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Abstract

Phosphate rock is a diminishing source of phosphorus (P) for crop production. Other sources are being investigated to replace it, for example bio-ash from wood combustion and biogas digestate from house hold wastes. Ash and digestate application have resulted in increased yields, but the effect on the soil biota has been somewhat varied. This study focuses on the effects of bio-ash and digestate on arbuscular mycorrhizal fungi (AM fungi), which live in symbiosis with the studied crop spring wheat (*Triticum aestivum*). Furthermore, it aims to determine the effect of AM fungi on the cadmium (Cd) uptake in wheat. Soil and plant samples were collected in a field in Östergötland county where seven different treatments had been applied (unfertilized control, digestate from two different production sites, ash, two combinations of digestate and ash, mineral fertilizer). There were three different types of samples collected: soil containing roots, soil without roots, and roots alone. These samples were studied using a quantitative polymerase chain reaction (qPCR) assay; the roots were furthermore studied for AM colonisation in a microscopy investigation and root, straw and kernel samples were also sent for Cd content determination at ALS Scandinavia. The qPCR assay showed that AM fungal biomass in the root samples and the soil samples including roots were positively affected by application of digestate in combination with ash, and negatively affected by mineral fertilizer and digestate alone. The biomass of wheat was affected by the treatments, and the mineral fertilizer addition resulted in the greatest total biomass. The AM fungal biomass in the root free soil was positively correlated with the wheat biomass, indicating that mineral fertilizer can indirectly be beneficial for some type of AM fungi. The microscopy investigation was only done for one block and was hence too small to give a definitive result. In the roots sent for Cd determinations contamination of soil could not be ruled out, instead Cd determinations of the straw and kernel were used for interpretations. The different treatments did not affect the Cd content in the wheat nor was there any correlation between amount of AM fungi and Cd content in the above ground plant parts. The same was true for the pH values in the soil. The conclusion of this investigation is that application of waste-based fertilizers affects AM fungi in various ways. According to my results soil-associated AM fungal biomass is associated with a large host plant biomass, and root-associated AM fungal biomass is positively affected by a combination of digestate and ash. More research is needed in this area, especially with focus on the effect of waste-based fertilizers on AM fungi with different life strategies and functional traits.

Keywords: digestate, ash, Cd, cadmium, circular economy, phosphorus, Glomeromycota, quantitative polymerase chain reaction, qPCR, microscope, field, pH, Quarna

Populärvetenskaplig sammanfattning

För växtproduktion av till exempel vete behövs diverse näringsämnen, därav fosfor (P) som vanligtvis utvinns från råfosfat. Det är en ändlig resurs vilket är anledningen till att andra källor undersöks som alternativ, till exempel bioaska från träförbränning och rötslam från biogasproduktion av köksavfall. Gödselmedel som dessa är ett steg i linje med Europeiska unionens mål att ha en mer cirkulär ekonomi där resurserna används flera gånger innan de tas ur systemet. Att lägga aska och rötslam på jordbruksmark har i studier resulterat i högre skördar, men effekten på jordlevande organismer har inte undersökts i lika stor utsträckning. Arbuskulära mykorrhizasvampar (AM-svampar) är marklevande organismer som lever i symbios med olika värdväxter genom att svampen levererar näringsämnen, såsom P, till växten och växten i sin tur ger svampen energirika kolföreningar. Svampens ”rötter”, så kallade hyfer, tar sig in i växtens rot och sedan kan utbytet börja. Alla AM-svampar har delar av sin biomassa inuti värdplantans rötter, men beroende på svampart och livsstrategi har de olika proportioner av sin biomassa inuti roten respektive ute i jorden.

I denna studie undersöktes effekterna av bioaska och rötslam på arbuskulära mycorrhizalsvampar i ett vårvetefält i Östergötland. Kadmium-halten (Cd) mättes också för att undersöka om AM-svampen påverkade upptaget i vetets rötter, halm och kärna Om så var fallet skulle den kunna påverka hur mycket Cd vi får i oss vid förtäring. För att undersöka detta gjordes ett försök med sju olika behandlingar i fyra upprepningar (block): ogödslad kontroll, rötslam från två olika biogasanläggningar (Uppsala och Linköping), aska från en förbränningsanläggning (Mora), två kombinerade gödselmedel med rötslam och aska (en från varje biogasanläggning), och en mineralgödselbehandling. I mitten av juni 2017 togs tre typer av jord- och rotprover i fält (jord med rötter, rotfri jord och rena rotprov) och dessa undersöktes sedan med kvantitativ polymeraskedjereakion (qPCR). Detta är en metod där specifika gener kopieras med hjälp av bland annat ett enzym och primers (en liten bit av en DNA sträng som fungerar som en startsträcka för DNA-syntes). Under kopieringen märks generna med ett fluorescerande ämne som kan mätas och därmed visar mängden gener i provet. Dessa olika mängder gener användes sedan i analysen för att hitta skillnader mellan de sju behandlingarna. Det var en stor variation av genkopior inom proverna vilket gör att små skillnader mellan behandlingar kanske inte syntes. Dessutom gjordes en mikroskopiundersökning för att undersöka storleksordningen på AM-svamparnas kolonisering. För att se svampstrukturerna tydligt i rötterna färgades svampstrukturerna med blått bläck innan de undersöktes under mikroskopet. Koloniseringsgraden var låg (0-1.09 %) och det fanns endast tid till att göra ett av de fyra blocken. Det medförde att mängden prover inte var tillräcklig för att göra en statistisk undersökning och resultaten kunde inte säga något definitivt om AM koloniseringen i veterötterna. Rot, halm och kärn-prover skickades till ALS Scandinavia där de undersöktes för Cd-innehåll. Rotproverna misstänks ha varit förorenade av jord och uteslöts därför från analysen, men halm och kärn-proverna användes. Kadmium-halten var alltid

högre i strået än i kärnan, men inga skillnader kunde urskiljas mellan behandlingarna. Jordens pH-värden och skördedata från huvudprojektet kunde utnyttjas.. När all data var insamlad gjordes statistiska analyser på dem i form av variansanalys (ANOVA) och regressionsanalys i programmet JMP® Pro 13.

Antalet genkopior i qPCR-analysen visade att de delar av svampens biomassa som är inne i värdplantans rötter (de rena rotproven) och den svampbiomassa som fanns i jordprover innehållandes rötter (alltså svampbiomassa både i rot och i jord) påverkades positivt av kombinationen av rötslam och aska. Däremot påverkades svampens biomassa inuti rötterna negativt av mineralgödselmedel, och för jordprovet som innehöll rötter var det rötslam på egen hand som hade en negativ effekt. Det fanns ett positivt samband mellan vetets biomassa och de olika behandlingarna, där mineralgödseln resulterade i den största vetebiomassan. Svampbiomassan i det rotfria jordprovet var positivt korrelerad med vetets biomassa, vilket indikerar att mineralgödsel indirekt kan ha en positiv effekt på vissa AM-svampar. De olika behandlingarna påverkade inte Cd-halten i vetets halm och kärna och det fanns heller inget samband mellan Cd-halten och mängden AM-svampar. Detsamma gällde för jordens pH och AM-svamparna. Jordens pH-värden ganska neutrala, vilket ska vara optimalt för AM-svampar.

Resultaten från denna studie indikerar att avfallsbaserade gödselmedel kan påverka AM-svampar på olika sätt. Svampens biomassa inuti rötterna påverkades negativt av mineralgödsel medan vetet själv påverkades positivt av gödselmedlet. Den AM-svampbiomassa som fanns i jorden (rotfritt prov) påverkades i sin tur positivt av en stor värdväxtbiomassa. De flesta AM-svamparna påverkades positivt av kombinationen av rötslam och aska. Mer forskning behövs inom detta område, särskilt med fokus på effekten av avfallsbaserade gödningsmedel på sammansättningen av AM-svamparnas samhälle.

Table of contents

Abbreviations

AM Fungi	Arbuscular Mycorrhizal Fungi
ANOVA	Analysis of Variance
DM	Dry Matter
DW	Dry Weight
qPCR	Quantitative polymerase chain reaction
SD	Standard Deviation
SQ	Starting Quantity

1 Introduction

Plants need inorganic phosphorus (P) to grow (Schachtman et al., 1998; Smith and Read, 2008). This mineral has been applied through different sources but since the middle of the 20th century mined phosphate rock is the most common source. However, phosphate in rock is a finite source of P (Cordell et al., 2009) and a circular economy is an important goal in the society. One step towards achieving prolonged use of resources is the usage of organic and waste-based fertilizers in agriculture (European Commission and Secretariat-General, 2015). Examples of nutrient-dense waste are biogas digestate and bio-ash (Garg et al., 2005). Garg et al., (2005) found that by-products from energy production, such as ash (from coal burning) and biogas slurry (from agricultural waste), increased wheat yield compared to the control treatment which was given no nutrient supplement at all. They furthermore found that such residues could be used not only for increased production but also for soil improvement, such as increased moisture retention capacity. Some by-products from bioenergy production, such as anaerobic digestate and bioethanol residue, are efficient nitrogen (N) fertilizers (Galvez et al., 2012). On the other hand, the available P content is rather low, especially if the soil is slightly acidic (Galvez et al., 2012); calling for an additional source of P to make a more complete plant fertilizer. When comparing the nutrient content, bio-ash contains more P while digestate contains more N (Demeyer et al., 2001; Nkoa, 2014). Theoretically, by combining N-rich digestate and P-rich bio-ash it would be an interesting option to substitute conventional fertilizers and thereby contribute to a more sustainable agriculture. However, the quality of these by-products vary (European Commission and Secretariat-General, 2015) and care need to be taken not to build in unwanted or yet unknown side-effects in the circular economy.

Application of anaerobic digestates can affect more than just the yield; it can also affect microbes in the soil (Smith and Read, 2008; Walsh et al., 2012b). The organic portion of P in the soil needs to be converted to inorganic P to be available for plant

uptake. Arbuscular mycorrhizal (AM) fungi is one of the actors in the soil that mobilize P (Smith and Read, 2008) and they play an important role in absorbing nutrients from the soil and transporting them to the plant roots (Smith and Read, 2008). They can enhance plant growth and production (shoot weight) for a number of different plant species (Lee and George, 2005; Medina et al., 2003; Vázquez et al., 2000). Different AM fungi have different strategies when it comes to root colonisation rate, mycelial extension in the soil and how they allocate their biomass between host roots and soil (Hart and Reader, 2002). Sheng et al. (2013) discovered that long-term P-fertilization reduced the AM fungal genetic diversity. Various AM fungi are affected by farming practices (fertilizers, biocides, tillage and crop rotations) in different ways and it is often difficult to predict the outcome of a specific action in such a complex system (Gosling et al., 2006), and the full potential for how AM association may benefit plants under different conditions is not yet clarified.

Digestate and bio-ash are complex fertilizers that contain a number of different nutrients as well as pollutants, cadmium (Cd) being one of them (Makádi et al., 2008; Gupta et al., 2012; Alburquerque et al., 2012). Cadmium have numerous negative effects on human health, for example the kidneys and bones are greatly affected (Nawrot et al., 2013). Dahlin et al. (2016) showed that adding recycling products rich in chloride (Cl^-), such as some biogas digestates, can increase the Cd concentrations two or three-fold in wheat grain whereas an addition of ash derived from virgin wood decreased Cd concentrations in the grain by increasing soil pH. Hence, these effects, as well as the plant nutrition and soil affecting aspects should be taken into consideration before using such residues as fertiliser replacements (Dahlin et al., 2016). Guo et al. (2013) investigated sorghum grown in soil from rare earth mine tailings and found that the shoot concentration of Cd was significantly lower when sorghum was inoculated with AM fungi (*G. mosseae*, *G. versiforme*) compared to non-inoculated plants. Arbuscular mycorrhizal fungi in cultivated fields could hence considerably reduce the amount of Cd ending up in the final food products, such as grains of rice (Luo et al., 2017). However, further studies focusing on other important food crops, for example wheat, are needed to clarify whether this effect is true for those specific crops.

1.1 Aim

This report will look into the effects of bio-ash and biogas digestate application on AM colonisation in spring wheat roots, AM fungi biomass in soil and roots, and if wheat Cd concentrations are affected by the different fertilizers. The following hypotheses will be tested:

- The mineral fertilizer will have a negative effect on the degree of AM colonisation and the number of AM fungi gene copies in the roots due to high concentration of easily available mineral nutrients.
- The amount of AM fungi will decrease the amount of Cd in the roots and aboveground biomass of wheat due to AM fungi functioning as a "filter" for further transport in the plants.
- The AM fungi that are mostly soil dwelling will be positively affected by mineral fertilizer and high pH values while the AM fungi that are mostly root dwelling will be positively affected by the waste based fertilizers and low pH, resulting in a shift in root-to-soil AM biomass ratio with the treatments.

