visible organic material were removed. The water content was adjusted by addition of deionized water to approx. 60% of water holding capacity (WHC). The soil was spread in boxes to a maximum soil depth of about 8 cm, to achieve satisfactory oxygenation, and pre-incubated at 20˚C under lid to maintain the desired water content.

2.1.2 Nutrient additions

The fully fertilized treatment was included as a positive control for the crops in the original pot experiment, where it was prepared according to a standard protocol used at the Department of Soil and Environment, with the aim of supplying the plants with 12 nutrients (Table 2). For the present experiments, a fully fertilized treatment was prepared from laboratory grade salts as in the pot experiment. The majority of nutrients were added in solution. However, for P and Ca, for which the salts have low solubility, the dry salts were added to the soil samples. In addition, individual solutions were prepared for each nutrient studied, using the same application rates of each salt per 100g dry soil as in the combined fully fertilized treatment. Since the nutrient ions were accompanied by other ions in the

salts, it was not possible to add just one element at a time, and at least one other element was added in addition to the target nutrient. Because of the difficulty in dissolving the $Ca(HPO_4)*2H_2O$ and $CaCO_3$ in water, two other treatments with P and Ca in soluble form, as KH_2PO_4 and $CaCl_2$, were included in the secondary experiment.

Table 2. Amounts of nutrients added to soil samples, singly or in combination (in the fully fertilized treatment) except for x-Ca and x-P, which were used only in the secondary experiment.
dw = dry weight

Nutrient	Added as	Amount (mg/100g dw soil)
N	NH_4NO_3	50.00
K	KCl	333.3
Mg	$MgCl_2*6H_2O$	66.67
Mn	$MnCl_2*4H_2O$	0.2883
S	H_2SO_4	4.904
Cu	$CuCl_2*2H_2O$	0.0093
P	$Ca(HPO_4)*2H_2O$	30.96
Ca	$CaCO_3$	83.33
Fe	$FeCl_3*6H_2O$ +HCl	1.336
B	$Na_2B_4O_7*10H_2O$	0.3333
Zn	$ZnCl_2$	0.2600
Mo	$(NH_4)_6Mo_7O_{24}*4H_2O$	0.0583
x-Ca	$CaCl_2$	3.219
x-P	KH_2PO_4	1.078

2.2 Procedure used for the main experiment

As the homogeneous, friable structure of the soil was destroyed upon addition of water, the soil samples were sieved once more after two weeks and divided into jars with 140 g (fresh weight) in each. The pre-incubation then continued in the jars for another 20 days, after which the nutrients were added to the soil according to Table 2, each soil was carefully mixed and the jars were incubated at 20˚C.

The functional profiles of the soil microbial communities was evaluated with MicroResp (Campbell et al., 2003) after 2 and 13 weeks of final incubation (Figure 3). For this, soil was transferred from the jars to deepwell plates according to the MicroResp manual (www.microresp.com). After 6 days of further pre-incubation, the substrates were added and the respiration was measured. The distribution of soil samples into the plates was randomized within each block. Each block was assessed on each of three consecutive days,

but all nutrients were added on the same day, which meant that the incubation time differed by 1 or 2 days for the different blocks.

Figure 3. a) Deepwell plate filled with soil, b) deepwell plate with soil, c) detection plate before the assay, d) deepwell plate and detection plate assembled with silicone seal, e) detection plate after assay. (Photo: Atefeh Ramezanian, Kajsa Söderström)

For the assay, 15 substrates (L-alanine, L-arabinose, ascorbic acid, L-cysteine HCl, citric acid, D-fructose, D-galactose, D-glucose, gamma-aminobutyric acid (GABA), L-lysine, L-malic acid, N-acetyl glucosamine (NAGA), oxalic acid, alpha-ketoglutaric acid (AKGA) and trehalose) were used, plus water, with three replicate determinations for each substrate and the water (Campbell et al., 2003). An incubation time of 6 hours from substrate addition was used before reading the detection plates, during which all CO_2 produced was trapped in the indicator gels of the detection plates.

One plate with the laboratory´s reference soil was included in each batch as a quality control of the MicroResp assay.

2.3 Procedure used for the secondary experiment

The soils used in the secondary experiment were pre-incubated together with those in the main experiment. They were then gently dried to bring the water content close to 40% of WHC, in order to allow addition of substrate solution for four MicroResp assays.

All treatments from the main experiment were replicated and, in addition, P and Ca were added in solution. The positions within the plates were randomized for each treatment, although each three replicates were held together to minimize the risk of error during nutrient addition. For the same reason the distribution was also the same for the two plates. Only one substrate was used in the secondary experiment, AKGA. It was chosen because it gave the highest respiration rate when the MicroResp assay was carried out on soil samples from the original pot experiment.

Two deepwell plates were filled with Hollsby soil on one side and Rådde soil on the other, apart from 3 x 3 wells on each side that were left empty for the $Ca(HPO_4)*2H_2O$ treatment,

$CaCO_3$ treatment and the fully fertilized treatment to be added later on. Due to a delay, the plates then had to be incubated for one night and the work resumed the following day/morning.

