The Environmental Impact due to the Use of Cu and Zn in the Pig Industry

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- http://www.thebellamossfoundation.com/pet-carers/treatment/mrsa-in-pigs/
- http://www.cidrap.umn.edu/news-perspective/2013/09/mrsa-studies-incidence-down-field-manure-may-be-risk-factor
- https://www.bozo.coop/co-op-food-blog/natural-allies-healthy-soil-and-healthy-plants

Title Page

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SUMMARY

In recent years copper and zinc has replaced the use of antibiotic growth-promoters in the pig farming industry. This is due to the fact that in 2000 the use of antibiotics as growth-promoters was forbidden in Denmark. It is well documented that the adding of cobber and zinc to the feed has a positive influence on the feed uptake and thereby an increased growth. At the same time cobber and zinc reduces the prevalence of diarrhoea in the weaning period. The problem with this is that only a small part of these metals is absorbed from the feed in the pig's body. That means that large amounts of heavy metals end up in the environment when manure is used as fertiliser on the soil.

The purpose of the project was therefore to examine the environmental consequences of the high amount of heavy metals being used in the pig farming industry, especially copperer and zinc. In connection with this urine samples were examined for heavy metal content. No extraordinary amounts were found. A few select foods (including pork) were likewise tested for heavy metal content because this is a potential source of heavy metals. Toxic metals such as aluminium, lead and nickel were found. Of these aluminium were found in the highest concentration of 9 mg per kg in canned tuna. The content of copper and zinc in pig- and cattle manure from local farms were examined. In the cattle manure 185 mg Cu and 190 mg Zn pr. kg. dry matter was found and in the pig manure it was determined to be 149 mg Cu and 640 mg Zn pr kg dry matter, respectively. Samples from the soil where the manure was spread was also analysed. On the field were the pig manure was spread the copper content was determined to be 8.3 mg and zinc content was 25 mg per kg soil. Accordingly, the content of copper and zinc on the field where cattle manure was spread was determined to be 2.5 mg Cu and 11 mg Zn per kg soil.

A series of plant experiments was conducted to demonstrate how an increase in copper and zinc concentration in soil affects the concentration in plants. In experiments with radishes a copper concentration of 7.37 mg per dry matter was proven after growth in soil, which contained 60 mg per kg a zinc concentration of 79.3 mg per kg was likewise proven. Furthermore, it was proven that increasing concentrations of zinc in soil decreases the absorption of copper in radish plants and vice versa. For comparison with controls, the addition of pig and cattle manure increased absorption of copper and zinc in cress significantly. The study showed an increase in copper and zinc absorption of 79 % and 136% after application of manure and 66 % and 143 % for cattle manure.

RESUMÉ

I de seneste år har kobber og zink erstattet anvendelsen af antibiotiske vækstfremmere i svineindustrien. Dette skyldes at brugen af antibiotika i år 2000 blev forbudt som vækstfremmer i Danmark. Det er desuden veldokumenteret at tilsætning af kobber og zink til foderet har en positive indflydelse på foderoptaget og dermed en forøget vækst. Samtidig reducerer kobber og zink forekomsten af diarré i fravænningsperioden. Problemet med dette er, at kun en lille del af

disse metaller optages fra foderet i grisens krop. Det betyder, at store mængder tungmetaller havner i miljøet, når gyllen anvendes som gødning på landbrugsjorden.

Formålet med dette projekt var derfor at undersøge de miljømæssige konsekvenser ved det høje forbrug af tungmetaller, herunder særligt kobber og zink i svineindustrien. I den forbindelse blev en række urinprøver undersøgt for indholdet af tungmetaller. Der blev dog ikke påvist bemærkelsesværdige niveauer af disse. Ligeledes blev nogle få udvalgte fødevarer (herunder svinekød) screenet for indholdet af tungmetaller, da dette er en potentiel kilde til tungmetaller. Her blev de toksiske metaller aluminium, bly og nikkel fundet. Af disse blev aluminium fundet i den højeste koncentration på 9 mg pr kg i dåsetun. Det aktuelle indhold af kobber og zink i svine- og kvæggylle fra lokale landbrug blev undersøgt. I kvæggyllen blev dette bestemt til 185 mg Cu og 190 mg Zn pr kg tørstof, mens det i svinegyllen blev bestemt til henholdsvis 149 mg Cu og 640 mg Zn pr kg tørstof. Tilhørende jordprøver fra marker, hvor den pågældende gylle var blevet spredt blev også analyseret. På marken, som var tilført svinegylle, blev der i gennemsnit påvist et indhold af kobber og zink på henholdsvis 8.3 og 25 mg pr kg jord. Tilsvarende blev indholdet bestemt til 2.5 mg Cu og 11 mg Zn pr kg jord på marken tilført kvæggylle.

En række planteforsøg blev udført for at demonstrere, hvordan stigende jordkoncentrationer af kobber og zink påvirker koncentrationen i planter. I forsøg med radiser blev en kobberkoncentration på 7.37 mg pr kg tørstof påvist efter vækst i en jord med 60 mg Cu pr kg. Ligeledes blev der påvist en zinkkoncentration på 79.3 mg pr kg. Det blev desuden påvist at stigende zinkkoncentrationer i jorden nedsætter optagelsen af kobber i radiseplanter og omvendt. Til sammenligning med kontroller, viste tilførsel af svine- og kvæggylle at forøge optaget af kobber og zink i karse markant. Forsøget viste et forøget optag af kobber og zink på 79 og 136% efter tilførsel af svinegylle samt henholdsvis 66 og 143% ved tilførsel af kvæggylle.

PREFACE

This report has been produced as a Master Thesis on the Master's degree programme in *Chemical Engineering* at Aalborg University Esbjerg. It has been created during the spring semester of 2015 and reflects the individual student work and ideas during this period.

This project is also of special interest because it is the approach to a new area, which there will be more focus on at the university in the future. Previously, the focus mainly has been on remediation of sites contaminated with heavy metal. This project is part of a new area that combine environmental chemistry, food and human/animal welfare based on agricultural activities with heavy metals. The consequences and reactions of the large amount of heavy metals in the pig's body are not fully understood. These reactions will be an indication of reactions that will be recognised in the human body because of increasing consume of heavy metals, because they presumably via meat and manure ends up in the food chain. It is not only the human consequences to be on the agenda though; animal welfare is certainly an important point as well.

The project and appertaining laboratory work has been carried out at Aalborg University in Esbjerg. The project concerns a characterisation of some soil and manure samples, which have been collected at local farmers.

Figures and tables are denoted with one of these prefixes as well as the chapter number and chronological number in italics. Unless otherwise indicated, figures and tables are own material.

References are denoted with brackets and a number – e.g. (14). This number refers to the bibliography in the back of the report, where all references are listed and numbered. These references are found at the end of each text paragraph and refer to part of, or the entire paragraph. In cases in which it refers to a fact, the reference is found directly in the text after the claim.

After the conclusion, a number of appendices are enclosed. They contain detailed information regarding the execution of the experiments as well as raw experimental data of the concerned experiments. In the report, mainly graphical representations of the overall results are found.

I would like to thank supervisor Erik Gydesen Søgaard for guidance during the project period. Great thanks should also be given to the laboratory personal Linda Madsen and Dorthe Spangsmark for their help with the experimental analyses. Last but not least, great thanks to farmers Flemming Kjær, Mary Kjeldsen and Ebbe Wind for supply of soil and manure samples.

Enjoy!