2 Literature review

2.1 Fertilizers from bio-energy residues

With the current rate of phosphate rock utilization it is estimated that the reserves will be exhausted within 50-100 years (Cordell et al., 2009; Bertilsson et al., 2005). The high quality phosphate rock is being depleted first while the remaining sources have a higher level of metallic contaminants (Driver et al., 1999). This calls for new sources of P as it is an essential nutrient for plant growth (Smith and Read, 2008). The workable part of the phosphate rock reserve is decreasing while much of the P added to the ecosystems (through fertilization of soils, depositions etc.) will end up in different water bodies (Bertilsson et al., 2005). It has been proposed by the European Commission and Secretariat-General (2015) that in order to lessen the dependence upon phosphate rock recycled nutrients should be used instead, such as waste based fertilizers.

Anaerobic digestate is a rather wide concept including digestate from household waste, manure and sewage sludge etc. (Nkoa, 2014). The digestate can be divided into a dry and a liquid fraction, and is a by-product from the anaerobic process of biogas production (Murphy and Thamsiriroj, 2013; Walsh et al., 2012). The properties of the digestate is dependent upon the properties of the type of biomass used in the biogas production (Provenzano et al., 2011). Walsh et al. (2012b) found that both digestate and mineral fertilizer application had a similar effect on fungi and bacteria. However, their trial indicated that bacteria are more favoured by digestate application than fungi, with a microbial decomposing community dominated by bacteria as a result. Multiple studies around the globe have shown that anaerobic digestates are as efficient, or even more so, than mineral fertilizers when it comes to releasing N (Nkoa, 2014). Anaerobic digestate from household waste was shown by Odlare et al. (2008) to be a superior fertilizer when compared to cow manure,

pig slurry and household compost when it came to supplying the plant with readily available N and promoting biological activity in the soil. Another study, made by Makádi et al. (2008), showed in an experiment on an acidic sandy soil in Hungary that biogas digestate increased soybean yield without causing any drastic changes in the soil quality. Furthermore, a study by Walsh et al. (2012a), showed that usage of liquid digestate on grasses yielded equally or even better than N or NPK fertilizer treated grasses.

Digestate often has a high pH and therefore they are prone to lose ammonia (NH_3) (Nkoa, 2014) but it also has a high concentration of NH_4^+-ions (Table 1) which promote nitrification (a process in which NH_4^+ is oxidized to NO_3^- that releases H^+-ions to the soil) (Eriksson et al., 2011; Möller and Müller, 2012) and can therefore have an acidifying effect on the soil.

Ash derived from combustion of biomass (bio ash), contains essential plant nutrients. The nutrient content varies with the type of biomass being combusted and under what conditions the biomass is combusted (Pandey and Singh, 2010, Demeyer et al., 2001). Cruz-Paredes et al. (2017) concluded that biomass ash is a viable alternative to conventional fertilizers (Triple superphosphate fertilizer, TSP) for barley production in soils with P-AL levels in class III (adequate level). In the treatments where they used a high ash application dose, the P availability was comparable to treatments with high TSP application (300 kg P/ha). The total P content in wood ash can vary a lot (6.9-14 g P/kg), but P is generally the least available nutrient of the macronutrients found in wood ash (Demeyer et al., 2001). Bio ash increases the pH in the soil while NPK can have a decreasing effect on pH if the N is in ammonium-form (Ohno and Susan Erich, 1990, Dahlin et al., 2016). Some typical biochemical properties of anaerobic digestate and wood ash are listed in Table 1.

Table 1. Typical biochemical properties of anaerobic digestate and wood ash.

Parameter	Value range		Value range
Unit	Anaerobic digestate	Unit	Wood ash
Total N (% DM*)	3.1-14.0	Total N (g/kg)	0.6-0.9
NH4+ (% Total N)	35-81	-	-
Total P (% DM)	0.2-3.5	Total P (g/kg)	6.9-14
pH	7.3-9.0	pH	8.9-13.5
*dry matter	(Nkoa, 2014)		(Demeyer et al., 2001)

Riding et al. (2015) proposed that a fertilizer consisting of a combination of digestate and ash could counteract the depletion of micronutrient and, furthermore, offer a balanced fertilizer of macro- and micronutrients. Bougnom et al. (2012) found that after application of digestate and ash the basal respiration and microbial biomass was not affected, in short term at least, by the amount or type of digestate (manure or biogas sludge). They concluded that the combination of digestate and ash had a positive effect on both yield and soil chemistry, but long-term experiments are needed to verify that the effects remain over time.

2.2 Cadmium

The term "heavy metal" has been debated whether it is applicable or not, but an example of such "heavy metals" is cadmium (Cd) which can be found naturally in all soils and is not essential for plant growth. The amount of Cd in soils is largely dependent upon the parent bedrock (Söderström and Eriksson, 2013), and areas such as Östergötland in Sweden are naturally rich in Cd (Mark- och grödoinventeringen, 2018). Some of the greatest sources of Cd in plants come from P fertilizers and deposition from the atmosphere (Smolders and Mertens, 2013). Cadmium uptake in plants is affected by soil pH since if soil pH is increased the availability of Cd is decreased (Eriksson, 1989). The release of cadmium (Cd) in wood ash is dependent on the pH, where acidic conditions (pH <6.0) leads to more release of Cd (Zhan et al., 1996).

In humans Cd can cause damage to the kidneys and softening of bones (Smolders and Mertens, 2013). Human intake of Cd predominantly comes from food consumption, especially potatoes, wheat and other grains because people consume them in such great quantities (Baird and Cann, 2012). Long-term intake of Cd can increase the risk of different kinds of cancer and fractures (Thomas et al., 2011). A guidance value of 7 µg Cd per kg body weight per week has been proposed, where as in Sweden the estimated average total daily intake is around 15 µg for women and 19 µg for men (European Food Safety Authority (EFSA), 2009; Julin et al., 2012; Thomas et al., 2011) which results in a rather high mean value for the population and measures should be taken to not increase the Cd-content in food crops.

2.3 Arbuscular mycorrhiza

Mycorrhiza has a long history; the first land plants were likely colonized by hyphal fungi and this association is still very common in the terrestrial plant kingdom today (Smith and Read, 2008; Chagnon et al., 2013). Arbuscular mycorrhiza is one of the

most ancient types of mycorrhiza and forms associations with many different plants, for example the majority of the Angiospermae (Smith and Read, 2008). They are found in over 85 % of all plant species, including cereals (Campbell et al., 2015). The fungi can provide many services for the plant in return for the sugar it receives from the plant. These services include increased surface area in the soil for nutrient and water uptake, stimulation of root and branch growth by excretion of growth factors and substances that protect the host plant from pathogens. The host plant can give up to 20 % of its photosynthesized C to the fungi in return for nutrients and water (Parniske, 2008). Mycorrhiza-inoculated plants take up more P and grow better than non-inoculated plants (Mosse et al., 1976). At the same time, the activity of all mycorrhiza in agricultural systems are not always beneficial for the plants (Gosling et al., 2006).

The rate and extent of colonisation differs between different AM fungi families, which results in different growth patterns. Members of *Glomeraceae* generally colonize roots fast and have an extensive biomass inside the roots, which is consistent with a stress-tolerant life strategy. Members of *Gigasporaceae*, on the other hand, have low root colonisation rate but an extensive biomass in the soil which is a competitive life strategy (Chagnon et al., 2013; Hart and Reader, 2002). Stress-tolerant AM fungi are efficient with the C allocated to them and they would benefit plants that are stress-tolerant (e. g. long-lived and slow growing). Competitive AM fungi would benefit plants that have a high P need and that are good carbon-fixers (as the AM fungi requires a lot of C to grow an extensive mycelium in the soil). The great extraradical mycelium can potentially transfer a great amount of nutrients (Hart and Reader, 2002). They are also benefited by N-fertilizer when P is a limiting factor (Chagnon et al., 2013). Arbuscular mycorrhizal fungi that have a parasitic effect on the plant are thought to have a slow colonisation rate and thereby require more resources for a longer time compared to fast colonizers, but there could be great long-term benefits (Chagnon et al., 2013; Hart and Reader, 2002). AM fungi with “fine” hyphae (such as *Glomaceae*) have been reported to dominate in low pH soils while AM fungi with “coarse” hyphae (such as *Gigasporaceae*) are more common in soils with high pH (Clark, 1997). Thus, it seems warranted to look further at the community composition to see which species dominate in which substrates and under what conditions to draw conclusions on how AM fungi are affected by different fertilizers, and how different AM fungi may affect plant growth. In the study conducted by Cruz-Paredes et al. (2017) they found that AM colonisation was not affected by the P application rate (30, 60 and 300 kg P/ha) but there were seasonal differences with the highest colonisation in June. Though, they did find that the colonisation was greater in the high ash applications compared to the TSP applications. In contrast to these results, the study of Breuillin et al. (2010) found that P can inhibit both the

colonisation and gene expression of AM fungi. Farming practices (organic and conventional practices) can also affect the composition of the AM fungal communities (Gast et al., 2011; Säle et al., 2015). Luo et al. (2017), furthermore, showed that AM fungi can reduce Cd uptake in plants. In their study they found that Cd-concentrations in grains were reduced by AM fungi, and in soils with 10 mg Cd kg^{-1} added, the AM fungi significantly increased the yield. The soil contamination of Cd did not hinder AM colonisation by *Rhizophagus intraradices* of rice roots.

Taken together, AM fungi can be beneficial for plant production in nutrient deficient soils and in contaminated soils. A circular economy encourages the use of waste-based fertilizers and it is therefore interesting to investigate how AM fungi are affected by various types of nutrient applications (European Commission and Secretariat-General, 2015).

3 Material and methods

3.1 Experiment design

This study is part of a greater research project ("Biogas digestate and bio ash as fertiliser replacements") that during 2017-2019 will be exploring how digestate (from household waste) and bio ash (from incineration of wood) affects crop yield and quality, element mobility and balance, soil biology, and lastly soil carbon balance and climate effects. In addition to the results produced in this study, other results from the main research project are included. These results are Cd content in above ground parts of the spring wheat, amount of spring wheat biomass and soil pH values and bulk density. Three below-ground fractions were studied (soil with roots, root-free soil and roots) to see how AM fungi with different life strategies were affected by the different treatments.

The sampling of soil and roots took place at Öjebro, west of Linköping, Östergötland county (Coordinates: 58°23'04.2"N 15°11'27.5"E; Figure 1 and Figure 2). The average annual precipitation in the area is 500 mm and the average temperature is +8 to +10 °C (SMHI, 1961a, 1961b). The bedrock was formed 1.8 billion years ago and consists of granite, granodiorite, syenitoid, quartz monzodiorite and metamorphic equivalents (SGU, n.d.a). The soil types in the area are a mix of post-glacial and glacial silt (SGU, n.d.b).

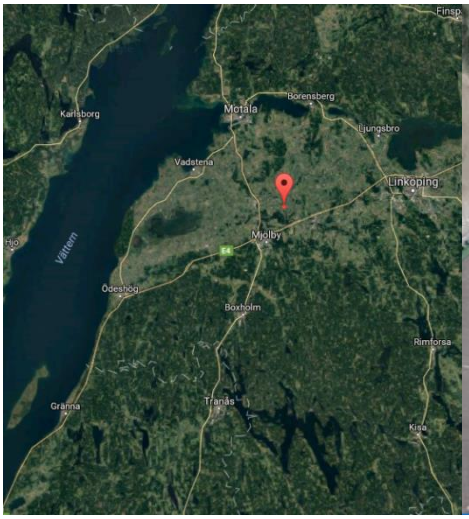

Figure 1. Site of sample collection, west of Linköping in Östergötland County.

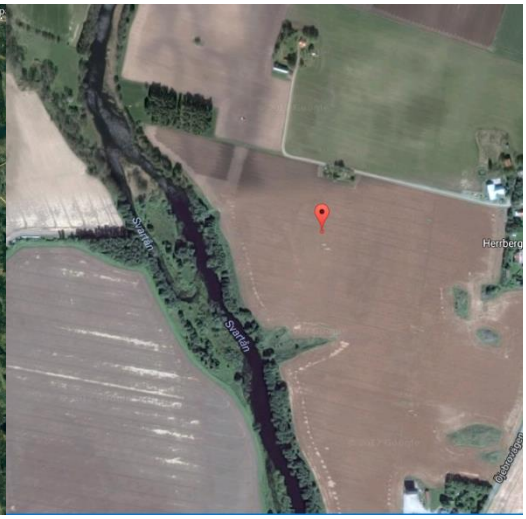

Figure 2. Collection site in field.

The experiment consisted of 7 treatments arranged in 4 replicate blocks (28 plots in total). Each treatment plot size was 18 m^2. Every plot received NS 27-4 (54 kg N ha^{-1}, 8 kg S ha^{-1}) fertilization at straw elongation. The treatments were the following: a control with no added fertilizer; ash application (from Mora district heating plant, applied amounts equivalent of 13,75 kg P ha^{-1}); biogas digestate application (one digestate from Uppsala and one from Linköping, applied amounts equivalent of 100 kg total N ha^{-1}); combined ash and digestate application (digestate Uppsala+ash and digestate Linköping+ash, applied amounts equivalent of 100 kg total N ha^{-1} and 20 kg P ha^{-1}); and mineral fertilizer application (NPK 27-3-3 at establishment, applied amounts equivalent of 100 kg total N ha^{-1} and 11 kg P ha^{-1}). The nutrient content of the waste-based fertilizers varied (Table 2), which is why the applied amounts are given in equivalents.