For addition of $Ca(HPO_4)*2H_2O$ and $CaCO_3$, 10-g portions of soil were mixed with the dry salts first, and subsamples of the mixture were then added to the appropriate wells of the deepwell plates. The same procedure was used for the fully fertilized treatment, since it included both salts. The nutrient solutions were pipetted onto the soils and the same volume (25 µL) of water was pipetted onto the soils that had received solid salts only. After this, the plates were shaken to simulate soil mixing and to achieve a similar disturbance of the soil in the treatments with solution only and those with solid salt.

MicroResp assays were carried out on four occasions; directly after nutrient application, 24 h later, and after 1 and 2 weeks. On each occasion, AKGA solution was added to one of the two plates, whereas only water was added to the other plate.

The plate with the AKGA added was read after 6 hours of incubation and the control plate with water added was read after 24 hours of incubation.

As in the main experiment, one plate with the laboratory´s reference soil was included in each batch as a quality control of the MicroResp assay.

2.4 pH and electrical conductivity measurements

Electrical conductivity (EC) and pH (determined in water (pH_{H2O}) and in $CaCl_2$ solution (pH_{CaCl2}) were measured after 16 weeks. For EC, 15 g of soil were mixed with 30 mL reversed osmosis (RO) water and shaken for 1 h and EC was measured on the following day. The same suspension was then used to measure pH_{H2O} after it been stirred for 30 s. Thereafter, 0.3 mL of 1M $CaCl_2$ was added and the samples shaken for additional 30 min before pH_{CaCl2} was measured (Sumner, 1994).

2.5 Statistics and data treatment

Two-factorial analysis of variance (ANOVA) was used to assess treatment and soil differences in SIR derived from individual substrates, using log or square root transformed data as appropriate (JMP 9, © 2011 SAS Institute Inc.) and using $p<0.05$ as the limit for statistical significance. Whenever the ANOVA indicated significant differences, pair-wise comparisons were made with Tukey´s HSD. The values presented are back-transformed LSMeans.

Statistical analyses of CLPP data were based on SIR data that had been calculated by subtracting the values of the CO_2 respired in control (deionized water) from the respiration after addition of the 15 substrates. The data were analyzed by canonical variate analysis using the computer programme "Canonical Analyses of Principal coordinates" (CAP) (Anderson & Willis, 2003). The analyses were based on Euclidean distances. Data were log transformed and standardized by dividing each variable by its standard deviation before analysis.

3 Results

3.1 Main experiment

3.1.1 pH and electrical conductivity

The EC after 16 weeks was significantly higher for the fully fertilized, N, K and Mg treatments compared with the other treatments, with the fully fertilized treatment having the highest values (Fig. 4). The N treatment had the lowest pH and the Ca treatment the highest, both as pH_{H2O} and pH_{CaCl2}. The other treatments showed similar, intermediate values. The Rådde and Hollsby soils were significantly different, with Rådde having the highest pH and Hollsby the highest EC (Table 3). For both soils, the pH in the control after 16 weeks was lower than the original value (Table 1).

Table 3. EC and pH, as mean values of all 14 treatments for Hollsby and Rådde after 16 weeks of incubation (given as LSMeans). Values within columns followed by different letters are significantly different

	EC	pH H_2O	pH $CaCl_2$
Rådde	561.0[b]	5.27[a]	4.97[a]
Hollsby	618.1[a]	4.69[b]	4.44[b]
Prob > F	<.0001	<.0001	<.0001