Annette Jensen
Esbjerg, June 2015

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1 Introduction

The agriculture industry for many years has been subject to constant criticism due to the supplement of nitrogen and phosphorus from pig manure to nature as well as the use of pesticides. Subjects, which are still under control. The discussion on these issues has taken attention away from the fact that large amounts of heavy metals such as copper and zinc are found in pig manure too. In pig production, increasingly amounts of heavy metals such as copper and zinc are used as 'growth-promoters' in the feed to replace the antibiotic growth-promoters, which was banned for a number of years ago. It is well documented that supplementation of 2000-3000 ppm of dietary zinc in a period of 1-2 weeks after weaning results in increased feed intake, increased growth rate and reduces the occurrence of diarrhoea. In Denmark, it is recommended to supply pigs in all categories with 100 ppm of dietary zinc. The metabolism of zinc is connected to the metabolism of copper. Copper is also added in high concentrations (170 ppm) to weaning diets until pigs are 12 weeks old and upwards addition of 6 ppm are recommended. The high doses of copper has also shown great effect on the feed uptake and increasing growth rate in weaned piglets.

The problem is post-weaning diarrhoea. The response of the piglets-stomachs to weaning at the age of just four weeks. It is long before the pigs' intestinal flora is ready to let go sow milk and switch to solid foods. Nature has set stomachs to fend for themselves after about 12 weeks. Nevertheless, in industrial pig production the piglets are weaned from the sow as soon as the law allows it. This is done, for the sow to reach to deliver a new litter of a fast rate, which is necessary to keep the pig producers economy viable.

Both copper and zinc are essential nutrients classified within the group of trace minerals and heavy metals as well. However, at elevated concentrations both copper and zinc becomes toxic to living organisms. This becomes a problem when the manure containing copper and zinc is applied to fields and hereby causes an accumulation of these metals.

To understand this entire problem the first part of the project deals with a brief theoretical study of the background of the use of metals in the pig industry with emphasis on copper and zinc. In addition, the environmental impact of the use of metals in the pig industry are evaluated based on surveys investigating copper and zinc in Danish agricultural soils. The consequences of high levels of heavy metals in the environment are briefly described as well. Based on this theoretical study a plan for the experimental laboratory work is designed. The object of this is to screen some real soil and manure samples for heavy metals, as well as demonstrating the environmental fate of metals.

2 Application of metals in the pig industry

Pigs have a metabolic requirement for several minerals including some heavy metals. A lack of minerals in the diet can result in deficiency signs, such as reduced or low feed intake, reduced rate of growth, soft or brittle bones, hairless piglets, breeding and reproductive problems, poor milk production and death. Therefore, pig diets are supplemented with minerals. Supplementation of high amounts of zinc and copper is known to exert positive influences on feed uptake, growth rate and cases of diarrhea. Therefore, zinc and copper are used in increasing amounts as growth promoters as an alternative to the antibiotic growth promoters which were banned in Denmark in year 2000.(1) Therefore, the description of these metals is elaborated. (1,2)

2.1 Physiological need and effects of minerals

Minerals are important for the metabolism because they perform important functions in the pig body and thus are essential for optimal growth, reproduction and lactation. Many of the minerals are part of a series of enzymes. They also have influence on various properties such as bone formation, oxygen transport and cell structure. Minerals required in large amounts are called macro minerals, while minerals required in small amounts are called micro or trace minerals. They are categorised as shown in *Table 2-1*. The macro minerals needed by the pig includes some salts, calcium and phosphorus. Salt is needed to maintain a healthy appetite and fluid balance. Calcium is important to bone formation. Phosphorus assists in utilising energy and is also involved in bone building. (2,3)

Table 2-1: Minerals required by pigs are divided in macro and micro minerals. (3)

Macro minerals	Micro minerals
Calcium	Iron
Phosphorus	Iodine
Sodium	Copper
Potassium	Manganese
Chloride	Selenium
Magnesium	Zinc

The micro mineral iron is a constituent of haemoglobin in the red blood cells and myoglobin in the muscles. Iron is found in several enzymes, the liver, spleen and other tissues as well. Iron is necessary for the oxygen to be transported around the body via the blood and delivered to the cells. The symptoms of iron deficiency are anaemia, which is characterised by a low blood percent and a lower haemoglobin concentration than normal in the red blood cells. Some characteristic sign are pale pigs that easily become breathless and overstrained. The symptoms also include poor growth, enlarged heart, spleen, and fatty liver. The first three weeks after birth the weight of the suckling pig are increases fourfold. This means that the suckling pigs must produce a lot of blood in this period. The daily iron requirement is 7 mg, which is significant higher than the supply from the sow milk (ca 1 mg). The inherent iron depot (ca 50 mg) is therefore used up after about one week. Thus, iron supplementation is very important for the baby pig. (4)

Iodine is required for a normal function of the thyroid gland as a constituent of the thyroid hormones. These are required to maintain a normal basal metabolic rate. An iodine deficiency causes goitre (enlarged thyroid). Therefore, iodine is added to pig diets as iodised salt. (4,5)

Manganese is a constituent of enzymes involved in carbohydrate metabolism and in the synthesis of polysaccharides and glycoproteins. The lipid metabolism is also dependent on manganese. Manganese is required for the formation of chondroitin sulphate, which is a component of the bone matrix. Signs of manganese deficiency include abnormal skeleton growth, decreased growth rate and feed efficiency. (4,5)

Selenium is a component of the enzyme glutathione peroxidase, which break down the peroxides that are otherwise destructive to the body's cell membranes. Selenium works in synergy with vitamin E in maintaining the integrity of cell membranes. Selenium also plays a role in the immune system and for the production of thyroid hormones, which are important for metabolism, growth and brain development and function. Selenium deficiency in pigs in growth means degeneration of muscles, prominent and strongly red-coloured eyes, damage to the liver and acute cardiac death. In the following, the physiological effects of the micro mineral iron are described because it is important in the prevention of anaemia. The physiological effects of copper and zinc are presented as well due to the use as growth promoters.(4,5)

2.1.1 Physiological effects of copper and zinc

Copper is essential for the activity of several enzymes known as cuproenzymes. The cuproenzymes regulate various physiologic pathways, such as energy production, iron metabolism, connective tissue maturation, and neurotransmission. Cytochrome *c* oxidase is a copper-dependent enzyme that plays a critical role in the cellular energy production. The enzyme catalyses the reduction of molecular oxygen (O_2) to water (H_2O) hereby generating an electrical gradient. This gradient is used by the mitochondria to create the vital energy-storing molecule, ATP. Copper is also required with iron for normal red blood cell formation. This is explained by the four cupro-enzymes, known as ferroxidases. Those enzymes are able to oxidise ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}), the form of iron that can be loaded onto the protein transferrin for transport to the site of red blood cell formation. Similar the activities of other enzymes are influenced by copper. Those mechanisms are not discussed further. (4,6)

Zinc is present in many enzyme systems and is required for the activity of more than 300 metalloenzymes. The enzymes are involved in the metabolism and are required for normal protein synthesis. Zinc is also important for the maintenance of cell membrane structure and function. It is a component of insulin and is therefore important for carbohydrate metabolism. Zinc is also important in relation to immune function. A study (7) showed that zinc is important for optimal growth, due to the increased level of the growth hormone IGF-1 (Insulin-like Growth Factor-1). The study also showed that the intestine epithelium become less sensitive to toxin from pathogenic bacteria (eg. *E. coli*). The toxins cause diarrhea by initiating a cascade of reactions, which results in secretion of chloride from the intestinal cells. The result of this is that the pig gets diarrhea. (5-7)