Table 2. Concentrations of total N, total P and Cd (digestion by conc. HNO_3 and HF) in the waste-based fertilizers, and the soil from Öjebro.

	Digestate (Linköping)	Digestate (Uppsala)	Ash (Mora)	Soil Öjebro
N (% DW)	5.95	5.49	0.11	0.13
P (% of DW)	1.41	1.45	1.33	0.06
Cd (mg kg^{-1} DM)	0.485	0.357	13.3	<0.2
pH (H_2O)	8.11	8.14	12.74	-

The applied fertilizers were applied according to the recommendations of the Swedish Board of Agriculture (Albertsson et al., 2016) and practice by the local farmers.

The ash in the combined applications was added to balance the N input from the digestate. The digestate and ash were applied at establishment.

The examined crop was spring wheat (*Triticum aestivum*) of the variety Quarna, which was sown on the 11th of April 2017 with 12.5 cm between rows. The tillage procedures can be seen in appendix 1. The wheat was harvested on the 12th of September.

3.2 Sampling

Samples of soil and roots were collected on the 19th-20th of June 2017. They were taken when the accumulated wheat biomass was large and the nutrient uptake was still high (DC 51-59, ear emergence) (Hirzel and Undurraga, 2013, Yara, n.d.). During the days of sampling the conditions were sunny and windy.

For each plot the following steps were taken: 12 soil core samples (down to 10 cm depth) were taken in a zigzag pattern across each plot. The 12 subsamples were gently homogenized into one big sample, and was then put in a portable cooler until reaching the facilities at SLU for further sample preparation.

To collect root samples 0,3 running metres of a wheat row was dug up in each plot with corresponding volume. The hole was dug in a row 50 cm in from the border of the northwest corner of the plot (to avoid external influence), except in plot 4 where the sample was taken in the northeast corner due to a patch of poor growth in the northwest corner. This volume was decided because most of the roots were suspected to be within that depth since wheat usually have more than 60% of its root mass in the upper 30 cm of the soil (Fan et al., 2016). Furthermore, the width 12,5 cm was the middle between two rows and therefore the most of one row was included without getting too much of another row's roots in that sample. The intact root samples were put in a portable cool box until arriving at SLU where they were put in the refrigerator awaiting to be cleaned from soil. The root samples were disturbed as little as possible to minimize the effect of the time until the roots were prepared.

3.3 Sample preparation

The sample derived from pooled cores was allowed to retain the roots included and freeze-dried in its entirety. After 3-4 days the samples were ground to a fine powder using a pestle.

Samples of root free soil was collected from the soil removed from the root samples (the volume of 30 cm x 12.5 cm x 10 cm). A subsample of this soil was taken by pooling small samples from the whole sample and sieved through a 250 µm sieve to remove as many roots as possible. If any root bits were visible they were removed with a pair of pincers. The samples were frozen, freeze-dried for 3-4 days and then pestled to a fine powder. During freeze-drying the bags had to be open and there is a possibility that contamination can occurred as the air could have moved the finest particles in the samples.

From each root sample the soil was removed by shaking the roots gently and using a 2 mm sieve. The roots were cut as closely as possible to the crown (1-2 mm away from the crown). The roots were then washed and rubbed softly with deionized water. The cleaned roots were gently dried using a paper towel and then weighed. About 1 gram of roots were put in Falcon tubes with 25 ml of ethanol (70 %), the remaining roots were intended for qPCR and Cd determination and put in plastic bags and put in the freezer. The frozen roots were then freeze-dried for 3 days and weighed before being ground in a Lab-Wizz 320 micro ball mill (Laarmaan Group B.V., Roermond, Netherlands). Approximately 50 mg was used for the DNA extraction and the remainder was used for Cd determination at ALS Scandinavia AB.

In total there were 28 root samples for Cd determination and 84 root and soil samples for qPCR, and 28 root samples for the microscope investigation.

3.4 Laboratory work

3.4.1 DNA-extraction

Both the DNA extraction, and the subsequent qPCR (quantitative polymerase chain reaction) were carried out at the laboratory at the Dept. of Forest Mycology and Plant Pathology, SLU, Ultuna. DNA from 300 mg soil or 50 mg roots were extracted using the NucleoSpin® 250 Soil kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. The only deviation from the protocol was in step 3 where a Precellys® 24 (Bertin Technologies, Rockville, USA) was used (5000 rpm, 2 x 30 seconds) instead of vortexing horizontally for 5 minutes. A few trials with both roots and soil samples were made before the real extraction to optimize the extraction and find a suitable buffer for these specific samples. The

ones which showed the strongest bands in the electrophoresis was deemed the optimal buffer for this extraction (SL1 in combination with the enhancer SX).

3.4.2 Production of qPCR standard

DNA was extracted from a clean culture of *Rhizophagus irregularis* colonizing roots of *Plantago lanceolata* and *Trifolium repens*, by using the NucleoSpin® 250 Soil kit. A PCR was performed for three different roots infected by isolates of AM fungi using primers NS31 and AML2 (Lee et al., 2008; Eurofins Genomics GmbH, Ebersberg, Germany, see Figure 3) with the thermal profile: 95 °C for 10 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and finishing with 74 °C for 9 minutes. The master-mix contained reaction buffer, 200 µM dNTP mix, 750 µM salt ($MgCl_2$), 0,025 U/µl DreamTaq, ddH_2O and 0,2 µM of each primer. After PCR amplification the product was cleaned from salt, unused nucleotides etc. using the E.Z.N.A Cycle Pure Kit (Omega bio-tek, Georgia, USA), following the manufacturer's instructions.

Next step in production of the standard was to amplify the PCR product using a bacterial host. The cleaned PCR product was ligated into bacteria using the TOPO TA kit (vector PCR® 2.1-TOPO with 3931 base pairs). The solution was spread out on Petri dishes with LB-medium, ampicillin and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The dishes were incubated overnight (37 ºC). This produced white and blue colonies that were resistant to the ampicillin. Only the white colonies were selected since they had taken up the PCR-product successfully. They were white because the gene on the plasmid vector needed for converting X-gal was disrupted by the PCR-product insert. These white colonies were streaked on a new Petri dish. The blue colonies contained resistance against ampicillin in the medium but did not contain the PCR-product as they exhibited the blue X-gal colour. The blue colonies were therefore discarded. The new Petri dishes were incubated overnight and the next day one clone was picked for further cultivation. The clone was put in 3 ml LB-medium with 3 µL ampicillin. Once again, the clone was incubated overnight in 37 ºC but this time on a shaker. Plasmids from the clone cultivation were then extracted using the GeneJET plasmid miniprep kit (Thermo Scientific, Massachusetts, USA) and put in glycerol for preservation. The DNA-concentration was measured in NanoDrop.

3.4.3 qPCR

The qPCR, also known as Real-time PCR because it measures the amount of DNA continuously during the PCR (Freeland et al., 2011) was carried out using Sso-Fast™ EvaGreen® Supermix (Bio-Rad Laboratories, California, USA) and the primers: AMV4.5NF (AAG CTC GTA GTT GAA TTT CG) and AMDGR (CCC AAC TAT CCC TAT TAA TCA T) in semi-skirted plates. The primers used for the standard (AML2 and NS31) covered a larger segment than the primers AMV4.5NF and AMDGR (Sato et al., 2005) while still including the segments covered by them (Figure 3).

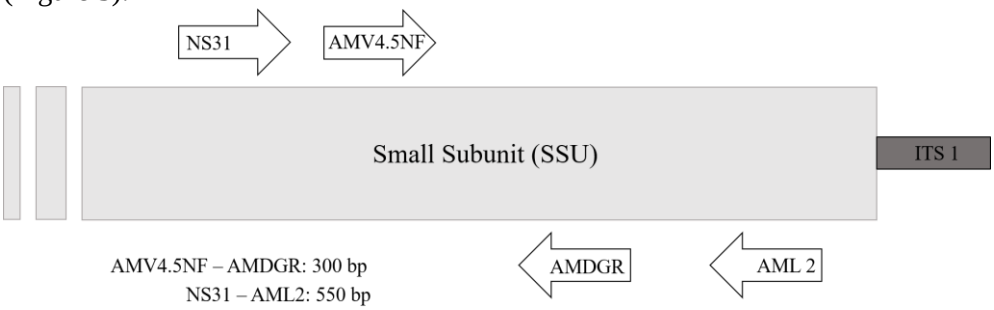

Figure 3. Schematic picture of the primers used in the qPCR. The NS31 and AMV4.5NF are the forward primers while the AMDGR and AML2 are the reverse primers. The number of base pairs for each primer pair is stated in the left corner of the picture. Adapted from Van Geel et al. (2014).

Using *Nucleotide BLAST* search towards all available sequences in GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for the primer sequences confirmed that the vast majority of matching gene sequences belonged to arbuscular mycorrhizal fungal species. The qPCR was run for 40 cycles using a iQ™5 Multicolor Real-Time PCR machine (Bio-Rad Laboratories, California, USA) with the following thermal profile: a start up with 95 °C for 10 min, and then the cycle scheme of 40 cycles of 95 °C for 15 s, 55 °C for 20 s, 60 °C for 45 s. The DNA was quantified by fluorescence at the end of the 60 °C period of each cycle. Three technical replicates were run per sample and then the average number of gene copies was determined from those replicates.

The DNA extracts of the root and soil samples were diluted 10 times. Before the qPCR assay an inhibition test was carried out on two soil samples and one root sample with x10 and x100 dilution to determine if there were inhibitors in the DNA extractions that would counteract the amplification. No inhibition was found and the dilution (x10) was kept for the qPCR assay for all samples. Six different dilutions were used for the Standard curve: 300-30 000 000 gene copies (x10 in each dilution step) per reaction. Since the original number of gene copies in each dilution step

was known, a standard curve of fluorescence against starting quantity was constructed and could be used to calculate gene copy numbers in the samples. A mean linear equation was calculated from all the standards and used in the calculations. The attained cycle threshold (C_T) in each technical replicate was used to calculate the mean starting quantity in the samples. The C_T is the cycle where the amount of fluorescence in a sample exceeds a certain threshold. An example of a standard curve and a calculation can be seen in appendix 2 and 3. If the standard deviation (SD) for the C_T was equal or less than 0.5 for at least two of the technical replicates they were approved. If more than one technical replicate exceeded the 0.5 limit they were all rerun. A baseline of 100 was set for all the plates to be able to compare between plates. The baseline is based on the initial cycles of the assay where there are only small changes in the fluorescence and this is then subtracted from the results as it represents only background fluorescence.

The gene copy numbers as calculated from the qPCR results were compensated for the different dilution steps in DNA-extraction and qPCR-preparation. Then the weigh-in amount of the samples was taken into the equation to finally arrive at the number of gene copies g^{-1} DW sample.

3.4.4 Microscope investigation

The roots were washed with water and stored in Falcon tubes with 70 % ethanol in the fridge until staining. The ink used for staining was Royal Blue (Pelikan Ink 4001®, Hannover, Germany) and it was done according to the ink-vinegar method by Vierheilig et al. (1998). The roots were stained by boiling them in 5 % ink-vinegar solution for 3 minutes after which they were rinsed with acidified water (a few drops of acetic acid) for 20-30 minutes. They were then stored in tap water in room-temperature until the microscope investigation (Vierheilig et al., 1998).

For each subsample 16 of the finest roots were chosen and mounted vertically on a microscope slide, with a maximum of three small roots taken from the same root. The finest roots were chosen as the coarser ones did not allow a tight seal between slide and cover slip. Water was used as mounting liquid. The roots were mounted vertically along the long axis of the slide. The estimation of colonisation was done with a light microscope (Zeiss Axioplan, Germany) using the magnified intersections method at magnification x250 (McGonigle et al., 1990) with ca 200 intersects per subsample. The cross-hair eye piece was moved from left to right and back again, looking at intersects during 10 passes over each subsample (Figure 4). The presence or absence of AM structures (arbuscules and vesicles) were noted. This was repeated for totally two subsamples per sample. The microscope investigation

was only done for block one, i.e. 7 samples. Lastly, the AM colonisation in percent was obtained using the formula: (number of intersects with noted AM structures/total number of intersects)x100.

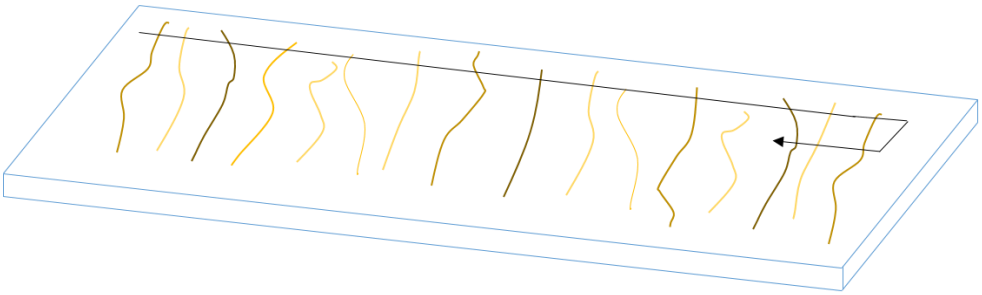

Figure 4. Schematic picture of the microscope slide with placing of roots and procedure.