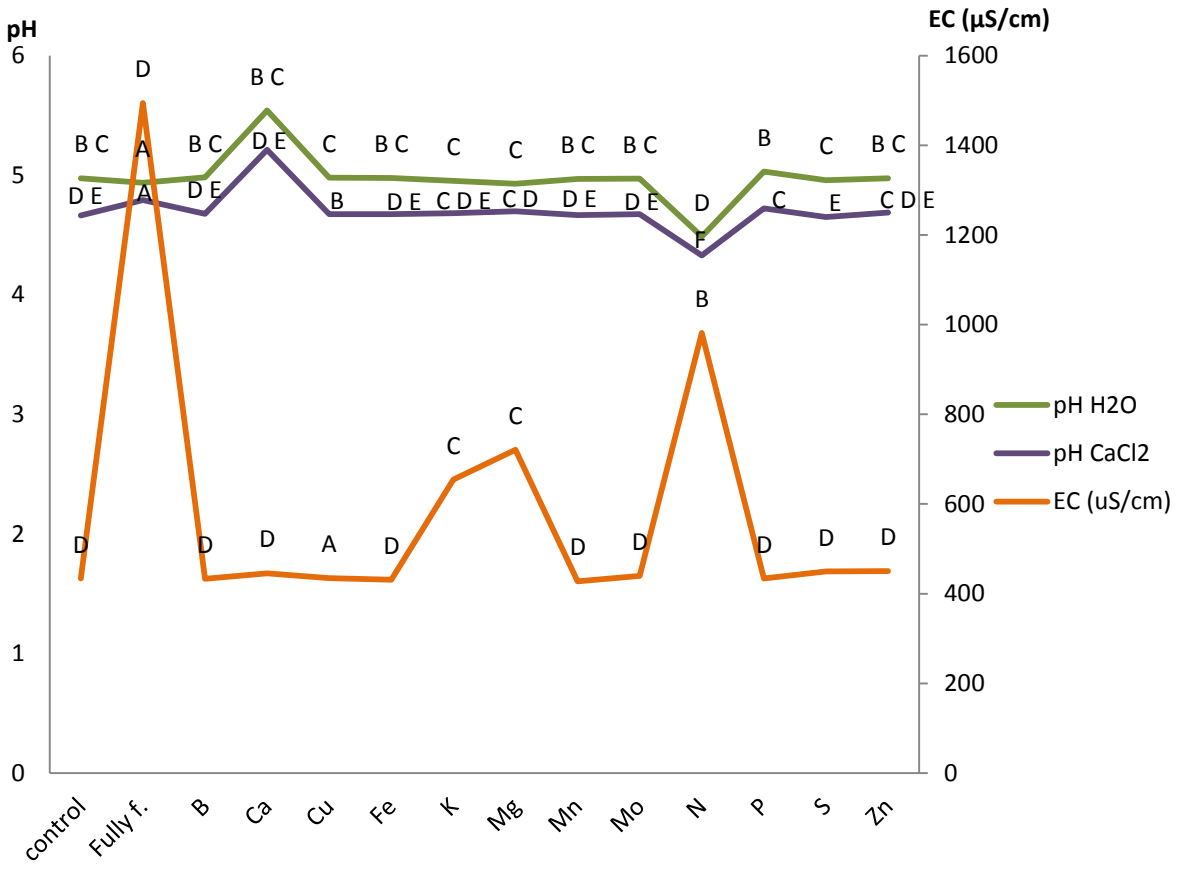

Figure 4. EC and pH for the 14 different treatments and both soils after 16 weeks of incubation (given as LSMeans). Different letters within a line indicate a significant difference.

3.1.2 Carbon dioxide evolution

The CO_2 evolution at 2 weeks was consistently higher for the $CaCO_3$ treatment than for the other treatments (Appendix 1). In contrast, the fully fertilized treatment did not have significantly higher CO_2 evolution than the other treatments. The remaining treatments were not different from each other. After 13 weeks, the CO_2 evolution for the $CaCO_3$ treatment was no longer significantly higher than for the other treatments. For the N, Mg, K and fully fertilized treatments, the CO_2 evolution was lower than average for AKGA, citric acid and malic acid, although the differences were only significant compared with some of the other treatments. The CO_2 evolution rates differed significantly between the Rådde and Hollsby soils for most of the carbon sources tested. For AKGA, citric acid and malic acid, Hollsby had a significantly higher CO_2 evolution rate than Rådde, whereas Rådde had a higher respiration rate for all the other substrates.

3.1.3 Microbial functional profile

The results of canonical analysis based on SIR showed that most of the treatments, after both 2 and 13 weeks, formed main clusters that were clearly separated from each other for the Rådde and Hollsby soils (Figs. 5 and 6). This implies that the functional profiles of the microbial communities differed between these two soils. After 2 weeks, there were no specific differences between the different treatments within each soil, except for the $CaCO_3$ treatment (shown with a triangle in Fig. 5), which was clearly separated from the others. However, after 13 weeks the $CaCO_3$ treatment had moved into the main cluster, while the Mg, K, N and fully fertilized treatments were separated from the other treatments. This indicates that there were differences in the functional profiles of the microbial communities at this time, particularly for the Hollsby soil, while for the Rådde soil this was only the case for the N and fully fertilized treatments.

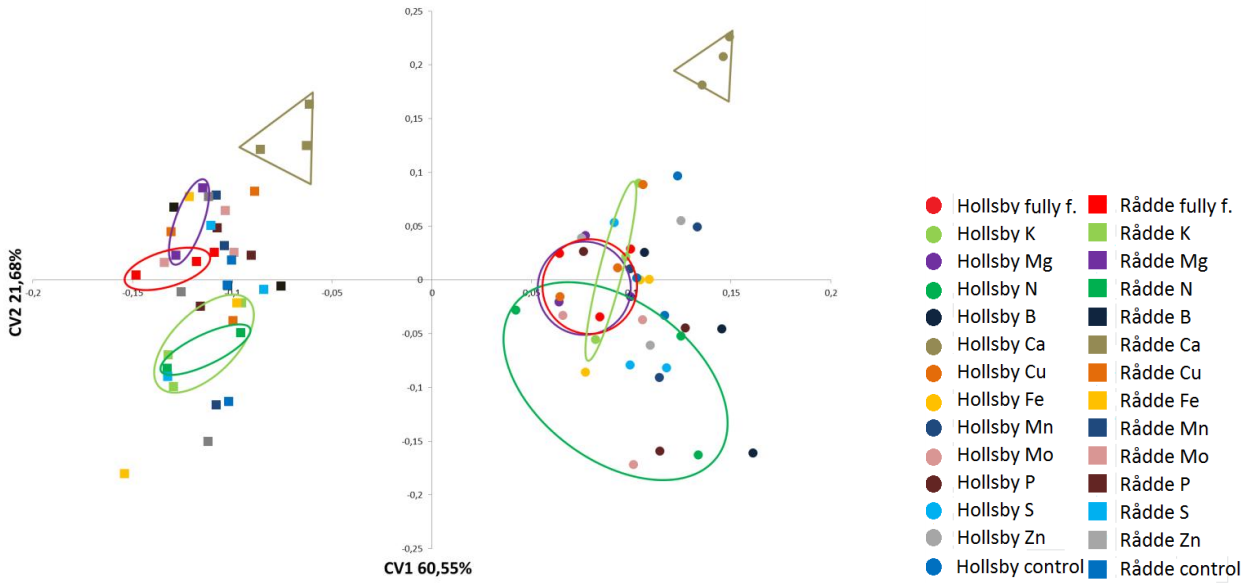

Figure 5. Ordination diagram of first and second coefficients of variation (CV) for the MicroResp CLPPs with the 15 different C sources at week 2 in the main experiment, based on CAP analysis.