2.2 Regulation of the use of mineral additives

In Denmark, law regulates the use of additives. A limiting value specifies the permitted maximum levels for certain micro minerals as shown in *Table 2-2*. The maximum levels indicates the content that total must be in complete feed. That is the natural as well as the added level. Therefore, the natural content must be taken into account in the dosing of additives. (8)

Table 2-2: The maximum value of the micro minerals in complete food. (8)

Limit of admixture of the micro minerals	Complete food [mg/kg]
Copper (Up to 12 weeks)	170
Copper (Older than 12 weeks)	25
Zinc	150
Iron (maximum level until a week before weaning)	250 mg per day
Iron	750
Cobalt	2
Manganese	150
Molybdenum	2.5
Selenium	0.5

Compound feed for piglets up to 12 weeks of age must not exceed 170 mg of copper per kg. Feed for pigs older than 12 weeks must not exceed 25 mg of copper per kg. Compound feed for pigs may contain a maximum of 150 mg zinc per kg independent of the age of the pigs. Veterinarians can prescribe 2,500 mg of zinc per kg feed for piglets 14 days after weaning. There are a number of terms that must be met in order to get zinc prescribed; those are not covered in this project. Zinc, which is prescribed by the veterinarian, is not regarded as an additive, but as medicine. The maximum level of iron supplied to the piglets is 250 mg per day until a week before weaning and 750 mg per kg feed for older pigs. (8)

As already mentioned the limits above indicate the maximum permitted amount admixed to feed. This is primarily to avoid poisoning of pigs by overdose. The addition of metals to the pigs' feed does also have an adverse impact on the environment. In order to reduce the consumption of minerals some recommended norms of the minerals is therefore established. The recommended norms for copper and zinc are the same for piglets, sows and young pigs, as seen in *Table 2-3*. FEsv is an abbreviation of feed units for growing pigs and FEso stands for feed units for sows in their entire cycle. However, the norms for some other minerals (not shown in the table) are different depending on the age and size of the pigs. (9)

Table 2-3: Recommended norms for some minerals to piglets, sows and young pigs, total amounts per feed unit (FEsv/FEso). (9)

Standards	Piglets 6-30 kg	Sows[1)] (pr. FEso)	Young pig (pr. FEsv/FEso)
Copper [mg]	6	6	6
Zinc [mg]	100	100	100

1) Includes gestating, lactating and sows in mating unit.

The nutrient standards are continuously revised. They are defined from the pigs' physiological needs plus a safety margin. It is not recommended to add minerals beyond the norm, because some of the minerals might interact with the other minerals. This means that a high amount of some mineral could inhibit the uptake of some other minerals. (9)

2.3 Copper and zinc as growth promoters

In Denmark, zinc and copper are added to all pig feed. In recent years, there has been focus on the use of copper and zinc as growth promoters. The growth-promoting effect are therefore described in the following section. Copper is absorbed in the first part of the small intestine. The main part of the feed copper content (90-95 %) passes through the intestine without being absorbed.(10) Contrary, zinc is absorbed in the whole small intestine. Zinc competes with copper for the transport proteins, but it is copper, which are believed to have first priority. If the transport proteins are occupied by copper, zinc instead is absorbed directly into the bloodstream through the intestinal cells. (11)

2.3.1 Growth-promoting effects of copper and zinc

The levels of copper and zinc in order to meet the pig's nutrient requirement are 4-6 and 100 mg per FFsv respectively as shown in *Table 2-4*. Pigs have, however, a large tolerance to copper and zinc. When supplied at high concentrations (100 to 250 ppm for copper and 2000 to 3000 ppm for zinc), these two minerals are known to exert positive influences on feed uptake, growth rate and cases of diarrhea. This is probably due to the physiological effects described in the previous. In addition, it also is due to the strengthen effect on the intestinal flora.(10-12)

Table 2-4: Pig's requirement of copper and zinc. (10,11)

Mineral	Requirement [mg per FEsv]
Copper	4-6
Zink	100

Therefore, copper and zinc are increasingly used as growth promoters. Especially after antibiotic growth promoters were banned, the consumption of these metals has increased significantly. Increased addition of zinc has shown an increasing effect on the content of zinc in plasma, increased feed intake and daily gain as well as a reduction in the incidence of diarrhea. Zinc is not very toxic, and pigs can withstand up to 1,000 mg of zinc per kg feed with no evidence of zinc poisoning. (11,12)

Several studies have shown that a higher supply of copper may result in an increased daily gain in growing pigs. The mechanism behind this is not fully understood. A study has shown that the positive effect of a high copper content (30 vs. 175 mg/kg) in feed for weaned pigs probably is due to a better absorption of zinc. However, the interaction effects are not fully understood. (10)

2.3.2 Copper and zinc sources

The most commonly used copper sources in feed is copper sulphate, see *Table 2-5*. Copper oxide has very low availability and is therefore not suitable as a source of copper. Additionally the following sources can be used: copper carbonate, copper chloride and organic copper sources, in which copper is bound to an amino acid (chelated). The content of copper in the organic copper products is dependent on the manufacturer/company. Pigs have a great tolerance to high levels of copper in the feed, and as long as the concentration stays below 250 mg of copper per FEsv, there are no symptoms of poisoning.

Table 2-5: Copper and zinc content in different copper and zinc sources. (10,11)

Source	Formula	Content [%]
Copper sulphate pentahydrate	$CuSO_4 \cdot 5\ H_2O$	25
Copper oxide	CuO	80 Low availability
Copper amino acid chelate	$Cu\ (X)_{1-3}\ nH_2O$ X=anion of amino acid	Product dependent
Zinc sulphate monohydrate	$ZnSO_4,\ H_2O$	36
Zinc sulphate heptahydrate	$ZnSO_4,\ 7\ H_2O$	23
Zinc carbonate	$ZnCO_3$	52
Zinc oxide	ZnO	80
Zinc amino acid chelate	$Zn\ (X)_{1-3}\ nH_2O$ X=anion of amino acid	Product dependent

Zinc is added to the feed by use of the following zinc sources: Zinc oxide, zinc acetate, zinc sulphate, zinc lactate, zinc chloride, zinc carbonate, and organic zinc sources, wherein the zinc is bound to an amino acid (chelated). The content of zinc in the different sources is shown in *Table 2-5*. Zinc is not very toxic, and pigs can withstand up to 1,000 mg of zinc per. kg feed with no signs of zinc poisoning. (11)

3 Heavy metals in the environment

Only a small fraction of the added metals is retained in the body of the pigs. (13) A large proportion of the metals are thus excreted with their urine and faeces and therefore end up in the slurry. The farmers use the slurry as organic manure to the agricultural soils and thus large amounts of metals end up in the nature. As well as pigs have a physiological need for metals, several metals are essential for plants too. However, only very small amounts are needed to meet the needs. In excessive concentrations, the compounds can be harmful to plants and other living organisms. Continuous use of the pig manure may cause an accumulation of the metals in the soils, which will have adverse effects on the fertility of the soil. In the following surveys regarding the concentration of copper and zinc in Danish agricultural soil are evaluated. In addition, regulation of the application of pig manure is briefly discussed. Finally, the impact of heavy metals with focus on copper and zinc are briefly described.