3.4.5 Cd content determination in roots

Eight samples of the finely ground roots were sent to ALS Scandinavia AB for determining content of Cd. These 8 samples were sent as test samples, and if they were found to be contaminated with soil the remaining samples would not be analysed. 0.5 g of plant material from each sample was first digested in 5 ml concentrated HNO_3 and 20 µl concentrated HF in open containers in a microwave oven, after which they were filtered and the Cd content was measured on ICP-SFMS. The result was attained in milligrams per gram air dry sample. Soil contamination could not be ruled out which is why the root results were not used in the analysis.

3.4.6 Cd content determination in above ground plant parts

Information concerning the content of Cd in the straws and kernels were carried out in the original project (by ALS Scandinavia) and was included in this study. The method for Cd determination was the same as for the roots, with the difference that the amount of analysed kernels were doubled to enable digestion of whole kernels instead of ground.

3.5 Statistical analysis

The calculations were made in Microsoft Excel 2013 and the statistical analysis was done using JMP® Pro 13 (SAS Institute Inc., Cary, USA). One-way ANOVAs and regression analyses were performed to find differences between treatments and possible correlations. The factors used in these ANOVAs were Treatment – all the seven different treatments; Fertilizer – treatments with any kind of fertilizer. The

experiment design allowed for separating the ash and digestate as two independent effects and explore their interaction statistically. In that two-way ANOVA the following factors were included: Ash only; Organic material – treatments containing digestate; Interaction - treatments with a combination of ash and digestate. In all cases, the blocks were included in the analysis to account for the possible block effect. When significance was found in the ANOVAs based on the seven treatments, a Tukeys test was performed to determine which treatment gave the significant result (α=0.05). For the regression analyses the following variables were used: Biomass – above ground, below ground and total wheat biomass (g dry mass m^{-2}); Cd concentration – mg Cd kg^{-1} DW straw or kernel; pH-values. When needed, the response variables were log-transformed to achieve normal distribution.

4 Results

4.1 Wheat biomass

The maximum total DW biomass was found in the mineral fertilizer treatment while the least was found in the control treatment (Figure 5). Bulk density did not differ among treatments.

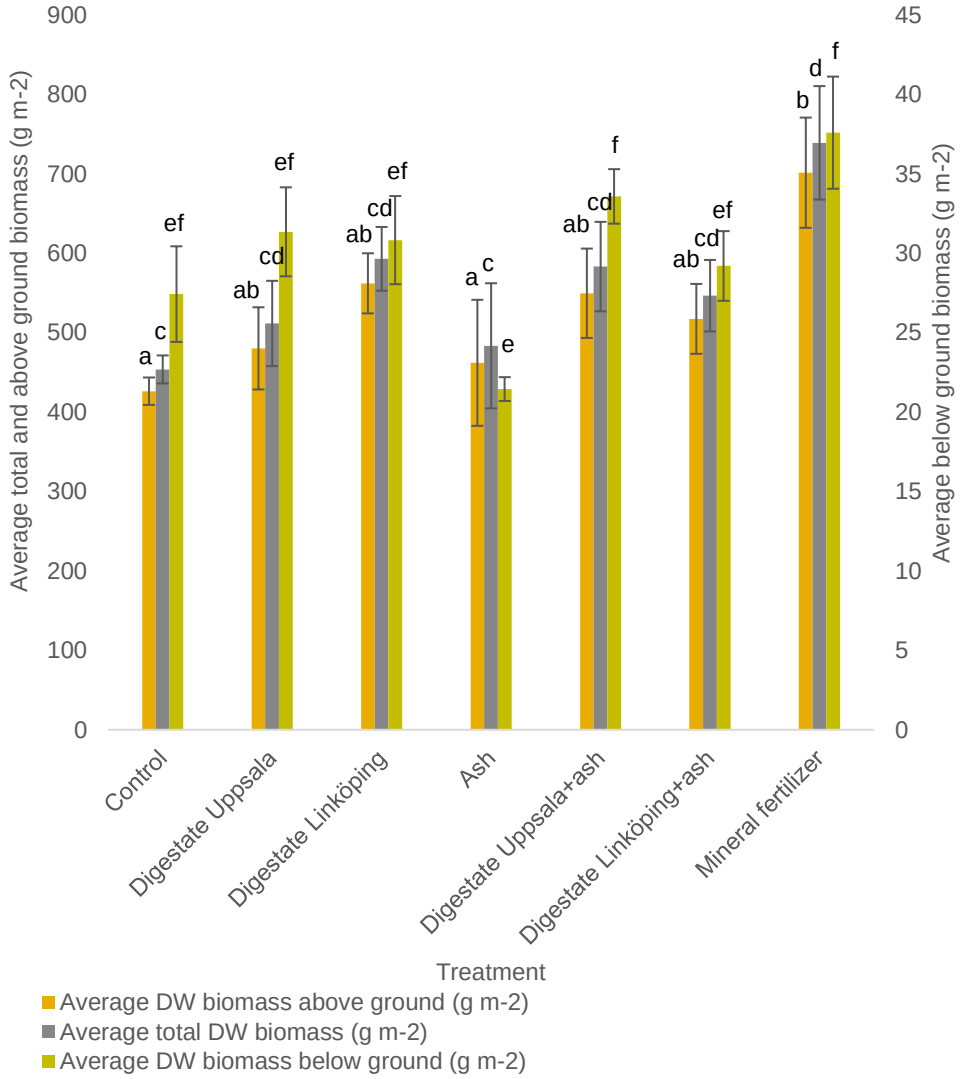

Figure 5. The effect of organic material, ash and mineral fertilizer application on wheat biomass. The response is divided into total DW biomass, and above ground DW biomass (left axis) and below ground DW biomass (right axis). SE-bars are displayed in diagram. N=28.

The wheat plants' biomasses were significantly affected by the treatments (Figure 5). A Tukeys HSD showed that the mineral fertilizer treatment had a larger biomass than the ash application and the control for both the total and the above ground biomass. For the below ground biomass, the mineral fertilizer as well as the digestate (Uppsala)+ash had a larger biomass than the ash application.

The ash treatment had the highest ratio of above ground:below ground biomass. The treatment of digestate (Linköping) had the highest ratio of below ground:above

ground biomass. The Cd contents of straw and kernel were not affected by the treatments. Neither the pH values nor the percentage of root-associated AM fungal biomass were affected by the different treatments (Table 3).

Table 3. ANOVA results of the treatments' effects on wheat biomass, Cd content, pH values and percentage of root-associated AM fungal biomass. Significant values are marked with a. N=28.

One-way ANOVAs		Main effect Treatment
Total biomass (g m^{-2})	F-ratio	3.4574
	p-value	0.0189[a]
Biomass above ground (g m^{-2})	F-ratio	3.3056
	p-value	0.0226[a]
Biomass below ground (g m^{-2})	F-ratio	3.9891
	p-value	0.0103[a]
Cd content (mg kg^{-1} DW straw)	F-ratio	0.4881
	p-value	0.8087
Cd content (mg kg^{-1} DW kernel)	F-ratio	1.2989
	p-value	0.3072
pH H_2O	F-ratio	0.7313
	p-value	0.6307
pH $CaCl_2$	F-ratio	0.5672
	p-value	0.7511
% root-associated -AM fungal biomass	F-ratio	0.803
	p-value	0.5804

4.2 qPCR analysis

A comparison was made between the number of gene copies per square meter and the amount of wheat biomass as well as the Cd content of the wheat plant. The amount of Cd in the straw and kernel, and the soil pH value was not correlated to the amount of AM fungi in any of the samples regardless of substrate. For the roots and the soil with roots the wheat biomass was not correlated with the amount of AM fungi, but for the root free soil, the total biomass and above ground biomass of wheat was correlated with the amount of AM fungi (p=0.001, Table 4). Most of the correlation coefficients were close to zero. The highest correlation coefficients were the

total biomass and the above ground biomass (r^2=0.58). The variation found was only partly explained by the relationship between biomass and amount of fungi.

Table 4. Regression analysis with pairwise comparison of amount of AM fungi with wheat biomass, Cd content and pH values. Significant values are indicated with [a]. N=28 for each substrate.

	Number of gene copies per gram DW sample of:					
Variable	Soil with roots p-value	Correlation coefficient	Root free soil p-value	Correlation coefficient	Roots p-value	Correlation coefficient
Total biomass (g m^{-2})	0.8806	-0.0297	0.0014[a]	0.5750	0.6615	0.0144
Biomass above ground (g m^{-2})	0.8484	-0.0378	0.0012[a]	0.5803	0.6134	0.0003
Biomass below ground (g m^{-2})	0.4729	0.1414	0.3020	0.2023	0.1610	0.3076
Cd content (mg kg^{-1} DW straw)	0.6505	0.0895	0.9459	0.0138	0.4276	0.1597
Cd content (mg kg^{-1} DW kernel)	0.7882	0.0531	0.5766	0.1086	0.2679	-0.2168
pH H_2O	0.7154	0.0721	0.5795	0.1094	0.7941	0.0517
pH $CaCl_2$	0.7550	0.0617	0.5381	0.1215	0.8792	0.0301

The pH was rather similar between the treatments and was rather neutral (Figure 6). However, the values of the individual plots were rather different within treatments. As it could not be determined if there were outliers or not, all the values were kept. The same was true for the wheat biomass determination.

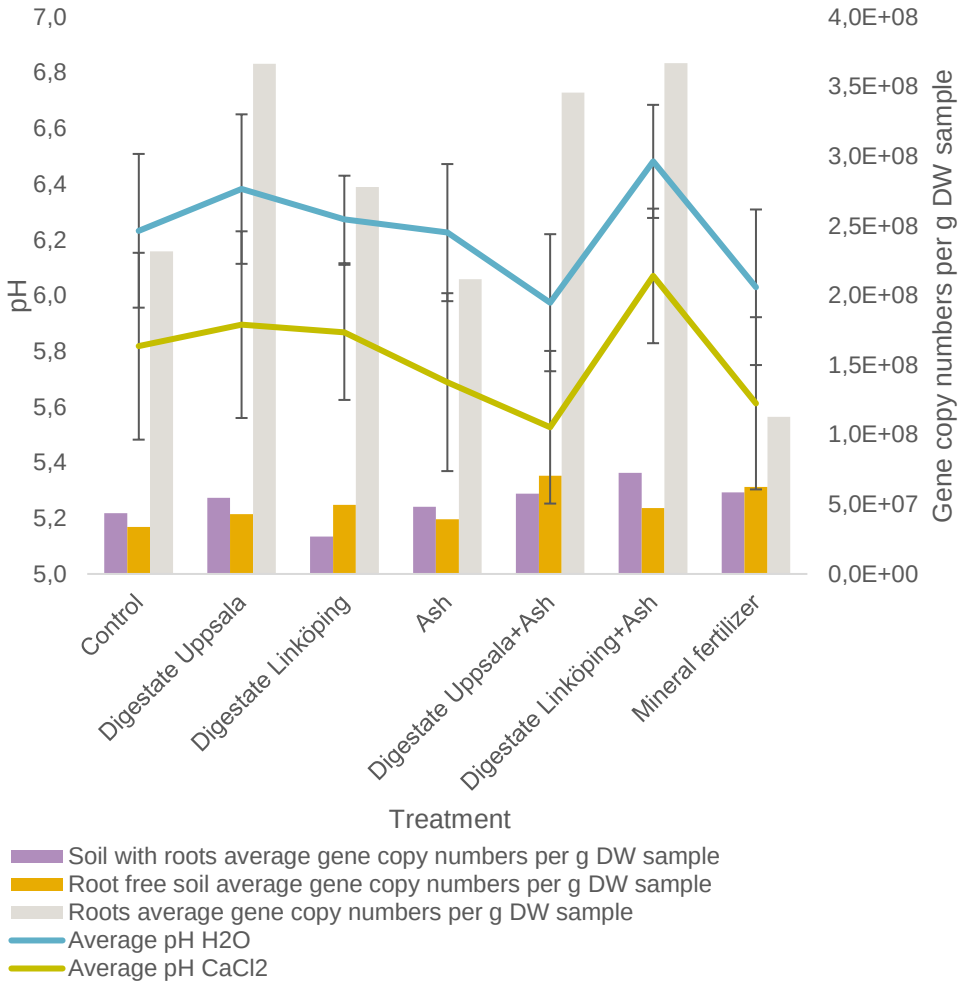

Figure 6. Average pH values (left axis) in the different treatments using two methods, displayed against the average number of gene copies per treatment in the three substrates (right axis). SE-bars for pH values are displayed in diagram. N=28.

When comparing the treatments for the soil with roots an effect was found and Tukeys HSD showed that it was digestate (Linköping)+ash that had more gene copies compared to the digestate (Linköping) treatment (p=0.02, Figure 7).