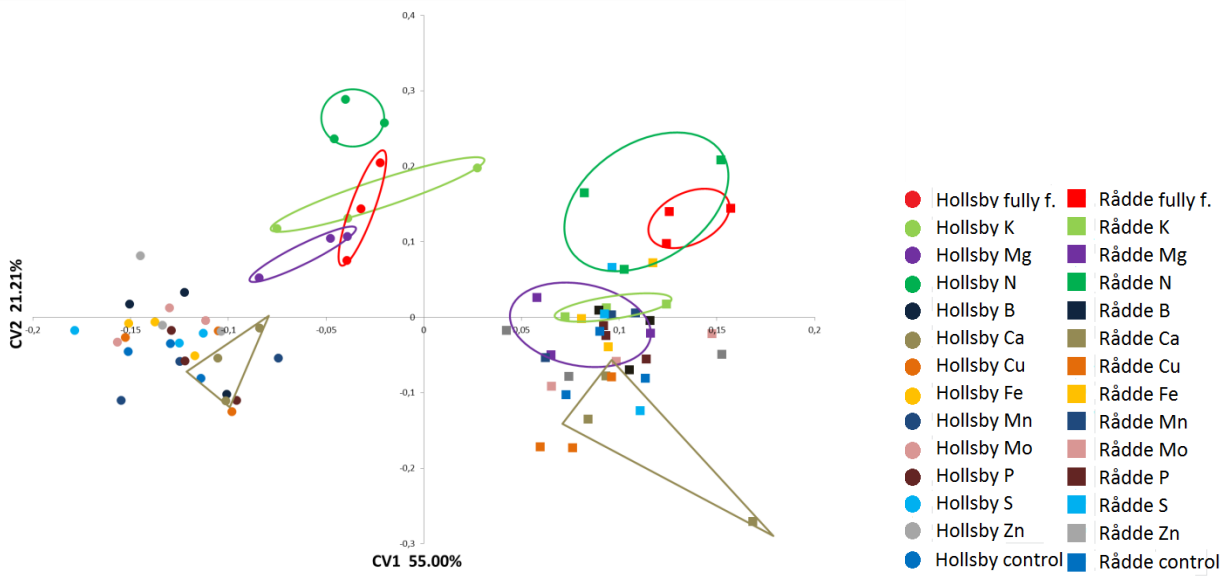

Figure 6. Ordination diagram of first and second coefficients of variation (CV) for the MicroResp CLPPs with the 15 different C sources at week 13 in the main experiment, based on CAP analysis.

3.2 Complementary experiment

Respiration after adding AKGA to the soils did not show a clear and consistent effect or tendency for any treatment over time (Table 4). For the plate with only water added, there was a significantly higher CO_2 evolution rate during the initial 24 hours from the treatments that received dry salts (Ca, P and fully fertilized treatments), and thus had been mixed at the start of the experiment, than from the other treatments. The CO_2 evolution was particularly high in the $CaCO_3$ treatment at the first two measurements, but then subsided, giving a lower and similar average CO_2 evolution rate as the other treatments for both soils at 1 and 2 weeks. Except for the first days´ differences due to the $CaCO_3$ and the effect of mechanical disturbance, it is possible to state that the CO_2 evolution rates were relatively stable for the Hollsby and Rådde soils when no substrate was added.

Table 4. Substrate-induced respiration after addition of alpha-ketoglutaric acid (AKGA) and respiration after addition of water in the complementary study. All values given as LSMeans. Values within columns followed by different letters indicate a significant difference due to nutrient addition.