3.1 Copper and zinc in Danish agricultural soils

The content of copper and zinc in pig manure may be high due to the previously mentioned use as growth promotors. In the pig body, about 3 mg Cu and 30 mg Zn is deposited per kg growth.(13) Pig manure contains approximately 600 g Cu per tonne dry weight and about 1500 g Zn per tonne dry weight. The application of pig manure is the most significant anthropogenic source of Cu and Zn. The contribution of Cu and Zn are 575 and 1290 g ha^{-1} each year to agricultural land via manure only. In addition, Cu and Zn are supplied to agricultural soils from fertilisers and lime, sewage sludge and atmospheric deposition as seen in *Table 3-1*. (14,15)

Table 3-1: Average supply and removal of Cu and Zn to Danish soils from different sources. (16)

	Copper		Zinc	
	[g/ha/year]	Total [t/year]	[g/ha/year]	Total [t/year]
Fertilisers and lime	40	90	20	25
Sewage sludge	220	20	700	55
Atmospheric deposition	8	20	80	185
Pig manure	600	500	1300	1200
Removal:				
By crops	0.02-0.1		0.25	
Leaching from clay soils	0.005		0.09	

The copper and zinc from the manure can be absorbed in the arable crops (transferred to food), bound in the soil or transferred with the surfaces water. Normally both metals are bound strongly in the soil, however zinc is more mobile than copper since copper absorbs more readily in soils. The conditions in the soil affect the concentration in the soil liquid, such as the texture of the soil, the content of organic matter and iron oxides as well as the pH. The metals bound usually strongest in soils with either high pH or high content of organic matter. At low pH, the solubility of the metals is increased and they are transported more easily by leaching. Copper and zinc can also be transported bound to soil particles trough macro-pore flow. Loss of the metals also happens by erosion and surface water runoff. If the metals are accumulated in the soil, they may be poisonous to microorganisms, higher plants, and fauna. (17)

The concentrations of copper and zinc in Danish agricultural soil have been investigated in two national surveys in 1986 and 1998. Several soil samples were taken at fixed points based on the Danish Square Grid system. The samples taken in 1998 were compared with samples taken at the same sites in 1986. The samples were analysed for total copper and total zinc as well as plant-available copper and zinc. The survey included 60 sites, which had received varying amounts of pig manure and fertiliser during the 12-year period. The samples were collected across the country. The amount of supplied manure was evenly distributed across all levels from approximately 10 to over 400 tonnes per ha during the 12-year period. The samples were collected in 3 layers; in 0-25, 25-50 and 50-75 cm's depth. The investigation showed only influence of supply of manure/fertiliser on the two top layers, which is why results from these are included only. The results from the surveys are shown in *Table 3-2*. (14,16)

Table 3-2: Average values for copper and zinc in the Danish soil in 1986 and 1998. Only 47 sites with pig manure and 11 sites with fertiliser is included, due to some missing soil in the samples from 1986. The bolded values indicate statistical significant differences between 1986 and 1998. (16)

		Copper				**Zinc**			
		Total		**Plant-available**		**Total**		**Plant-available**	
Sites	**Depth** [cm]	[mg/kg soil]				[mg/kg soil]			
		1986	1998	1986	1998	1986	1998	1986	1998
Pig Manure	0-25	**8.2**	**9.6**	**2.4**	**3.0**	36.6	32.9	5.7	4.6
	25-50	**6.2**	**7.4**	**1.2**	**1.7**	32.2	28.9	2.8	2.2
Fertiliser	0-25	7.0	7.7	1.7	1.8	**33.0**	**27.3**	4.5	2.1
	25-50	**4.9**	**6.7**	0.9	1.3	31.2	25.3	3.2	1.3

The surveys showed that fields, which are receiving pig manure, both in 1986 and 1998 had higher concentrations of copper and zinc than the fields only supplied with fertilisers. This is consistent with the fact that higher amounts of copper and zinc are supplied to the soil with pig manure than fertilisers (*Table 3-1*).

The addition of pig manure caused an increase in the concentration of copper in both 0-25 cm's and 25-50 cm's depth. In addition, the increase in the layer of 25-50 cm's depth indicated that the compound is transported through the soil. In this layer, the relative increase was the highest for plant-available copper, which is consistent with the higher mobility of this copper fraction than of total copper. By contrast, there was no statistical significant change for zinc in any of the layers on the fields supplied with pig manure. On the fields supplied with fertiliser, a decreasing tendency was observed. This indicates a loss of zinc from the soil. (16)

Since pig manure was the main source of copper and zinc supply, a correlation between supplied amounts of copper and zinc and the concentrations of copper and zinc in the soil was expected. This correlation was investigated by calculations of the measured concentrations of copper and zinc in the soil on selected field in 1998 relative to the total amount of pig manure added during the 12-years period. In addition to added amount of pig manure, the concentration of copper and zinc also is affected by the organic matter, clay and silt in the soil. Therefore, this was taken into account in the calculations. (14,16)

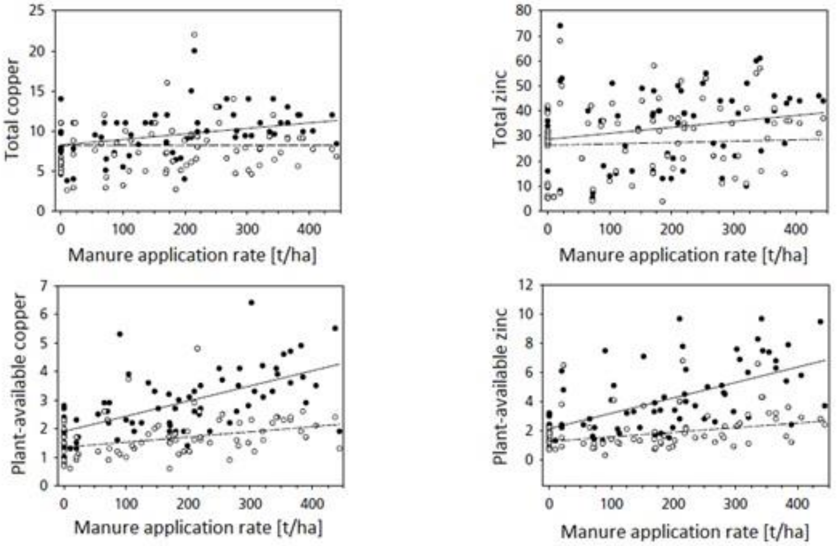

Figure 3-1: Concentrations in soil (mg/kg soil) of total copper, plant-available copper, total zinc and plant-available zinc recorded in 1998 at the depth 0-25 cm (●—) and 25-50 cm (o---) related to the applied manure in the period 1986 to 1998.(14)

A statistical significant relation between added amount of pig manure and the concentrations in the soil measured in 1998 was observed from this, as seen in *Figure 3-1*.(14)

3.1.1 Conclusion on surveys

The surveys indicated that the application of pig manure causes an accumulation of total and plant-available copper and zinc in the soils. This accumulation corresponds to the amount of copper and zinc added from the pig manure, which is attributed to the copper and zinc addition in the pig fed. Total copper and zinc accumulates primarily in the topsoil while the plant-available copper and zinc accumulates in both the topsoil and the 25-50 cm soil layer.