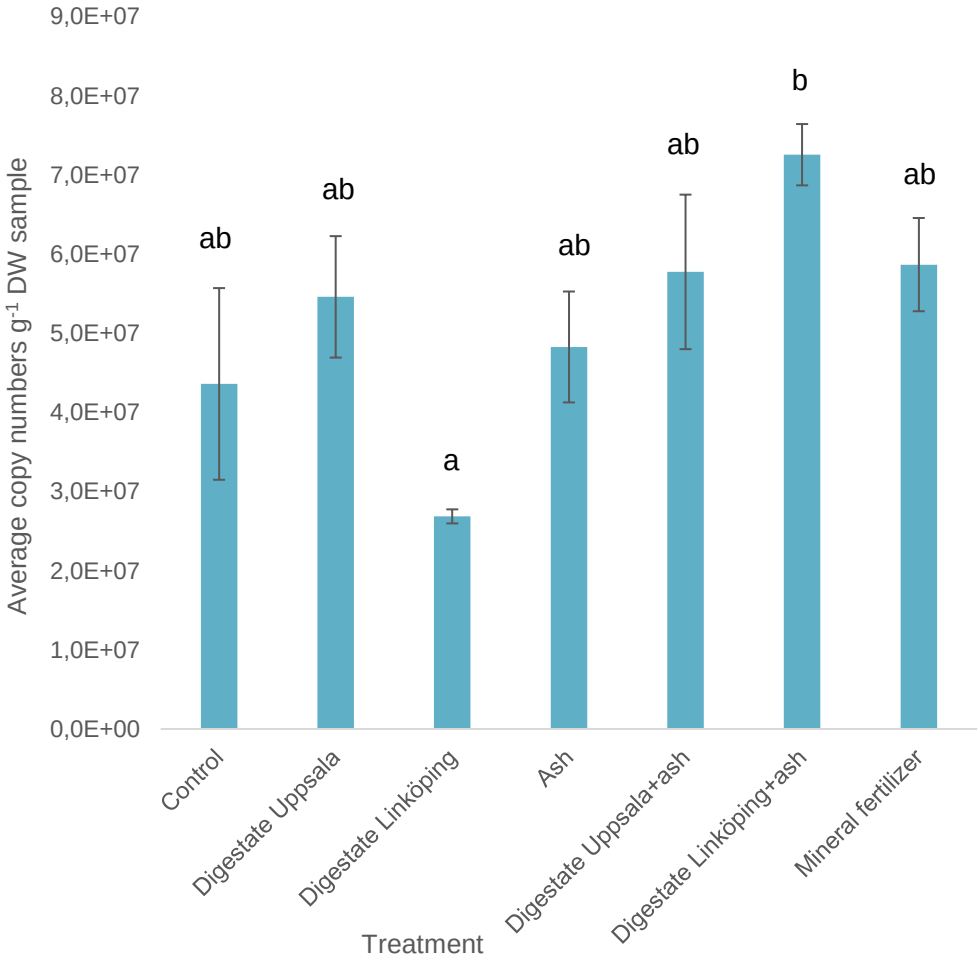

Figure 7. ANOVA results of the effect of treatments on the number of SSU copy numbers per gram dry weight sample Soil with roots, non-transformed data. SE-bars are displayed in diagram. Treatments sharing the same letter were not significantly different. Lettering is based on a Tukeys HSD. N=28.

The root free soil did not show any significant results from the different treatments (Table 5).

The AM fungi in the roots were affected by the treatments (p=0.04). When a Tukeys HSD was performed Digestate (Linköping)+ash showed higher numbers of gene copies in the wheat roots than the mineral fertilizer treatment (Figure 8).

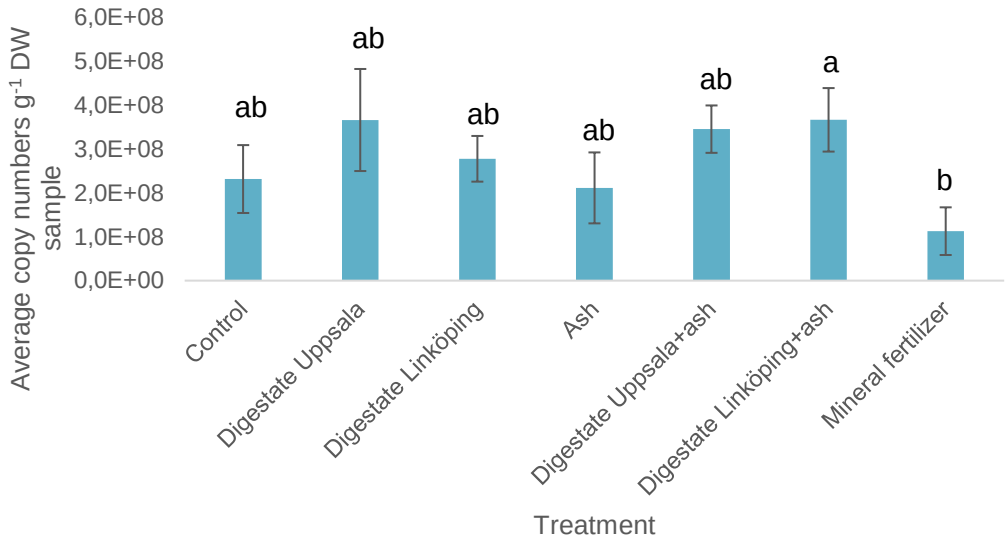

Figure 8. ANOVA results of the effect of treatments on the number of SSU copy numbers per gram dry weight sample in Roots, non-transformed data. SE-bars are displayed in diagram. Treatments sharing the same letter were not significantly different. Lettering is based on a Tukeys HSD. N=28.

When investigating the effect of organic material or ash application in the soil with roots, there was a trend that the ash treatment had an effect, but this was not the case when the effect of fertilization (of any sort) was investigated (Table 5).

For the root-free soil there was no effect of the different treatments. The mere application of fertilizer did not have a significant effect neither had the application of ash, organic material or combination of the two (Table 5).

In the Roots' ANOVA, when the effect of ash and organic addition and their interaction was tested the organic material had a significant effect on the amount of AM fungi. However, when the factor of fertilizing the soil was added, there was no significant effect of fertilizing (Table 5).

*Table 5. Summarized results from ANOVA for all substrates: Soil with roots (non-transformed data), Root-free soil and Roots (log-transformed data). Significant values are marked with [a], and trends are marked with *. N=28 for each substrate.*

			Soil with roots		**Root free-soil**		**Roots**	
	Levels	**Main effects**	**F-ratio**	**p-value**	**F-ratio**	**p-value**	**F-ratio**	**p-value**
ANOVA	7	Treatment	3.6574	0.015[a]	0.6875	0.6624	2.849	0.0395[a]
	4	Ash	3.631	0.0738*	0.5175	0.4817	0.0032	0.9553
		Organic material	0.8432	0.3713	2.2463	0.1523	5.5033	0.0314[a]

		Interaction ash*org	1.6734	0.2131	0.0081	0.9294	0.3797	0.5459
	2	Fertilizer	0.8321	0.3711	1.9899	0.1717	0.1791	0.6761

A t-test was performed to see if the total number gene copies m^{-2} in roots and root free soil (from the two samples derived from the same initial soil sample) was different from the gene copies in soil with roots (collected and handled as an intact sample) and identified no significant difference between the treatment means ($p>0.05$).

The percentages of AM fungal biomass found inside the roots compared to outside in the soil (Table 6) was calculated from the combination of the two substrates roots and root free soil since these were derived from the very same field samples. There were no significant treatment differences (Table 3).

Table 6. The percentage of AM fungal biomass found inside the roots in the different treatments, compared to the percentage of AM fungal biomass found in the soil. The percentages were calculated from the combined results of gene copies m^{-2} in the substrates roots and root free soil.

Treatment	Percentage of AM fungal biomass found inside the roots (%)
Control	0.28
Digestate Uppsala	0.35
Digestate Linköping	0.24
Ash	0.20
Digestate Uppsala+ash	0.37
Digestate Linköping+ash	0.37
Mineral fertilizer	0.12

The ash treatment and the control had the smallest wheat biomass, and they both had low percentages of root-associated AM fungal biomass. Mineral fertilizer resulted in the highest amount of total wheat biomass but had the least proportion of root-associated AM fungal biomass.

4.3 Microscope investigation

The microscope investigation was only conducted for one block (seven plots) due to time limitations and very low colonisation rates (Table 7).

Table 7. The number of AM fungal structures found under the microscope per treatment in block 1. The number of arbuscules and vesicles are divided with the total number of observations to receive the colonisation of the roots. Two subsamples were done per treatment.

Block	Treatment	Number of arbuscules and vesicles	Number of empty intersects	Total number of observations	AM colonisation of roots (%)
1	Control	2	268	270	0.74
	Digestate Uppsala	4	362	366	1.09
	Digestate Linköping	2	338	340	0.59
	Ash	0	321	321	0.00
	Digestate Uppsala + ash	2	314	316	0.63
	Digestate Linköping + ash	2	297	299	0.67
	Mineral fertilizer	1	327	328	0.30

The colonisation (number of arbuscules and vesicles) was small in all samples, as was the difference between the plots. The colonisation ranged between 0-1.1 %. An example of arbuscules can be seen in Figure 9. The microscopy findings (even though there are few observations) do not correspond with the qPCR findings. The highest individual colonisation numbers were found in the digestate (Uppsala) and the control (1.1 and 0.74 % respectively), while the highest percentage of root-associated AM fungal biomass (0.37 %) in the qPCR was found in the two digestates in combination with the ash. No colonisation was found in the ash treatment which does not correspond with the qPCR findings.

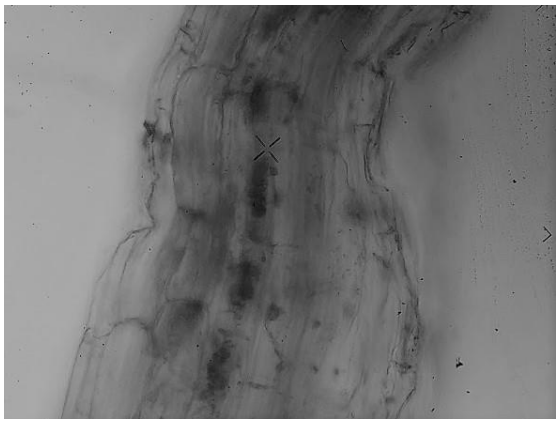

Figure 9. Example of arbuscules (the darker spots that runs through the middle of the root) in a wheat root sample.

The mineral fertilizer treatment had both a low colonisation (0.3 %) and a low proportion of AM biomass inside the roots (0.12 %).

4.4 Cd content in above ground biomass

The Cd content is on average 1.4 times higher in the straw than in the kernel (Figure 10). There was no effect of different treatments on the Cd concentration in the plant ($p>0.05$, Table 3).

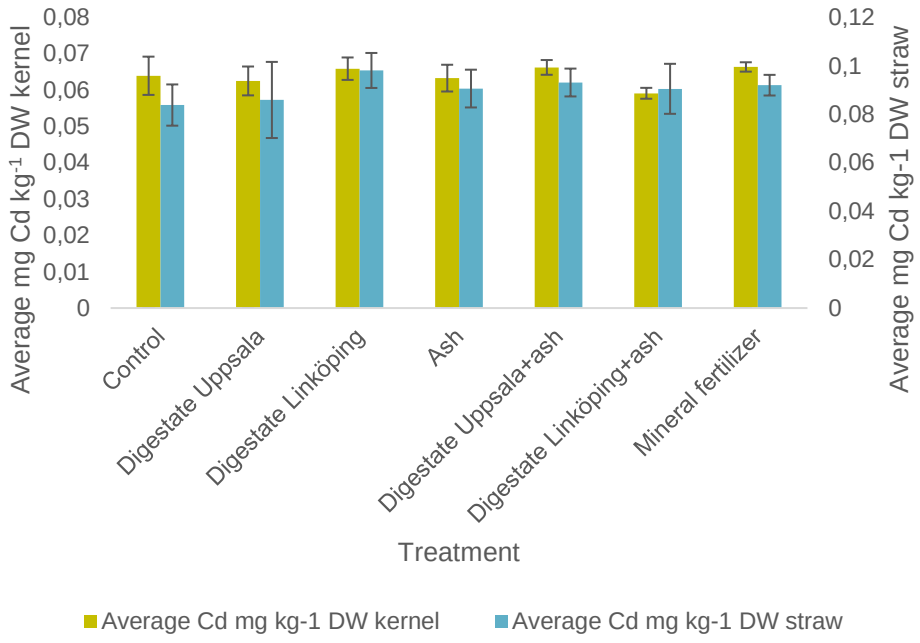

Figure 10. Cd content in DW kernel (left axis) and straw (right axis) of wheat. SE-bars displayed in diagram. N=28.

5 Discussion

5.1 Overall abundance of AM fungi in roots and soil

In this study I used the SSU gene copy number to quantify abundance of AM fungi in soil and wheat roots in a spring wheat field. As suspected the highest copy numbers of the AM gene g^{-1} DW sample was found in the roots. This substrate had about 5 times as many gene copies as the soil with roots and the root free soil. However, when the results were re-scaled to gene copies per square meter the two soil fractions had the most AM fungi as the roots constituted such a small part of the whole area.