	AKGA				WATER			
	6h	24h	1 w	2w	6h	24h	1w	2w
Treatment								
B	1.052^{abc}	0.917^{abc}	3.053	2.477^{abcde}	0.126^{cde}	0.114^{c}	0.109^{abcd}	0.111^{bcde}
Ca	1.008^{bc}	1.625^{a}	3.579	1.561^{e}	0.436^{a}	0.251^{a}	0.117^{a}	0.122^{abcd}
Cu	1.466^{ab}	0.861^{bc}	3.566	3.123^{ab}	0.117^{e}	0.111^{c}	0.104^{cd}	0.108^{de}
Fe	1.438^{ab}	0.963^{abc}	3.051	2.969^{abc}	0.133^{cd}	0.108^{c}	0.103^{cd}	0.112^{bcde}
Fully f.	1.775^{a}	1.204^{abc}	3.415	2.817^{abcd}	0.361^{b}	0.139^{b}	0.109^{abcd}	0.114^{bcde}
control	1.242^{abc}	1.523^{ab}	3.390	2.668^{abcd}	0.124^{de}	0.105^{c}	0.108^{abcd}	0.130^{a}
K	1.208^{abc}	1.355^{abc}	2.988	2.718^{abcd}	0.128^{cde}	0.105^{c}	0.104^{cd}	0.111^{bcde}
Mg	1.086^{abc}	1.123^{abc}	2.978	1.918^{de}	0.124^{de}	0.099^{c}	0.099^{d}	0.110^{cde}
Mn	1.046^{abc}	1.290^{abc}	3.390	2.829^{abcd}	0.129^{cd}	0.108^{c}	0.108^{abcd}	0.119^{abcde}
Mo	1.196^{abc}	1.157^{abc}	3.318	2.702^{abcd}	0.124^{de}	0.103^{c}	0.103^{cd}	0.111^{bcde}
N	1.457^{ab}	0.787^{c}	2.665	3.393^{a}	0.122^{de}	0.108^{c}	0.101^{d}	0.105^{e}
P	1.031^{abc}	1.179^{abc}	2.906	2.038^{cde}	0.138^{c}	0.116^{c}	0.115^{ab}	0.119^{abcde}
S	1.114^{abc}	0.921^{abc}	2.600	2.259^{bcde}	0.126^{cde}	0.108^{c}	0.105^{cd}	0.123^{abc}
x-Ca	1.256^{abc}	1.255^{abc}	3.445	2.571^{abcde}	0.130^{cd}	0.100^{c}	0.100^{d}	0.110^{cde}
x-P	1.097^{abc}	0.993^{abc}	3.003	2.256^{bcde}	0.127^{cde}	0.110^{c}	0.112^{abc}	0.125^{ab}
Zn	0.821^{c}	0.867^{bc}	3.338	2.102^{cde}	0.123^{de}	0.105^{c}	0.106^{bcd}	0.119^{abcde}
Soil								
Hollsby	3.071	1.506	3.076	2.564	0.151	0.118	0.107	0.116
Rådde	0.458	0.781	3.260	2.486	0.140	0.114	0.106	0.115
Prob > F								
Treatment	0.0019	0.0006	ns	<.0001	<.0001	<.0001	<.0001	<.0001
Soil	<.0001	<.0001	ns	ns	<.0001	ns	ns	ns
Soil*Treatment	<.0001	<.0001	<.0001	<.0001	<.0001	0.0009	<.0001	<.0001

Significance: ns: *not significant*

4 Discussion

4.1 Change in functional profiles

One of the objectives of the present study was to determine whether one or some of the elements in the fully fertilized treatment caused the change in function of the microorganism community observed in a previous pot experiment. According to the results, the Rådde and Hollsby soils differ from each other in terms of both their functional profile and respiration patterns for individual substrates. This is not at all surprising, since they are two different soils with different pedogenesis, structure, texture, etc. It also confirms by the results reported for the original soils (Ramezanian et al., unpublished). In the pot experiment the fully fertilized treatment resulted in a change in the functional profile of the Hollsby soil so that it fell into the same cluster as all treatments on the Rådde soil. In the present study, although no treatment effects could be seen during the early stages of the incubation, when looking at the results after 13 weeks it was possible to distinguish that as well as the fully fertilized treatment, addition of three single nutrients, Mg, K and N, had given rise to a change in the physiological profile for the Hollsby soil compared with the unfertilized control. For the Rådde soil, similar changes were observed for the fully fertilized treatment and the N treatment.

4.1.1 pH changes

Nitrogen fertilization can give indirect effects such as acidification, which is considered to have negative effects on soil organisms (Bünemann et al., 2006). In the present study the pH decreased in the N treatment, although the respiration activity for the N treatment did not show a consistent response, as it differed between measurement occasions and experiments. The Ca treatment, which had the highest pH, did not seem to have a consistent effect either, except for higher CO_2 evolution than the other treatments during the first days after Ca addition (see below). Judging by the small response to pH in this experiment, it may be possible to conclude that small changes in pH do not have an effect on the activity or functional profiles of soil microbial communities.

4.1.2 Electrical conductivity, chloride and cadmium

The EC was high in the treatments that showed a clear change in CLPP, i.e. the fully fertilized treatment and, to a lesser degree, the N, K, and Mg treatments. The EC may have affected

the MO directly by changing the osmotic potential of the soil solution. However, a number of studies have shown that the EC and chloride (Cl^-) concentration in soil are important in determining cadmium (Cd) availability to plants, with the concentration of Cd increasing exponentially as EC increases and linearly as Cl^- increases (Kamewada & Nakayama, 2011). In the present study, K and Mg were added as KCl and $MgCl_2{*}6H_2O$, both in rather large amounts compared with the other nutrients that were added as the chloride, while the N was added as NH_4NO_3, also increasing the EC. In the original pot experiment, plants grown on soil with the fully fertilized treatment had significantly higher values of Cd than all other treatments (Dahlin et al., unpublished). This increase was particularly large for the Hollsby soil, on which the clover Cd concentration was approx. 500% of that in the unfertilized control, whereas the perennial ryegrass Cd concentration was approx. 300% higher. In the same experiment, EDTA-extractable Cd was also significantly higher in the fully fertilized solution treatment than in all other treatments for the Hollsby soil, whereas concentrations did not differ significantly for the Rådde soil (Ramezanian et al., unpublished).