The concentration of copper increased from 1986 to 1998. In contrast, no average increase in the concentration of zinc in the soil during the 12-year period was ascertained. This is probably due to limited data and a higher mobility of zinc in the soil and hereby a greater loss than of copper. It is necessary to show caution with the use of copper and zinc as a growth promoter for pigs due to the possibility of accumulation in the soil in the long term. The continued use of copper and zinc in pig production increases the need for studies of mobility and binding of copper and zinc from fertilizer in the soil, and the temporal evolution of soil should be followed.(14,16)

3.2 Regulation of the application of pig manure

The overall use of manures as fertilisers is regulated by the harmony rule. There must be "harmony" between the number of livestock in a farm and the area which manure is applied on as a fertiliser for crops. The EU legislation provides that fields as a maximum is fertilised with 170 kg nitrogen from livestock manure per. Hectare (18). In Denmark, this obligation is tightened up for pig and chicken production, allowing application of maximum 140 kg nitrogen per hectare (18). In this context, you transform the livestock to "livestock units". Different amount of fertiliser are produced from a hen, a pig and a cow. Therefore, the authorities have made a conversion factor – a ‘livestock unit’ - as a common expression of the environmental impact. A livestock unit is equal to 100 kg nitrogen from the slurry tank or dunghill. For pigs, one livestock unit equals 4.4 sows with piglets up to 7.4 kg or 39 fattener pigs between 32 and 107 kg. For cattle one livestock unit equals 0.75 dairy cow of heavy race (black-and-white or red Danish dairy breeds) or 0.88 jersey cow (18). Thus, the application of manure as fertiliser is controlled by the nitrogen content only. However, there are strict rules on how much copper the industry must discharge. For example, the Danish Statutory Order on Sludge regulates the use of sludge on agricultural lands. The content of copper and zinc is restricted to maximum 1 g Cu and 4 g Zn per kg dry matter in the sludge, respectively (19). The content of copper and zinc in the manure may even be much higher, as described previously, in spite of this the harmony rule regulates the application of manures only.

3.3 The impact of copper and zinc on living organisms

Metals are found naturally in the soil. As already mentioned some metals are essential for humans, animals and plants. However, in too high concentrations the metals can be very harmful for the living organisms and the ecosystems. The rise in the consumption of metals in the Western world since the 1950s has resulted in large amounts of metals is being spread to the environment, including the agricultural land. The accumulation of heavy metals in the environment poses threat to living systems. Some consequences are briefly described in the following.

3.3.1 Heavy metals in humans

As trace elements, some heavy metals are essential to maintain the metabolism of the human body. However, at higher concentrations they can lead to poisoning. Similar a number of non-essential or toxic metals are not required by living organisms, instead they interfere with functions of essential metals and enzymes. High levels of toxic metals deposited in body tissues and subsequently in the brain, may cause significant developmental and neurological damage (20). An overview of some essential and toxic metals and elements are presented in *Table 3-3*.

Table 3-3: Some essential and toxic metals and elements.(21)

Essential	Toxic
Boron	Aluminium
Calcium	Antimony
Chromium	Arsenic
Cobalt	Barium
Copper	Beryllium
Iron	Bismuth
Lithium	Cadmium
Magnesium	Lead
Manganese	Mercury
Molybdenum	Nickel
Selenium	Platinum
Sodium	Thallium
Strontium	Thorium

Essential	Toxic
Sulphur	Tin
Vanadium	Tungsten
Zinc	Uranium

Heavy metal poisoning can be both acute and chronic. Acute poisoning occurs when a patient suddenly is exposed to a large amount of heavy metal. Acute metal poisoning can be diagnosed in a general blood or urine sample. Heavy metals are dangerous because they tend to bio-accumulate and chronic cumulative (accumulated) heavy metal poisoning occurs when the patient over a long period are exposed to small amounts of heavy metals, which are deposited in organs such as the liver, kidneys, heart and brain. Chronic metal poisoning cannot be diagnosed in a general blood or urine sample. The best way to diagnose a chronic heavy metal poisoning is by autopsy, but on living individuals the next best method must be used; a provocation test. In a provocation test, pills are taken or the patient is injected with a medical substance (a chelating agent) which causes the heavy metals to leave the deposits and adhere to the drug. It is then possible to diagnose the heavy metals either in a blood or urine sample (22).

Chelating agents are capable of binding to toxic metal ions to form complex structures, which mobilises the toxic metal mainly into urine. The chelation therapy is used as a tool for modifying metal concentration in the body. Metal toxicity may occur due to essential heavy metal overdose form various sources. The metal and metal compounds formed in the body may interfere with functions of various organ systems. By use of the chelating agents, the toxic effects of metals are reduced. However, there may be some side effects by the chelation therapy (23). Some normal values of concentration of metals in human urine are seen in *Table 3-4*. It is seen that both some essential as well as toxic elements might be found in the urine, however in only small levels.

Table 3-4: Reference values for normal concentrations in human urine of different metals.(17)

Element [µg/L]		Age	
Symbol	Name	**0-15**	**≥16**
Zn	Zinc	300-600	300-600
Mo	Molybdenum	22-173	22-173
As	Arsenic	0-35	0-35
Al	Aluminium	0-20	0-20
Ni	Nickel	0-6.0	0-6.0
Pb	Lead	0-4.0	0-4.0
Ti	Titanium	0-2.0	0-2.0
Co	Cobalt	0-1.9	0-1.9
Tl	Thalium	0-1.0	0-1.0
V	Vanadium	< 1.0	< 1.0
Fe	Iron	Not established	100-300
Cu	Copper	Not established	15-60
Se	Selenium	Not established	10-35
Hg	Mercury	Not established	0-9.0
Cr	Chromium	Not established	0-7.9
Mn	Manganese	Not established	0-1.2
Cd	Cadmium	Not established	0-1.3

There are several common features among the toxicity of heavy metals. One of the most widely studied mechanisms of action for toxic metals is oxidative damage of cells due to direct generation of free radical species and depletion of antioxidant reserves (24). Common mechanisms involve the Fenton reaction in which the superoxide radical and the hydroxyl radical are generated. A free radical is an atom or a compound, which has an odd number of electrons in its outer orbital, leaving this electron unpaired and reactive. These reactive species indiscriminately pick up electrons other atoms in their neighbourhood and convert those into secondary free radicals setting up a chain reaction causing random biological damage in the cell. One of the most reactive transition metals ions is iron; present at low levels in biological systems that catalysis the Haber-Weiss reaction generating hydroxyl (•OH) and superoxide ($\bullet O_2^-$) radicals. Iron catalysed by hydrogen peroxide generates the production of hydroxyl (OH^-) ions and hydroxyl radical (•OH) known as the Fenton's reaction.(24,25)

$$Fe^{3+} + \bullet O_2{}^- \rightarrow Fe^{2+} + O_2 \qquad Haber - Weiss\ reaction$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^- \qquad Fenton\ reaction$$

Several metals are therefore carcinogenic, because of their tendency to generate free radicals and therefore plays a role in many cancers. Many toxic heavy metals act as molecular “mimics” of nutritionally essential trace elements; as a result, they may compete with essential metallic cofactors for entry into cells and incorporation into enzymes. For example, cadmium can compete with and displace zinc from proteins and enzymes. Heavy metal toxicity is also due to their tendency to bind to the body's sulfur-containing enzymes, thereby being put out of action, so that they are missing in the metabolism. (24,26)

Copper is in a similar way included in the generation of hydroxyl and superoxide radical. In addition, metals such as copper play a central role in the development of diseases like Alzheimers and Parkinson (27). Non-metallic copper works especially irritating to the intestinal mucosa. The ingestion of even a few milligrams of copper can result in vomiting and diarrhoea. Consumption of copper in gram quantities can cause a lack of red blood cells, liver impact, low blood pressure and possibly death. (28)