5.2 Wheat biomass and AM fungi

The AM fungal biomass that was situated in the soil (root free soil fraction) was positively correlated with the total and above ground wheat biomass (Table 4), but was not affected by any of the fertilizers (Table 5). If the biomass affects the AM fungi or if the AM fungi affects the biomass is not completely clear. The total and above ground wheat biomass was higher in the mineral fertilizer application compared to ash and the control treatment (Table 3), this suggests that these fungal structures could indirectly benefit from mineral fertilizer while root-associated AM fungal biomass do not. The AM fungal biomass in the roots were instead more abundant in the digestate (Linköping) treatment (Table 5).

The roots that were used in this study were taken from the top 10 cm of the soil, leaving the rest of the root system undisturbed. Narayanan et al. (2014) found that the rooting depth of 296 different spring wheat cultivars ranged between 77-202 cm and the root:shoot ratio ranged between 0.18-4.1 compared to the results in this study where the root:shoot ratio ranged between 0.03-0.08. They also concluded that approximately 60% of the wheat root mass was situated in the top 30 cm of the soil. However, in this study the roots were cut from the crown, while in Narayanan

et al.'s (2014) study they included the base of the stem in the root ratio. If the base of the stem had been included and the same density of root biomass had been found downwards in the soil profile the root:shoot ratio would correspond to the lower end of the range 0.18-4.1. Nevertheless, even though only a small part of the root system was analysed in this study, the relative differences that were found between treatments are still valid, while the absolute amounts are not necessarily so.

5.3 Effect of treatment on AM fungi

The different types of fertilizers did affect AM fungi and the combination of digestate and ash seems to be particularly favourable for the AM fungi as there was significantly more gene copies g^{-1} DW sample in these treatments for two of the substrates (soil with roots and roots alone, see Table 5). The P in these fertilizers is not always easily available to the plant, and the plant therefore depends on the AM fungi to provide these nutrients (Demeyer et al., 2001; Nkoa, 2014; Smith et al., 2011). Adding organic material has been shown to have a positive effect on AM colonisation compared to inorganic P application (Mackay et al., 2017), which corresponds to the results in this study where the AM fungi in the root fraction was negatively affected by the mineral fertilizer treatment (Table 5, Figure 8). This treatment (mineral fertilizer) also showed the second lowest AM root colonisation in the microscopy investigation and the lowest average proportion of root-associated AM fungal biomass (Table 6 and Table 7). At the same time, the mineral fertilizer treatment had the greatest total wheat biomass which suggests that there was no problem for the plant to take up nutrition without the AM fungi in the roots. Hetrick et al. (1993, in Sawers et al., 2008) studied how wheat varieties developed before and after the turn of the 20th century responded to AM fungi and suggested that wheat breeding had decreased the dependence upon AM fungi. The fact that mineral fertilizer causes the host plant to be less dependent upon the AM fungi seems to have a negative effect on the AM fungi, at least the ones that have most of their biomass in the roots, which also corresponds to the findings of Cruz-Paredes et al. (2017). They furthermore observed that AM fungi were not affected by normal application of ash (in Denmark, equivalent of 30 kg P ha^{-1}), but positively affected by high ash application rates (equivalent of 300 kg P ha^{-1}) as there was higher arbuscular and hyphal colonisation in this treatment. These authors also found that there was less AM colonisation in the control (no fertilizer) and the Triple Superphosphate (TSP) application, than in the high ash application. The current study found that the ash never had a significant effect on its own (only a trend in the soil with roots) but only in combination with digestate (Linköping) (Figure 7 and Figure 8).

Fungi with extensive soil mycelium, such as Gigasporaceae, have been found to benefit from N-fertilization in P deficient environments compared to AM fungi that allocate more of their biomass to root colonisation and less to an extensive soil mycelium, such as Acaulosporaceae, that are more stress-tolerant (Chagnon et al., 2013; Hart and Reader, 2002). One of the hypotheses in this study was that this would result in a shift in root-to-soil AM biomass ratio with the treatments. The percentage of root-associated AM fungal biomass was not correlated with the treatments (Table 3) but the percentages (Table 6) mirror the results from the qPCR. However, when the percentage of soil-associated AM fungal biomass increased in the mineral fertilizer treatment, the actual amount of soil-associated AM fungal biomass might not have increased, rather it is likely that the root-associated AM fungal biomass was simply at a disadvantage in the soil with easily available nutrients. This may indicate a shift in AM fungal community composition, since all AM fungi have biomass both outside and inside the root, but with different proportions in the two phases. In order to draw further conclusions on the effects of fertilizers on AM fungi with different life strategies, the AM fungal community composition would need to be explored.

The number of identified AM structures (arbuscules and vesicles) in the microscope investigation were low in all treatments (Table 7). The low colonisation of the wheat roots was rather surprising as June was found to be the peak of root colonisation in Cruz-Paredes et al. (2017) investigation. In García and Mendoza's (2008) study the colonisation was found to be highest in the beginning of the growing season (late winter), and during the summer instead more vesicle (temporary storage organs for AM fungi) colonisation was found, which indicates that in this study AM fungi structures should have been found in the roots if there had been any.

5.4 pH and AM fungi

The pH values did not vary significantly between the treatments. The average pH of each treatment suggested that digestate (Linköping)+ash had the highest pH (pH 6.5) and the digestate (Uppsala)+ash treatment had slightly lower pH (pH 6.0, see Figure 6). The low pH value of digestate (Uppsala)+ash was rather surprising as the ash had such a high pH value (Table 2). The amount of AM fungi in the soil with roots and roots alone seemed to be slightly more abundant in the treatment with the highest pH, while the AM fungi in the root free soil seemed to be more abundant in the treatment with the lowest pH. This does not correspond with the literature where the AM fungi that have an extensive soil mycelium and “coarse” hyphae (competitive AM fungi such as Gigasporaceae), which would rather be expected to be found

in the root free substrate, have been found to be positively affected by high pH values. Furthermore, the AM fungi that have an extensive root mycelium and "fine" hyphae (stress-tolerant AM fungi such as Acaulosporaceae), which would be expected to be found in the root substrate, instead was supposed to have an advantage in more acidic conditions and other stressful situations (Chagnon et al., 2013; Hart and Reader, 2002). The reason that the results of this study did not conform with the previous studies regarding pH is most likely due to the pH-values being rather neutral and because potential treatment effects were smaller than the plot level variation, which make findings of possible correlations with pH rather difficult. Fungi generally prefer a pH close to neutral in their environment but can survive in lower pH as well. They do not compete well with other soil organisms if the pH is greater than neutral (Rousk et al., 2009, Arao, 1999). Clark (1997) reported that root colonisation decreased with decreasing pH. In the study by Cruz-Paredes et al. (2017), community composition of AM fungi was not affected by pH, but rather by the amount of available P. More experimental replicates and a wider pH range would therefore be needed to facilitate the interpretation of pH-related treatment effects on AM fungi.

5.5 Effect of AM fungi on Cd concentration

The cadmium concentration in the above ground plant biomass was not different between the treatments, neither was it correlated with the amount of AM fungi. Similarly, Gao et al. (2010) concluded that the AM fungi colonisation was not correlated to the Cd content of the grain. However, there have been studies where AM fungi can decrease the Cd-concentration in different plant parts, for plants such as maize and wheat (Liu et al., 2018; Shahabivand et al., 2012). Similar results were also shown in the study by Cruz-Paredes et al. (2017), which showed that the composition of the AM community, rather than total AM colonisation of roots, affected the Cd concentrations in the above ground biomass of the plant.

The previous crop (potatoes) in this experiment does not have a high dependency on AM fungi (Plenchette et al., 1983). However, Plenchette et al. (1983) compared crops planted in fumigated and non-fumigated soils. Such treatment might kill other organism than just AM fungi. A more recent study showed that inoculation with the AM mycorrhizal fungus *Rhizophagus irregularis* significantly increased the potato yield (Hijri, 2016).Even though it is not dependent upon the AM fungi the potato crop apparently benefitted from the symbiosis. Gao et al. (2010) found that wheat that was grown after a non-mycorrhizal crop (canola) had a lower colonisation than one being grown after a mycorrhizal crop (flax). According to Plenchette et al.

(1983) wheat is not dependent on AM fungi for its growth and can grow equally well in the presence or absence of AM fungi. On the other hand, Singh et al. (2012) found differences in dependency and compatibility with AM fungi between varieties of durum wheat. In this experiment the plants grew well regardless of the amount of AM fungi, which could be due to Quarna not being dependent upon AM fungi. However, in the study of Plenchette et al. (1983) the soil contained what was considered to be almost sufficient P for wheat, hence, there was no need for the crop to invest in a symbiotic relationship with the fungi. To the contrary Pellegrino et al. (2015) found in a meta-analysis that the grain yield of wheat, as well as the P concentration in the grain, was positively correlated to the root colonisation by AM fungi in field trials. This suggests that maintaining a high AM colonisation rate is nevertheless important for wheat grain production in general.

5.6 Methodological considerations

The number of target gene copies g^{-1} DW sample was used as an indication of the biomass of AM fungi in the different substrates. It was assumed that the differences in copy numbers was a sign of difference in abundance of AM fungi. Of course, the primers used in the qPCR did not amplify all AM fungi and there is no one primer pair that will amplify only the wanted species (Kojima et al., 2004; Redecker et al., 2000; Sato et al., 2005). However, Sato et al. (2005) found in a Nucleotide BLAST search that the specific primers used in this assay amplified almost all AM fungi. The Nucleotide BLAST search done in this study also only showed species of the Glomeromycota phylum, which form arbuscular mycorrhiza (https://blast.ncbi.nlm.nih.gov/Blast.cgi; Redecker et al., 2013). The variation in number of gene copies within each treatment was rather large and small differences between the treatments may not be possible to detect. This large range is possibly partly due to the standard deviation, SD≤0.5, that was allowed for the technical replicates in the qPCR assay. This SD allowed for a maximum of one qPCR cycle difference among the technical replicates run for a sample, which corresponds to a doubling in gene copies. The qPCR method is a very sensitive method since tiny amounts of DNA can be amplified (Bio-Rad Laboratories, n.d.) but might not be very precise as samples contaminated with foreign DNA during the laboratory processes could give misleading results. However, the blanks (control samples) were empty, supporting the validity of the results. The variation among replicates is most likely related to real biological variation in the system. There are a range of factors that could affect how well the samples reflect the plot-level AM fungal abundance e.g. sampling spatial organization, sample size, sub-sampling for analyses etc.

Another limitation was the microscope investigation since the staining was difficult to perfect and resulted in AM structures being difficult to discern, or simply my lack of experience made me miss structures. The latter is not very likely as several practice runs were made before the actual investigation and many pictures of AM structures were used to compare with. More likely there was low AM colonisation in the field.

Conclusions

The conclusion of this investigation is that application of waste-based fertilizers affects AM fungi in various ways. According to the results soil-associated AM fungal biomass are associated with a large host biomass, while root-associated AM fungal biomass are positively affected by a combination of digestate and ash. The mineral fertilizer had a positive effect on the wheat biomass but not on the root-associated AM fungal biomass. In order to explain the large variation within treatments, studies with more replicates could be conducted. Furthermore, community analyses could give further insight into the effects on AM life strategies.

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References

Åkesson, A., Julin, B., Wolk, A., 2008. Long-term Dietary Cadmium Intake and Postmenopausal Endometrial Cancer Incidence: A Population-Based Prospective Cohort Study. Cancer Res. 68, 6435–6441. https://doi.org/10.1158/0008-5472.CAN-08-0329

Alburquerque, J.A., de la Fuente, C., Ferrer-Costa, A., Carrasco, L., Cegarra, J., Abad, M., Bernal, M.P., 2012. Assessment of the fertiliser potential of digestates from farm and agroindustrial residues. Biomass Bioenergy 40, 181–189. https://doi.org/10.1016/j.biombioe.2012.02.018

Arao, T., 1999. In situ detection of changes in soil bacterial and fungal activities by measuring 13C incorporation into soil phospholipid fatty acids from 13C acetate. Soil Biol. Biochem. 31, 1015–1020. https://doi.org/10.1016/S0038-0717(99)00015-2

Baird, C., Cann, M., 2012. Toxic heavy metals, in: Environmental Chemistry. W. H. Freeman and Company, New york, pp. 519–572.

Bertilsson, G., Rosenqist, H., Mattson, L., Sverige, N., 2005. Fosforgödsling och odlingsekonomi med perspektiv på miljömål, Rapport / Naturvårdsverket, 5518. Naturvårdsverket, Stockholm.

Bio-Rad Laboratories, n.d. What is Real-Time PCR (qPCR)? | Applications & Technologies | Bio-Rad [WWW Document]. URL http://www.bio-rad.com/en-se/applications-technologies/what-real-time-pcr-qpcr (accessed 2.15.18).