It is thus possible that it was the fertilizer addition that indirectly, via increased solubility of Cd, produced the effect for the fully fertilized, N, K, and Mg treatments in the present experiment. However, it is also possible that the effect was at least partly caused by alleviation of K and Mg deficiency in the Hollsby soil. In the pot experiment it can be assumed that the plants had taken up the N provided and therefore that the rise in EC at the end of the growing season was probably not due to N addition. The EC values in the pot experiment were also overall much lower than in the present study, which indicates that leaching and uptake from plants occurred during the growing season (the control had EC of around 90 µS/cm and the fully fertilized solution EC around 200 µS/cm in the pot experiment, compared with around 440 and 1500 µS/cm, respectively, in the present study). This may explain why no effect was seen for the Rådde soil in the pot experiment, while an effect was apparent in this study. However, the change in the physiological profile that was seen in the Hollsby soil with the fully fertilized treatment in the pot experiment could also have been due to K and Mg addition, as the concentrations of these nutrients are very low in the original soil and a change was seen for these treatments only for the Hollsby soil in the incubation experiment. To investigate further whether K and Mg have a direct effect, an experiment could be carried out where these two elements are added in compounds with some element other than Cl and thus do not have such a high impact on EC.

4.1.3 Use of carbon source

The separation between the main cluster of treatments and the fully fertilized solution, N, K

and Mg treatments after 13 weeks of incubation could be linked to significantly different use of AKGA, citric acid and malic acid (Table 3). In the pot experiment, AKGA gave the highest respiration rate in the MicroResp assay and therefore was chosen as the single substrate in the secondary experiment. A previous study has observed a larger response to CO_2-C evolution associated with the carboxylic acids (citric acid, AKGA, α-ketobutyric acid, α-ketovaleric acid, DL-malic acid fumaric acid and L-ascorbic acid), with citric acid and AKGA at the top, compared with 18 other amines, amino acids, carbohydrates and carboxylic acids (Hoyle & Murphy, 2006). However, another study by those authors one year later found that for a number of C substrates tested, there was no obvious association between the response and the chemical structure of the substrate (Hoyle & Murphy, 2007). Neither of these studies mentions anything about different treatments and differences in the microbial communities in association with the chemical structure of the substrate, although this would be interesting to investigate.

4.2 Time impact

Another aim of the present study was to investigate the impact of time on the changes in soil microbial activity. It is important to remember that long-term experiments generally tend to go on for years and short-term often refer to a season. In this study, however, long-term was 13 weeks and short-term 2 weeks. In comparison with most studies, the 13 weeks measurements would thus be short-term and the 2 weeks measurement would count as more or less immediate. Comparing the results from the main experiment at weeks 2 and 13, it is evident that time made a difference, which indicates that the soil microbial community takes a little time to change in response to the new nutrient conditions.

In the secondary experiment the same soil was amended repeatedly, since new doses of substrates were added to the same soil sample at each assay. In the main experiment, on the other hand, the soil sample was replaced with new samples for each assay and therefore only received substrate once. A previous study in which soil had a 'trigger solution' consisting of glucose, amino acids or root extract, added once or with the same amount split into three doses, found greater total evolution of CO_2-C (about 3-fold) for the split dose approach than the single dose (De Nobili et al., 2001). The two experiments in the present study are therefore not fully comparable as there were two factors affecting the results of the secondary experiment: time since fertilizer addition and number of substrate additions. In the main experiment there was only the time factor.

For the secondary experiment the soil samples amended with $CaCO_3$, $Ca(HPO_4)*2H_2O$ and the fully fertilized solution had much higher CO_2 evolution rates than the other treatments and the rates decreased over time. The $CaCO_3$and $Ca(HPO_4)*2H_2O$ used in these treatments were mixed with soil as solid salts. The soils were subsequently added to the plates one day later than the soil for the other nutrients. That difference in the degree and time of disturbance of the soil clearly gave an effect during the first day of reading, even though the plates were shaken in an attempt to simulate the disturbance and to get a more equal situation.

The Ca and fully fertilized treatments in particular initially had significantly higher CO_2 evolution values than the other treatments, most likely due to evolution of CO_2 from CO_3^{2-} rather than an effect of the nutrient on MO respiration. After 1 week, however, the rate was at the same level as in the other treatments, indicating that the carbonate had been consumed. However, in the main experiment the CO_2 evolution at 2 weeks was higher for the $CaCO_3$ treatment, whereas it was not significantly higher for the fully fertilized treatment, which was also amended with $CaCO_3$. This could due to the fully fertilized treatment having a lower pH compared with the Ca treatment, as low pH dissolves carbonate faster (Coto et al., 2012), and therefore having caused the carbonate to disappear already by 2 weeks. The fact that the effect was still apparent after 2 weeks in the main experiment, while it disappeared after 1 week in the secondary experiment, might be due to the closed jars keeping the carbonate from dissolving, while the plates in the secondary experiment were aerated more often and therefore the carbonate could disappear faster.