3.3.2 Heavy metals in animals

The accumulation of heavy metals such as copper in the environment will have some consequences on animals as well. Sheep are known to be sensitive to copper since they accumulate copper in the liver more readily than other farm animal. A seriously impact of the increasing concentration of copper is observed by death of sheep grazing on fields that have been applied with pig manures (28). Rabbits are also known to be especially sensitive to copper. The decline in the past few years in the hare (whose physiology are very similar to rabbits) and roe deer populations in Denmark is suspected to be in association with the copper pollution.(29)

3.3.3 Copper and zinc in soil

The crops on the agricultural fields will absorb some of the nutrients and metals supplied from the pig manure. The uptake will depend on numerous factors such as soil properties (pH, organic matter) and the corresponding plant-available concentration of the metals. For instance, the availability of zinc decreases as pH increases. Cool, wet weather generally has a negative effect on zinc availability, while increasing soil temperatures increases zinc availability. Zinc is retained by soil particles on the cation exchange sites or as zinc cations in soil solution, including soluble zinc and organic matter complexes known as chelates. Zinc bearing minerals can dissolve and supply zinc to the soil solution. Once in the soil solution, zinc can be immobilized, taken up by plants, retained by soil particles, or chelated with soluble organic matter. Organic matter containing zinc must undergo mineralization before it becomes available for plant uptake. Copper, iron, manganese, and phosphorus can interfere with zinc uptake (30).

Copper is found bound in inaccessible compounds in the clay minerals and more accessible to the clay minerals surface. Copper binds strongly to not plant available form of soil organic compounds. Copper availability is lower in highly leached, coarse textured soils. The availability of copper decreases as pH increases primarily due to decreased solubility of copper minerals. Copper availability to plants may be reduced when zinc, iron, and/or phosphorus contents are high in the soil solution. Copper-containing minerals can dissolve and supply Zn to the soil solution. Like zinc, copper can be immobilized by microorganisms, taken up by plants, or exchanged on soil particle surfaces. Copper may also form chelates with soluble organic matter. Organic copper must be mineralized before it is available for plant uptake.(30)

4 Thesis Statement

In the previous chapters, the use of the heavy metals copper and zinc in the pig industry was investigated. The main purpose of copper and zinc are the use as growth promotors. Copper and zinc are added to the pig feed in order to ensure optimal growing pigs by preventing diarrhoea especially after weaning. The amounts mixed with the feed typically exceed the physiological needs. A lot of the added copper and zinc therefore passes through the pig's body and ends up in the manure. The manure is utilised as an organic fertiliser on the farmlands and considerable amounts of the heavy metals therefore ends up in the nature. There is no law governing the content of heavy metals in the manure. The concentration of copper and zinc in Danish agricultural soil has been investigated in two national surveys in 1986 and 1998. The surveys showed that the concentration of copper was increased in the period. Afterwards no surveys have followed up on the increase of copper caused by the use of manure. Therefore, there is a potential impact on the environment, which is overlooked.

The laboratory part in this project will based on the problem analysis includes the following subjects:

- Heavy metals in human urine
- Heavy metals in foodstuff
- Characterisation of manure
- Characterisation of agricultural and forest soils
- Plant experiments regarding uptake of Cu and Zn

Heavy metals in human urine

In the first part, a small test group provides urine samples that are screened for the content of heavy metals. The purpose is to investigate an eventual heavy metal accumulation in humans. If it turns out that there are detectable metals in the urine, additionally 20-25 persons provide a sample of urine. It will then be investigated whether there can be demonstrated correlations in the obtained concentrations with age, sex, diet, smoking, etc.

Heavy metals in foodstuff

Food and beverages consumed by humans represent a potential source of toxic metals. In order to identify potential sources of heavy metals some different foodstuff are screened for the content of heavy metals. This includes an investigation of the levels of copper and zinc in pork meat due to the use as growth promotors.

Characterisation of manure

Samples of pig and cattle manure are collected in this project. The manures are characterised and the content of heavy metals are determined with focus on copper and zinc. This study will only include the characterisation of manures from three farms. This section must be regarded as a limited screening of manures and their content of heavy metals from Danish farms. The manures are utilised in some plant experiment presented later in this chapter.

Characterisation of agricultural and forest soils

In this part, the purpose is an investigation of the metal levels in soils from two different farmlands compared to soil samples collected in a forest. The two farmlands include lands receiving pig manure and cattle manure respectively. The soil from the forest is used as a reference for normal concentrations of metals in a soil with minimal anthropological activity. This section should be regarded as a limited screening of the levels of heavy metals in Danish agricultural soils in 2015. Due to the time constraint in the project, only a very limited number of fields are examined.

Plant experiments

In this part of the project the uptake/accumulation of heavy metals from soils to plants are investigated. The purpose is to demonstrate the crops' uptake of heavy metals in agricultural soils due to the application of pig manure containing heavy metals. For this purpose, some pot experiments are carried out. In these experiments, copper and zinc are applied the soil and plants growing in the soils are analysed for the uptake of these metals. In addition, the effect on the uptake of metals in plants by application of manure is investigated. Full factorial design is used for the experimental planning in some of the experiments.

Experimental work

In this section, the experimental work in this project is presented.

The experiments are reviewed generally in this section. An appurtenant appendix goes through the details of experiments with regard to the execution. Similar the results are summarised in this section, while the experimental raw data is found in the appendix.

5 Heavy metals in human urine

Human urine might contain some toxic as well as nutritionally important metals. The content of metals in the urine originates from the ingestion of metals in food and beverages. If the intake of metals is greater than the need, some metals are excreted in the urine. In addition, the surplus metals are excreted in the faeces while some are absorbed in the bloodstream and accumulated in the body. This depends on the type of the metal, including the chemical and physical properties. Therefore, high concentrations of heavy metals in the urine either will be an indication of a heavy metal accumulation in the body or may be caused by a recent intake of foodstuff or beverage containing heavy metals. In *Table 3-4* some reference values for normal concentrations in human urine were presented. In general, the concentrations are very low. However, in (31) human urine from Nigerian people are screened for the content of As, Pb, Ni, and Cd, which are found in concentration as high as 1.2 mg/L. This is considerably higher than the reference values in *Table 3-4*. In this chapter, human urine samples from different persons are screened for the content of heavy metals and metalloids. Four different Danish test persons have provided a urine sample. The gender year of birth of the persons are listed in *Table 5-1*.

Table 5-1: Age and gender of persons who have provided a urine sample.

Identification	Gender	Year of birth
Person A	Female	1988
Person B	Female	1958
Person C	Female	1949
Person D	Male	1948

The four persons are coincidental chosen to represent persons with different age, because the heavy metal concentration in the urine may vary with the age, as presented in (31). Since the results did not show significant levels of heavy metals in the urine from the four test persons as presented in the following, additional urine samples were not collected. Reference is made to *Appendix A - Heavy metals in urine by ICP-OES* for detailed procedure and results.

5.1 Material and methods

Inductively Coupled Plasma - Optical Emission Spectroscopy (ICP-OES) was used for the element analysis of the urine. Reference is made to *Appendix A - Heavy metals in urine by ICP-OES* for a detailed procedure. Urine has several components such as uric acids, proteins, salts etc. including ionic compounds that are bound to coexist with your analyte of interest. Digestion of the urine samples in order to destroy the organic material therefore may be necessary. In (31) the preparation of the urine samples includes evaporation, lyophilisation, digestion with nitric acid etc. Due the type of sample material, the preparation of the urine in this project was requested to be as simple as possible.