Bougnom, B.P., Niederkofler, C., Knapp, B.A., Stimpfl, E., Insam, H., 2012. Residues from renewable energy production: Their value for fertilizing pastures. Biomass Bioenergy, Biorefinery 39, 290–295. https://doi.org/10.1016/j.biombioe.2012.01.017

Breuillin, F., Schramm, J., Hajirezaei, M., Ahkami, A., Favre, P., Druege, U., Hause, B., Bucher, M., Kretzschmar, T., Bossolini, E., Kuhlemeier, C., Martinoia, E., Franken, P., Scholz, U., Reinhardt, D., 2010. Phosphate systemically inhibits development of arbuscular mycorrhiza in Petunia hybrida and represses genes involved in mycorrhizal functioning. Plant J. 64, 1002–1017. https://doi.org/10.1111/j.1365-313X.2010.04385.x

Campbell, N.A., Reece, J.B., Urry, L., Chain, M.L., Wasserman, S.A., Minorsky, P.V., Jackson, R.B., 2015. 37. Plant nutrition, in: Biology: A Global Approach. Pearson Education Limited, Harlow, pp. 863–878.

Chagnon, P.-L., Bradley, R.L., Maherali, H., Klironomos, J.N., 2013. A trait-based framework to understand life history of mycorrhizal fungi. Trends Plant Sci. 18, 484–491. https://doi.org/10.1016/j.tplants.2013.05.001

Clark, R.B., 1997. Arbuscular mycorrhizal adaptation, spore germination, root colonization, and host plant growth and mineral acquisition at low pH. Plant Soil 192, 15–22. https://doi.org/10.1023/A:1004218915413

Cordell, D., Drangert, J.-O., White, S., 2009. The story of phosphorus: Global food security and food for thought. Glob. Environ. Change, Traditional Peoples and Climate Change 19, 292–305. https://doi.org/10.1016/j.gloenvcha.2008.10.009

Cruz-Paredes, C., López-García, Á., Rubæk, G.H., Hovmand, M.F., Sørensen, P., Kjøller, R., 2017. Risk assessment of replacing conventional P fertilizers with biomass ash: Residual effects on plant yield, nutrition, cadmium accumulation and mycorrhizal status. Sci. Total Environ. 575, 1168–1176. https://doi.org/10.1016/j.scitotenv.2016.09.194

Dahlin, A.S., Eriksson, J., Campbell, C.D., Öborn, I., 2016. Soil amendment affects Cd uptake by wheat — are we underestimating the risks from chloride inputs? Sci. Total Environ. 554, 349–357. https://doi.org/10.1016/j.scitotenv.2016.02.049

Demeyer, A., Voundi Nkana, J.C., Verloo, M.G., 2001. Characteristics of wood ash and influence on soil properties and nutrient uptake: an overview. Bioresour. Technol. 77, 287–295. https://doi.org/10.1016/S0960-8524(00)00043-2

Driver, J., Lijmbach, D., Steen, I., 1999. Why Recover Phosphorus for Recycling, and How? Environ. Technol. 20, 651–662. https://doi.org/10.1080/09593332008616861

Eriksson, J., Dahlin, S., Nilsson, I., Simonsson, M., 2011. 9. Oxidation och reduktion i marken, in: Marklära. Studentlitteratur AB, Lund.

Eriksson, J.E., 1989. The influence of pH, soil type and time on adsorbtion and uptake by plants of Cd added to the soil. Water. Air. Soil Pollut. 48, 317–335. https://doi.org/10.1007/BF00283334

European Commission, Secretariat-General, 2015. Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee of the Committee of the regions: Closing the loop - An EU action plan for the Circular Economy (No. COM(2015(614 final). Brussels.

European Food Safety Authority (EFSA), 2009. Cadmium in food - Scientific opinion of the Panel on Contaminants in the Food Chain: Cadmium in food - Scientific opinion of the Panel on Contaminants in the Food Chain. EFSA J. 7, 980. https://doi.org/10.2903/j.efsa.2009.980

Fan, J., McConkey, B., Wang, H., Janzen, H., 2016. Root distribution by depth for temperate agricultural crops. Field Crops Res. 189, 68–74. https://doi.org/10.1016/j.fcr.2016.02.013

Galvez, A., Sinicco, T., Cayuela, M.L., Mingorance, M.D., Fornasier, F., Mondini, C., 2012. Short term effects of bioenergy by-products on soil C and N dynamics, nutrient availability and biochemical properties. Agric. Ecosyst.

Environ., Recycling of organic resudues to agriculture 160, 3–14. https://doi.org/10.1016/j.agee.2011.06.015
Gao, X., Akhter, F., Tenuta, M., Flaten, D.N., Gawalko, E.J., Grant, C.A., 2010. Mycorrhizal colonization and grain Cd concentration of field-grown durum wheat in response to tillage, preceding crop and phosphorus fertilization. J. Sci. Food Agric. 90, 750–758. https://doi.org/10.1002/jsfa.3878
García, I.V., Mendoza, R.E., 2008. Relationships among soil properties, plant nutrition and arbuscular mycorrhizal fungi–plant symbioses in a temperate grassland along hydrologic, saline and sodic gradients. FEMS Microbiol. Ecol. 63, 359–371. https://doi.org/10.1111/j.1574-6941.2008.00441.x
Garg, R.N., Pathak, H., Das, D.K., Tomar, R.K., 2005. Use of Flyash and Biogas Slurry for Improving Wheat Yield and Physical Properties of Soil. Environ. Monit. Assess. 107, 1–9. https://doi.org/10.1007/s10661-005-2021-x
Gast, V.D., J, C., Gosling, P., Tiwari, B., Bending, G.D., 2011. Spatial scaling of arbuscular mycorrhizal fungal diversity is affected by farming practice. Environ. Microbiol. 13, 241–249. https://doi.org/10.1111/j.1462-2920.2010.02326.x
Gosling, P., Hodge, A., Goodlass, G., Bending, G.D., 2006. Arbuscular mycorrhizal fungi and organic farming. Agric. Ecosyst. Environ. 113, 17–35. https://doi.org/10.1016/j.agee.2005.09.009
Guo, W., Zhao, R., Zhao, W., Fu, R., Guo, J., Bi, N., Zhang, J., 2013. Effects of arbuscular mycorrhizal fungi on maize (Zea mays L.) and sorghum (Sorghum bicolor L. Moench) grown in rare earth elements of mine tailings. Appl. Soil Ecol. 72, 85–92. https://doi.org/10.1016/j.apsoil.2013.06.001
Gupta, A.K., Singh, R.P., Ibrahim, M.H., Lee, B.-K., 2012. Fly Ash for Agriculture: Implications for Soil Properties, Nutrients, Heavy Metals, Plant Growth and Pest Control, in: Agroecology and Strategies for Climate Change, Sustainable Agriculture Reviews. Springer, Dordrecht, pp. 269–286. https://doi.org/10.1007/978-94-007-1905-7_11
Hart, M.M., Reader, R.J., 2002. Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. New Phytol. 153, 335–344. https://doi.org/10.1046/j.0028-646X.2001.00312.x
Hetrick, B.A.D., Wilson, G.W.T., Cox, T.S., 1993. Mycorrhizal dependence of modern wheat cultivars and ancestors: a synthesis. Can. J. Bot. 71, 512–518. https://doi.org/10.1139/b93-056
Hijri, M., 2016. Analysis of a large dataset of mycorrhiza inoculation field trials on potato shows highly significant increases in yield. Mycorrhiza 26, 209–214. https://doi.org/10.1007/s00572-015-0661-4
Julin, B., Wolk, A., Johansson, J.-E., Andersson, S.-O., Andrén, O., Åkesson, A., 2012. Dietary cadmium exposure and prostate cancer incidence: A population-based prospective cohort study. Br. J. Cancer 107, 895–900. https://doi.org/10.1038/bjc.2012.311
Kojima, T., Sawaki, H., Saito, M., 2004. Detection of arbuscular mycorrhizal fungi, archaeospora leptoticha, and related species coloning plant roots by specific PCR primer. Soil Sci. Plant Nutr. 50, 95–101. https://doi.org/10.1080/00380768.2004.10408456

Lee, J., Lee, S., Young, J.P.W., 2008. Improved PCR primers for the detection and identification of arbuscular mycorrhizal fungi. FEMS Microbiol. Ecol. 65, 339–349. https://doi.org/10.1111/j.1574-6941.2008.00531.x

Lee, Y.-J., George, E., 2005. Contribution of Mycorrhizal Hyphae to the Uptake of Metal Cations by Cucumber Plants at Two Levels of Phosphorus Supply. Plant Soil 278, 361–370. https://doi.org/10.1007/s11104-005-0373-1

Liu, L., Li, J., Yue, F., Yan, X., Wang, F., Bloszies, S., Wang, Y., 2018. Effects of arbuscular mycorrhizal inoculation and biochar amendment on maize growth, cadmium uptake and soil cadmium speciation in Cd-contaminated soil. Chemosphere 194, 495–503. https://doi.org/10.1016/j.chemosphere.2017.12.025

Luo, N., Li, X., Chen, A.Y., Zhang, L.J., Zhao, H.M., Xiang, L., Cai, Q.Y., Mo, C.H., Wong, M.H., Li, H., 2017. Does arbuscular mycorrhizal fungus affect cadmium uptake and chemical forms in rice at different growth stages? Sci. Total Environ. 599, 1564–1572. https://doi.org/10.1016/j.scitotenv.2017.05.047

Mackay, J.E., Macdonald, L.M., Smernik, R.J., Cavagnaro, T.R., 2017. Organic amendments as phosphorus fertilisers: Chemical analyses, biological processes and plant P uptake. Soil Biol. Biochem. 107, 50–59. https://doi.org/10.1016/j.soilbio.2016.12.008

Makádi, M., Tomócsik, A., Eichler-Loebermann, B., Schiemenz, K., 2008. Nutrient cycling by using residues of bioenergy production - effects of biogas-digestate on plant and soil parameters. Cereal Res. Commun. 36, 1807–1810.

Mark- och grödoinventeringen, 2018. Kartor | Externwebben [WWW Document]. SLU.SE. URL https://www.slu.se/institutioner/mark-miljo/miljo-analys/akermarksinventeringen/kartor/ (accessed 2.12.18).

Medina, A., Probanza, A., Gutierrez Mañero, F.J., Azcón, R., 2003. Interactions of arbuscular-mycorrhizal fungi and Bacillus strains and their effects on plant growth, microbial rhizosphere activity (thymidine and leucine incorporation) and fungal biomass (ergosterol and chitin). Appl. Soil Ecol. 22, 15–28. https://doi.org/10.1016/S0929-1393(02)00112-9

Möller, K., Müller, T., 2012. Effects of anaerobic digestion on digestate nutrient availability and crop growth: A review. Eng. Life Sci. 12, 242–257. https://doi.org/10.1002/elsc.201100085

Mosse, B., Powell, C.L., Hayman, D.S., 1976. Plant Growth Responses to Vesicular-Arbuscular Mycorrhiza. New Phytol. 76, 331–342. https://doi.org/10.1111/j.1469-8137.1976.tb01468.x

Murphy, J.D., Thamsiriroj, T., 2013. 5 - Fundamental science and engineering of the anaerobic digestion process for biogas production, in: The Biogas Handbook, Woodhead Publishing Series in Energy. Woodhead Publishing, pp. 104–130. https://doi.org/10.1533/9780857097415.1.104

Narayanan, S., Mohan, A., Gill, K.S., Prasad, P.V.V., 2014. Variability of Root Traits in Spring Wheat Germplasm. PLoS ONE 9. https://doi.org/10.1371/journal.pone.0100317

Nawrot, D.T.S., Staessen, D.J.A., Roels, D.H.A., Cuypers, D.A., Smeets, D.K., Vangronsveld, D.J., 2013. Cadmium and Health Risks, in: Kretsinger,

R.H., Uversky, V.N., Permyakov, E.A. (Eds.), Encyclopedia of Metalloproteins. Springer New York, pp. 338–342. https://doi.org/10.1007/978-1-4614-1533-6_31

NCBI, n.d. Nucleotide BLAST: Search nucleotide databases using a nucleotide query [WWW Document]. URL https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch (accessed 8.18.17).