4.3 Direct or indirect effect

The present study investigated whether the changes to the soil microbial community caused by fertilizer addition were a direct effect on the microorganisms or an indirect effect via the clover and ryegrass used in the pot experiment. In the latter case, the microbial reaction to the fully fertilized solution could have come from the crops' reaction, i.e. an effect on plant growth and root exudation. Rhizobacterial communities shift due to changes in root exudation (Schallmach et al., 2000) and the condition of plants and roots has an effect on the quality of root exudation (Liljeroth et al., 1990). It has been shown in several studies that the microbial community in the rhizosphere differs from that in soils without any roots (Söderberg et al., 2004; Kim et al., 1999). In the pot experiment all the soil was enclosed by a pot and could more or less be regarded as rhizosphere, as the root density was high, while no plants were included in the present study. It is reasonable to assume that root exudation

had an impact when roots were present. However, the results from the present study still agree quite well with those of the pot experiment, indicating a possible direct effect of nutrient addition on the soil microorganisms.

4.4 Sources of error

Water content

In the main experiment the water content was mistakenly higher than the recommended water content for MicroResp (40-60% of WHC) at the 2 week assay. The actual water content was 60-70% of WHC, which may have impeded CO_2 transportation to the indicator gel/CO_2 trap. However, the water content was higher in all treatments and its effect was probably too small to have significantly affected the results.

5 Conclusions

This study investigated the causes for the shift in microbial community function observed after fertilizer addition in a previous pot experiment. In terms of the specific objectives:

a) Identify the nutrient or nutrients in the fully fertilized solution that caused the change in microorganism community function observed in the pot experiment.

It is possible that addition of Mg and K caused this change for the Hollsby soil. However, it could also have been an effect of EC, either directly or indirectly through its effect on Cd availability.

b) Investigate the impact of time on the change(s).

The response was significant at 13 weeks, which indicates that the change in the microbial community physiological profile developed over time.

c) Investigate whether the changes were caused by a direct effect on the microorganisms or an indirect effect via the plants used in the pot experiment.

An indirect effect on the physiological profile of the microorganisms via fertilizer effects on the plants in the pot experiment cannot be ruled out. However, since there were no plants in the present experiment, the effect observed must have been exerted directly on the microorganisms.

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Appendix 1

Table A1. Substrate-induced respiration (given as LSMeans) after addition of the 15 different substrates in the main experiment. Values within columns followed by different letters indicate significant differences due to nutrient addition

	AKGA		Ascorbic acid		Citric acid		Fructose		Galactose		Glucose		GABA		Alanine	
	2w	13w	2w	13w	2w	13w	2w	13w	2w	13w	2w	13w	2w	13w	2w	13w
Treatment																
B	3.387	2.105^{ab}	1.300^{b}	0.998	0.513^{b}	0.366^{abc}	0.259	0.184	0.091	0.130	0.290	0.231	0.082^{b}	0.088	0.141^{b}	0.081
Ca	3.643	2.145^{ab}	1.929^{a}	1.460	1.086^{a}	0.570^{a}	0.319	0.269	0.177	0.152	0.399	0.336	0.311^{a}	0.114	0.264^{a}	0.105
Cu	3.405	2.139^{ab}	1.460^{ab}	0.998	0.609^{b}	0.396^{abc}	0.306	0.185	0.167	0.114	0.304	0.213	0.103^{b}	0.083	0.153^{b}	0.072
Fe	3.067	2.800^{a}	1.125^{b}	1.036	0.462^{b}	0.368^{abc}	0.247	0.201	0.133	0.129	0.244	0.339	0.083^{b}	0.105	0.132^{b}	0.083
full s.	2.805	1.706^{b}	1.404^{b}	1.194	0.475^{b}	0.248^{bc}	0.295	0.171	0.126	0.102	0.324	0.259	0.101^{b}	0.079	0.145^{b}	0.074
K	3.072	1.945^{ab}	1.259^{b}	1.017	0.496^{b}	0.222^{bc}	0.265	0.179	0.119	0.132	0.237	0.276	0.083^{b}	0.082	0.125^{b}	0.071
Mg	2.881	1.790^{b}	1.335^{b}	1.053	0.403^{b}	0.334^{abc}	0.265	0.212	0.112	0.119	0.290	0.257	0.087^{b}	0.082	0.132^{b}	0.082
Mn	3.406	2.478^{ab}	1.304^{b}	1.067	0.558^{b}	0.468^{ab}	0.261	0.224	0.105	0.103	0.310	0.312	0.098^{b}	0.091	0.136^{b}	0.074
Mo	3.052	2.333^{ab}	1.255^{b}	0.950	0.423^{b}	0.378^{abc}	0.255	0.177	0.120	0.133	0.342	0.253	0.091^{b}	0.082	0.149^{b}	0.078
N	2.249	1.785^{b}	1.049^{b}	0.923	0.335^{b}	0.195^{c}	0.165	0.119	0.071	0.075	0.182	0.169	0.049^{b}	0.069	0.078^{b}	0.051
control	3.473	2.497^{ab}	1.336^{b}	1.227	0.566^{b}	0.462^{abc}	0.248	0.233	0.118	0.155	0.252	0.297	0.082^{b}	0.094	0.136^{b}	0.101
P	3.298	2.329^{ab}	1.224^{b}	1.076	0.417^{b}	0.424^{abc}	0.248	0.199	0.101	0.140	0.304	0.220	0.093^{b}	0.081	0.128^{b}	0.080
S	3.264	2.456^{ab}	1.165^{b}	1.121	0.448^{b}	0.424^{abc}	0.248	0.212	0.121	0.149	0.261	0.253	0.085^{b}	0.089	0.122^{b}	0.098
Zn	3.451	2.527^{ab}	1.287^{b}	1.143	0.622^{ab}	0.453^{abc}	0.313	0.230	0.137	0.119	0.308	0.302	0.085^{b}	0.100	0.140^{b}	0.080
Soil																
Hollsby	6.332	4.630	1.117	0.969	0.837	0.614	0.199	0.161	0.083	0.097	0.211	0.203	0.085	0.072	0.108	0.060
Rådde	1.085	0.670	1.517	1.212	0.276	0.190	0.343	0.239	0.160	0.157	0.367	0.328	0.120	0.107	0.175	0.105
Prob > F																
Treatment	ns	0.0026	<.0001	ns	<.0001	0.0009	ns	ns	ns	ns	ns	ns	<.0001	ns	<.0001	ns
Soil	<.0001	<.0001	<.0001	0.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0062	<.0001	<.0001	0.0008	ns	<.0001	<.0001
Soil*Treatment	ns	ns	ns	ns	ns	0.0023	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Significance: ns: *not significant*