In order to evaluate the need for digestion, a sample of urine was measured directly and after digestion at 120°C for 30 minutes with concentrated nitric acid, respectively. The results are seen in *Table 5-2*, in which only the detected elements are listed.

Table 5-2: Urine sample measured directly and after digestion with concentrated HNO_3, respectively. Entries are mean values based on three measurements.

Element	Directly [mg/L]	Digested [mg/L]
Boron	0.377	0.485
Barium	0.002	0.000
Chrome	0	0.005
Iron	0	0.055
Manganese	0.001	0.005
Molybdenum	0.013	0.043
Lead	0	0.008
Silver	0.002	0.006
Selenium	0.001	0
Zinc	0.176	0.205
Silicon	7.827	8.492

The results for the digested sample were a little higher than the directly measured sample. Therefor all urine samples in the following were digested with concentrated nitric acid prior to analysis. Hereby ensuring that all metals were available for detection with the applied method.

5.2 Results and discussion

Blanks with no urine were treated and analysed as the samples. The urine results were corrected for the concentrations obtained from the blanks and are represented in *Table 5-3*.

Table 5-3: Metal analysis of urine from four different persons. Entries represent the mean of three replicates.

Element [mg/L]	Person A	Person B	Person C	Person D
Aluminium	0	0.107	0.204	0.129
Antimony	0	0	0	0
Arsenic	0	0	0	0
Boron	0.485	0.036	2.929	4.745
Barium	0	0.007	0.052	0
Cadmium	0	0	0	0
Cobalt	0	0	0	0.001
Chrome	0.005	0.004	0.004	0
Copper	0	0	0	0
Iron	0.055	0.048	0.033	0.021
Manganese	0.005	0.006	0.002	0.000
Molybdenum	0.043	0	0.001	0.002
Nickel	0	0.005	0	0
Lead	0.008	0.002	0.026	0
Silver	0.006	0	0	0
Selenium	0	0	0	0.001
Titanium	0	0	0	0
Zinc	0.205	0.236	0.185	0.161
Strontium	0	0	0	0
Vanadium	0	0	0	0
Thallium	0	0	0	0
Silicon	8.492	6.208	8.294	9.477

In general, the concentrations of metals were very low and close/under the detection limit. The only elements detected in significant levels were iron, aluminium, boron, zinc and silicon. Of these boron and silicon were found in the highest concentration. These metals were found also in relative high concentrations in the blanks though. The laboratory staff reported that the method is quite uncertain for especially silicon as well. Therefore, this uncertainty must be taken in to account in the evaluation of the results.

In all urine samples, a low concentration of iron and some higher concentration of zinc are detected. Therefore, the results correspond in some way to the theoretical normal concentrations in urine given in *Table 3-4*. Here it was seen that of the listed elements, iron and zinc are found in the highest normal concentrations in urine. The normal concentrations of iron and in zinc in the urine are 0.1-0.3 mg/L and 0.3-0.6 mg/L, respectively. The detected concentrations are therefore somewhat lower than the theoretical values. In general, the observed concentrations were low and an acute poisoning (as mentioned previously) was not indicated. However, if some persons a suffering an acute poisoning, the person will have symptoms of this.

In (31) the concentration of As, Pb, Ni, and Cd were significant higher than the levels detected in this project. The level of heavy metals in the urine depends on the content in the consumed food and beverage, as previously mentioned. The deviation of the obtained results in this project compared to (31) might be explained by the composition of for instances the drinking water in Nigeria compared to Danish drinking water. Another reason might be some procedural and analytical deviations.

An issue in the analysis of the urine samples is the complex sample matrix. The samples contain relative high concentrations of potassium, sodium and calcium that results in an intensive plasma flame. This might interact with or dwarf the emission from the trace elements. However, it would have been difficult to concentrate the interesting metals only. By evaporation, both the heavy metals as well as the more common metals (K, Na, and Ca) concentrate. Since no heavy metals were detected in significant levels in the urine from the four test-persons, no further urine analysis was carried out.

5.3 Further work

There is a number of additional sampling and tests, which should have been carried out in order to make a complete screening of heavy metals in the body. Since working with human specimens, they do exceed the scope of this project though, since it would require some medical professionals:

- There are significant diurnal variation in the levels of metals in the urine due to the type and amounts of intake of food and beverages. Therefore, it had been optimally to collect urine from 24 hours. According to *Table 3-4* the normal values are so low that it is problematic to detect them with the applied method anyway.
- The metals, which are consumed, are excreted not only in the urine. In addition, they are excreted in the faeces while some are absorbed in the bloodstream and accumulated in the body. In order to make a complete screening of metals in the body it thus would have

required a number of analyses, which exceeds the scope of this project. This includes blood and faeces analysis.

- In addition, a way to extract the metals absorbed in the body to the urine is the use of a chelating agent as previously described. However, medical professionals should carry this out due to the side effects related to this.

6 Heavy metals in foodstuff

Food and beverages consumed by humans represent a potential source of toxic as well as nutritionally important metals. The concentrations of metals in food give important information about dietary habits of special groups, including the health situation of individuals and origins of elements. Therefore, it is important to determine the daily dietary intake of metals by evaluating the concentrations and sources. (32)

In this chapter, a very limited number of foodstuffs are screened for the content of heavy metals and metalloids. The purpose is to investigate whether ordinary foodstuffs are a source of heavy metals. A very limited group of fish and meat types are selected for the screening. Among this, some pork meat is analysed for the content of copper and zinc. Hereby, an investigation of the accumulation of those metals in pig meat due to the use as growth promotors is carried out.

Fish is typically thought to contain heavy metals; this includes especially mercury, which is not covered in this project though. On the other hand, the foodstuffs are screened for many other heavy metals by ICP-OES with the same method used in the previous chapter covering the urine analysis.

Reference is made to *Appendix B - Heavy metals in foodstuffs by ICP-OES* for detailed procedure and results.

6.1 Materials and methods

Since the foodstuffs are solid matters, all samples were dried in a stove at 105°C prior to analysis. After drying, the samples were ground to be homogenous in a mortar followed by digestion of an accurately weighed amount. Nitric acid (7 M) was used for the digestion in an autoclave at 120°C for 30 minutes. After cooling to room temperature the samples were filtrated and diluted with demineralised water to a fixed volume was reached. ICP-OES was used for the determination of metal content with the multi method described in the previous chapter concerning the urine analysis. All measurements were performed with a triple determination. Blanks with no foodstuff but only 7 M nitric acid were treated as the samples and the results of the foodstuff analyses were corrected for the levels obtained by analysis of the blanks. The concentrations of the metals were calculated based on the dry weight of the concerned foodstuff.

6.2 Results and discussion

In this section sample of fresh salmon, canned tuna, pig cutlet and minced beef were analysed for the content of different elements including heavy metals and metalloids. The results of the elements analysis are presented in *Table 6-1*.

Table 6-1: Element analysis of different fish and meat foodstuff. Entries are average on a triple determination with the standard deviation.