Nkoa, R., 2014. Agricultural benefits and environmental risks of soil fertilization with anaerobic digestates: a review. Agron. Sustain. Dev. 34, 473–492. https://doi.org/10.1007/s13593-013-0196-z

Odlare, M., Pell, M., Svensson, K., 2008. Changes in soil chemical and microbiological properties during 4 years of application of various organic residues. Waste Manag. 28, 1246–1253. https://doi.org/10.1016/j.wasman.2007.06.005

Ohno, T., Susan Erich, M., 1990. Effect of wood ash application on soil pH and soil test nutrient levels. Agric. Ecosyst. Environ. 32, 223–239. https://doi.org/10.1016/0167-8809(90)90162-7

Pandey, V.C., Singh, N., 2010. Impact of fly ash incorporation in soil systems. Agric. Ecosyst. Environ. 136, 16–27. https://doi.org/10.1016/j.agee.2009.11.013

Parniske, M., 2008. Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat. Rev. Microbiol. 6, 763–775. https://doi.org/10.1038/nrmicro1987

Pellegrino, E., Öpik, M., Bonari, E., Ercoli, L., 2015. Responses of wheat to arbuscular mycorrhizal fungi: A meta-analysis of field studies from 1975 to 2013. Soil Biol. Biochem. 84, 210–217. https://doi.org/10.1016/j.soilbio.2015.02.020

Plenchette, C., Fortin, J.A., Furlan, V., 1983. Growth responses of several plant species to mycorrhizae in a soil of moderate P-fertility. Plant Soil 70, 199–209. https://doi.org/10.1007/BF02374780

Provenzano, M.R., Iannuzzi, G., Fabbri, C., Senesi, N., 2011. Qualitative Characterization and Differentiation of Digestates from Different Biowastes Using FTIR and Fluorescence Spectroscopies. J. Environ. Prot. 83–89. https://doi.org/10.4236/jep.2011.21009

Redecker, D., Morton, J.B., Bruns, T.D., 2000. Ancestral Lineages of Arbuscular Mycorrhizal Fungi (Glomales). Mol. Phylogenet. Evol. 14, 276–284. https://doi.org/10.1006/mpev.1999.0713

Redecker, D., Schüßler, A., Stockinger, H., Stürmer, S.L., Morton, J.B., Walker, C., 2013. An evidence-based consensus for the classification of arbuscular mycorrhizal fungi (<Emphasis Type="Italic">Glomeromycota</Emphasis>). Mycorrhiza 23, 515–531. https://doi.org/10.1007/s00572-013-0486-y

Riding, M.J., Herbert, B.M.J., Ricketts, L., Dodd, I., Ostle, N., Semple, K.T., 2015. Harmonising conflicts between science, regulation, perception and environmental impact: The case of soil conditioners from bioenergy. Environ. Int. 75, 52–67. https://doi.org/10.1016/j.envint.2014.10.025

Rousk, J., Brookes, P.C., Bååth, E., 2009. Contrasting Soil pH Effects on Fungal and Bacterial Growth Suggest Functional Redundancy in Carbon Mineralization. Appl. Environ. Microbiol. 75, 1589–1596. https://doi.org/10.1128/AEM.02775-08

Säle, V., Aguilera, P., Laczko, E., Mäder, P., Berner, A., Zihlmann, U., van Der Heijden, M.G.A., Oehl, F., 2015. Impact of conservation tillage and organic farming on the diversity of arbuscular mycorrhizal fungi. Soil Biol. Biochem. 84, 38–52. https://doi.org/10.1016/j.soilbio.2015.02.005

Sato, K., Suyama, Y., Saito, M., Sugawara, K., 2005. A new primer for discrimination of arbuscular mycorrhizal fungi with polymerase chain reaction-denature gradient gel electrophoresis. Jpn. Soc. Grassl. Sci. 51, 179–181. https://doi.org/10.1111/j.1744-697X.2005.00023.x

Sawers, R.J.H., Gutjahr, C., Paszkowski, U., 2008. Cereal mycorrhiza: an ancient symbiosis in modern agriculture. Trends Plant Sci. 13, 93–97. https://doi.org/10.1016/j.tplants.2007.11.006

Schachtman, D.P., Reid, R.J., Ayling, S.M., 1998. Phosphorus Uptake by Plants: From Soil to Cell. Plant Physiol. 116, 447–453. https://doi.org/10.1104/pp.116.2.447

SGU, n.d.a. SGUs Kartvisare Berggrund 1:1 miljon [WWW Document]. URL https://apps.sgu.se/kartvisare/kartvisare-berggrund-1-miljon.html (accessed 8.18.17).

SGU, n.d.b. SGUs Kartvisare Jordarter 1:25000 - 1:100000 [WWW Document]. URL https://apps.sgu.se/kartvisare/kartvisare-jordarter-25-100.html (accessed 8.18.17).

Shahabivand, S., Maivan, H.Z., Goltapeh, E.M., Sharifi, M., Aliloo, A.A., 2012. The effects of root endophyte and arbuscular mycorrhizal fungi on growth and cadmium accumulation in wheat under cadmium toxicity. Plant Physiol. Biochem. PPB 60, 53–58. https://doi.org/10.1016/j.plaphy.2012.07.018

Sheng, M., Lalande, R., Hamel, C., Ziadi, N., 2013. Effect of long-term tillage and mineral phosphorus fertilization on arbuscular mycorrhizal fungi in a humid continental zone of Eastern Canada. Plant Soil 369, 599–613. https://doi.org/10.1007/s11104-013-1585-4

Singh, A. k., Hamel, C., DePauw, R. m., Knox, R. e., 2012. Genetic variability in arbuscular mycorrhizal fungi compatibility supports the selection of durum wheat genotypes for enhancing soil ecological services and cropping systems in Canada. Can. J. Microbiol. 58, 293–302. https://doi.org/10.1139/w11-140

Smith, S.E., Jakobsen, I., Grønlund, M., Smith, F.A., 2011. Roles of Arbuscular Mycorrhizas in Plant Phosphorus Nutrition: Interactions between Pathways of Phosphorus Uptake in Arbuscular Mycorrhizal Roots Have Important Implications for Understanding and Manipulating Plant Phosphorus Acquisition. Plant Physiol. 156, 1050–1057. https://doi.org/10.1104/pp.111.174581

Smith, S.E., Read, D.J., 2008. Mycorrhizal symbiosis, 3rd ed. Academic Press, San Diego.

Smolders, E., Mertens, J., 2013. Cadmium, in: Heavy Metals in Soils - Trace Metals and Metalloids in Soils and Their Bioavailability. Springer, Dordrecht.
Söderström, M., Eriksson, J., 2013. Gamma-ray spectrometry and geological maps as tools for cadmium risk assessment in arable soils. Geoderma 192, 323–334. https://doi.org/10.1016/j.geoderma.2012.07.014
Thomas, L.D., Michaëlsson, K., Julin, B., Wolk, A., Åkesson, A., 2011. Dietary cadmium exposure and fracture incidence among men: A population-based prospective cohort study. J. Bone Miner. Res. 26, 1601–1608. https://doi.org/10.1002/jbmr.386
Van Geel, M., Busschaert, P., Honnay, O., Lievens, B., 2014. Evaluation of six primer pairs targeting the nuclear rRNA operon for characterization of arbuscular mycorrhizal fungal (AMF) communities using 454 pyrosequencing. J. Microbiol. Methods 106, 93–100. https://doi.org/10.1016/j.mimet.2014.08.006
Vázquez, M.M., César, S., Azcón, R., Barea, J.M., 2000. Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (Azospirillum, Pseudomonas, Trichoderma) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants. Appl. Soil Ecol. 15, 261–272. https://doi.org/10.1016/S0929-1393(00)00075-5
Walsh, J.J., Jones, D.L., Edwards-Jones, G., Williams, A.P., 2012. Replacing inorganic fertilizer with anaerobic digestate may maintain agricultural productivity at less environmental cost. J. Plant Nutr. Soil Sci. 175, 840–845. https://doi.org/10.1002/jpln.201200214
Zhan, G., Erich, M.S., Ohno, T., 1996. Release of trace elements from wood ash by nitric acid. Water. Air. Soil Pollut. 88, 297–311. https://doi.org/10.1007/BF00294107

Appendix 1 – Tillage procedures

The preceding crop was potato. Ploughing was performed on the 31st of March with a depth of 25cm. Harrowing followed on the 7th of April (7m Väderstad NZA). The mineral fertilizer was applied on the 7th of June, with a weed control in between on the 23rd of May with Zypar 0,75 l/ha + Mangan 1 L. A fungi control was applied on the 15th of June with Ascra Xpro 0,5 L/ha.

Appendix 2 – Example of calculations

Example calculation to achieve number of gene copies/g DW sample:
The linear equation for this plate was y = -0,2569x + 10,229. The tables are read from left to right.

Table 8. Calculations of starting quantity of target gene for each of the technical replicates of sample 29.

Rep	Ct	Log SQ*	SQ	SQ mean	SQ SD ᴸ
29	20,67	-0,2569*20,67+ 10,229 = 4,919	10^4,919 = 82962		√((82962-71713)^2 +(64329- 71713)^2+(67847- 71713)^2)/(3-1)) = 9899
29	21,1	-0,2569*21,1+ 10,229 = 4,808	10^4,808 = 64329	(82962+64329+67847)/3 = 71713	
29	21,01	-0,2569*21,01+ 10,229 = 4,832	10^4,832 = 67847		

* *Log Starting Quantity*
ᴸ *Starting Quantity Standard Deviation*

The same calculations were made for the cycle threshold. If more than one of the three technical replicates had a C_T SD>0,5 the samples were rerun.

Table 9. Calculations to compensate for the dilutions made during DNA extraction and in preparation for the qPCR and receive the number of gene copies/ 50 uL DNA extract.

Rep	Dilution in DNA extraction	Dilution in qPCR	Number of gene copies/5uL	Number of gene copies/50 uL DNA extract
29	(1000/400*650/550) = 2,954545455	10	82962	82962/5*10/50 = 8296158
29	(1000/400*650/550) = 2,954545455	10	64329	64329/5*10/50 = 6432947
29	(1000/400*650/550) = 2,954545455	10	67847	67847/5*10/50 = 6784705

Table 10. Calculations including the weigh-in of samples and arriving at a mean number of gene copies/g DW sample.

Rep	mg DW* sample extract	Number of gene copies/mg DW	Number of gene copies/g DW sample	Mean number of gene copies/g DW sample
29	291,6	(8296158*2,954545455)/ 291,6 = 84058	84058*1000 = 84058214	(84058214 + 65179820 + 68743898)/3 = 72660644
29		(6432947*2,954545455)/ 291,6 = 65180	65180*1000 = 65179820	
29		(6784705*2,954545455)/ 291,6 = 68744	68744*1000 = 68743898	

**milligram Dry Weight*

Appendix 3 – Example of standard curve

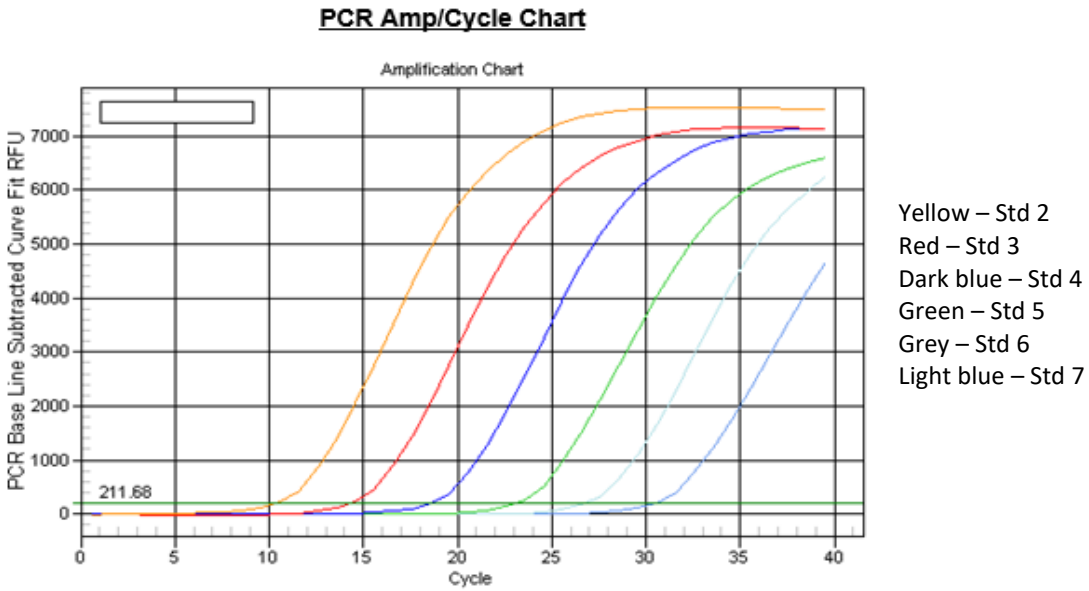

Figure 11. Standard of qPCR plate 5. Each dilution step was x10 gene copies with Std 7 being the most diluted one and Std 2 having the most gene copies.

Appendix 4 – pH values

Treatment	pH H2O	pH CaCl2
Control	5.55	5.08
Control	6.10	5.49
Control	6.41	6.11
Control	6.87	6.60
Digestate Uppsala	6.88	6.44
Digestate Uppsala	6.16	5.52
Digestate Uppsala	5.73	5.14
Digestate Uppsala	6.76	6.48
Digestate Linköping	6.05	5.44
Digestate Linköping	6.58	6.24
Digestate Linköping	5.96	5.46
Digestate Linköping	6.51	6.33
Ash	6.06	5.42
Ash	5.86	5.31
Ash	6.03	5.38
Ash	6.95	6.65
Digestate Uppsala+Ash	5.40	4.96
Digestate Uppsala+Ash	6.60	6.28
Digestate Uppsala+Ash	5.92	5.45
Digestate Uppsala+Ash	5.98	5.42
Digestate Linköping+Ash	6.77	6.46
Digestate Linköping+Ash	6.52	6.10
Digestate Linköping+Ash	5.90	5.38
Digestate Linköping+Ash	6.75	6.35
Mineral fertilizer	5.90	5.42
Mineral fertilizer	5.40	5.06
Mineral fertilizer	6.07	5.47
Mineral fertilizer	6.75	6.50