Table A1 continued.

	Arabinose		Cysteine		Lysine		Malic acid		NAGA		Oxalic acid		Trehalose	
	2w	13w	2w	13w	2w	13w	2w	13w	2w	13w	2w	13w	2w	13w
Treatment														
B	0.140	0.107	0.190^{b}	0.230	0.042^{b}	0.050	0.414^{bc}	0.287^{abc}	0.185^{ab}	0.160	0.644	0.732	0.228	0.207
Ca	0.187	0.135	0.590^{a}	0.321	0.155^{a}	0.079	1.033^{a}	0.461^{a}	0.311^{a}	0.217	0.923	0.638	0.228	0.267
Cu	0.169	0.086	0.247^{ab}	0.222	0.060^{b}	0.058	0.461^{b}	0.399^{ab}	0.204^{ab}	0.179	0.591	0.670	0.274	0.241
Fe	0.126	0.100	0.219^{ab}	0.224	0.044^{b}	0.063	0.363^{bc}	0.348^{abc}	0.165^{b}	0.193	0.493	0.934	0.230	0.248
full s.	0.115	0.083	0.281^{ab}	0.244	0.070^{b}	0.066	0.420^{bc}	0.191^{cd}	0.198^{ab}	0.124	0.498	0.684	0.262	0.195
K	0.111	0.075	0.230^{ab}	0.233	0.046^{b}	0.047	0.377^{bc}	0.224^{bcd}	0.166^{b}	0.150	0.479	0.625	0.225	0.229
Mg	0.113	0.106	0.194^{b}	0.231	0.047^{b}	0.049	0.374^{bc}	0.237^{abcd}	0.171^{b}	0.184	0.439	0.696	0.225	0.202
Mn	0.140	0.107	0.200^{b}	0.249	0.047^{b}	0.055	0.426^{bc}	0.344^{abc}	0.189^{ab}	0.203	0.547	0.821	0.234	0.210
Mo	0.148	0.092	0.195^{b}	0.206	0.048^{b}	0.046	0.397^{bc}	0.303^{abc}	0.174^{ab}	0.169	0.538	0.775	0.237	0.184
N	0.058	0.049	0.125^{b}	0.197	0.018^{b}	0.047	0.221^{c}	0.142^{d}	0.099^{b}	0.084	0.365	0.763	0.170	0.138
control	0.129	0.120	0.212^{ab}	0.264	0.046^{b}	0.052	0.403^{bc}	0.387^{ab}	0.199^{ab}	0.211	0.630	0.843	0.240	0.284
P	0.130	0.099	0.186^{b}	0.232	0.053^{b}	0.043	0.418^{bc}	0.316^{abc}	0.192^{ab}	0.179	0.545	0.665	0.255	0.214
S	0.122	0.112	0.195^{b}	0.273	0.054^{b}	0.047	0.385^{bc}	0.303^{abc}	0.166^{b}	0.181	0.483	0.836	0.233	0.244
Zn	0.137	0.105	0.219^{ab}	0.240	0.048^{b}	0.075	0.432^{bc}	0.363^{abc}	0.166^{b}	0.184	0.527	0.929	0.244	0.285
Soil														
Hollsby	0.093	0.085	0.169	0.190	0.0489	0.053	0.482	0.339	0.136	0.141	1.037	1.405	0.186	0.186
Rådde	0.167	0.111	0.286	0.301	0.0621	0.059	0.372	0.256	0.233	0.202	0.279	0.402	0.292	0.264
Prob > F														
Treatment	ns	ns	0.0001	ns	<.0001	ns	<.0001	<.0001	0.0068	ns	ns	ns	ns	ns
Soil	<.0001	0.0110	<.0001	0.0006	ns	ns	0.0004	0.0003	<.0001	0.0277	<.0001	<.0001	<.0001	0.0007
Soil*Treatment	ns	ns	ns	ns	ns	ns	0.0201	ns	ns	ns	ns	ns	ns	ns

Significance: ns: *not significant*