Element [mg/kg]	Salmon	SD	Canned tuna	SD	Pig cutlet	SD	Beef	SD	Mean	SD
Aluminium	**4.88**	1.962	**9.89**	5.82	**7.36**	5.21	**4.52**	0.547	**6.66**	3.38
Antimony	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0
Arsenic	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0
Boron	**0.320**	0.554	**2.54**	2.23	**0**	0	**2.10**	3.63	**1.24**	1.60
Barium	**0**	0	**0.370**	0.641	**0.437**	0.349	**1.47**	0.008	**0.569**	0.250
Cadmium	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0
Cobalt	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0
Chrome	**0**	0	**0.175**	0.303	**0**	0	**0.388**	0.109	**0.141**	0.103
Copper	**1.61**	0.016	**2.46**	0.187	**1.16**	1.11	**2.50**	0.520	**1.93**	0.458
Iron	**18.7**	0.819	**69.8**	4.63	**21.1**	2.94	**67.3**	8.77	**44.2**	4.29
Manganese	**0.598**	0.051	**1.29**	0.346	**0.477**	0.180	**0.427**	0	**0.698**	0.144
Molybdenum	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0
Nickel	**0.540**	0.384	**1.47**	1.32	**0.496**	0.645	**0.080**	0.12	**0.647**	0.617
Lead	**1.13**	0.459	**0.442**	0.710	**1.72**	1.75	**1.48**	1.25	**1.19**	1.04
Silver	**0**	0	**0**	0	**0.036**	0.062	**0**	0	**0.009**	0.016
Selenium	**1.01**	0.567	**1.64**	0.517	**0.148**	0.162	**3.00**	0.646	**1.45**	0.473
Titanium	**0**	0	**0.067**	0.117	**0.631**	0.225	**0.165**	0.073	**0.216**	0.104
Zinc	**11.6**	0.420	**28.5**	0.799	**37.8**	4.32	**120**	16.0	**49.5**	5.38
Strontium	**1.0**	0.150	**1.23**	0.103	**0.564**	0.212	**1.15**	0.392	**0.986**	0.214
Vanadium	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0
Thallium	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0
Silicon	**1449**	2348	**707**	921	**5003**	2760	**2597**	2916	**2439**	2236

The most dominating element found in the highest concentration seems to be silicon, which is found as high as 5003 mg/kg dried pig cutlet. However, the standard deviation on the triple determination was very high and some uncertainty is related to the silicon values. As described in the previous chapter regarding the urine analysis, the laboratory staff reported that the method is quite uncertain for especially silicon. Therefore, this uncertainty must be taken in to account in the evaluation of the results. The average metal content from the table are presented in *Figure 6-1*. Please note that silicon is not shown due to the high and uncertain results.

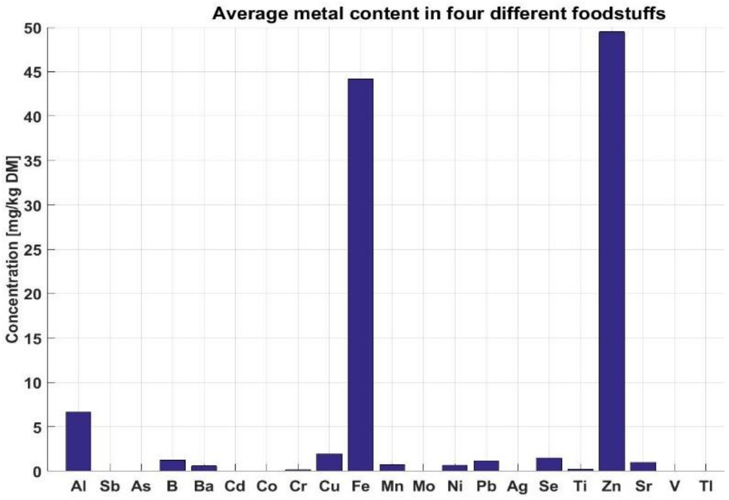

Figure 6-1: Average metal content in the four different foodstuffs. OBS: silicon is not shown.

Besides silicon, the dominating elements were iron and zinc. Iron and zinc are essential nutrients as described previously in this report and the high concentrations might therefore not be a problem. Both copper and zinc are found in all four foods and the concentration is highest in the beef. An accumulation of copper and zinc in the pork meat due to the use as growth promoters is therefore not indicated. Of the toxic metals, aluminium is found in the highest concentration. In average of the four food types, it is found in 6.66 mg per kg dry foodstuff. The highest concentration is 9.89 mg per kg found in canned tuna. The toxic metals lead and nickel are found in some levels as well.

As previously mentioned some metals in small amounts are essential for survival and health of animals and humans. In just a little higher concentration, these metals instead are toxic to the organism. Therefore, it can be difficult to determine in what concentrations a metal is toxic. For the essential metals, this is typically established in order to ensure amounts needed to prevent clinical or biochemical deficiency. The effect of any substance on living organisms is always a consequence of the concentration available to the cells. Therefore, the dose-response relationship is needed for the evaluation, since all substances might be toxic in sufficient amounts. In addition, the metal toxicity is also dependent on form at which the metals is present. For instance, silver in itself is not toxic, but some silver salts are very toxic. It will therefore require a comprehensive study of the individual metals toxicity to living organisms to assess what concentrations, which might be considered harmful.

The above investigation is just a limited screening of a few selected foodstuffs. To identify the importance of foodstuff as a source of heavy metals, many more food products must be analysed. Certain types of foodstuff are already known to contain large quantities of specific heavy metals, for example, sunflower seeds contain much cadmium. The content of heavy metals in food will also be determined by where and how they are grown, harvested and processed. Therefore, these factors also should be taken in to account in a more thorough investigation of this.

7 Characterisation of manures

In this chapter, pig and cattle manures are analysed and characterised. The purpose is to investigate the content of heavy metals in the manures as well as any differences in the manures originating from pigs and cows, respectively. The content of metals in the manures originates, just like humans, from the fodder and water given to the pigs and cows. If the fodder contains more metals (such as copper) than the physiological needs, a small amount is absorbed in the body while the rest are excreted in the urine and excrements. Additionally, some metals in the manure may originate from the products used in the pigsty and cowshed. As manures are utilised as fertilisers the metals are transferred to the farmlands and the nature. The crops also have a need for some metals but if the supply exceeds the needs, a potential pollution may occur. Thus, the environmental impact depends on the quantities supplied to the farmlands as well as content of metals.

Reference is made to *Appendix C - Manure analysis* for detailed procedure and results.

7.1 Manure characterisation

In this project, one sample of cattle manure and two samples of pig manure have been analysed. However, one of the samples of pig manure has been analysed by ICP-OES only. This is because a small amount was received only, and no other analyses were carried out before drying of the sample for ICP-OES analysis. In the following sections, analyses of one sample of pig manure and one sample of cattle manure therefore are presented only. Except for the section with ICP-OES where the two different samples of pig manure are included as well as the cattle manure. Otherwise the missing results will be indicated by *NA*. The cattle and pig manure were collected from local farmers and are seen in *Figure 7-1*.

Figure 7-1: Cattle manure to the left and pig manure to the right.

The samples was not homogeneous. In general, the cattle manure was thicker than the pig manure, which was almost thin dark urine. If big straws or similar was found in the manure, they were discarded before any analysis. In order to minimise the effects from inhomogeneity the samples were always shaken/stirred meticulous before analysis.

7.2 Material and methods

In order to evaluate the dry matter in the samples, a known amount of the manures was dried in a stove in a fume hood at 105°C until a constant weight was obtained. The dried manures were cooled in a desiccator and weighed after cooling. A known amount of the dried manures was ignited at 550°C for the determination of organic matter.

Some of the dried manure was used for the determination of metals as well by ICP-OES. The samples were prepared in the following way: A small amount of the dried manures was accurately weighed into an autoclave bottle and added nitric acid (7 M). The solution was digestion at 120°C for 30 minutes. After cooling to room temperature the samples were filtrated and diluted with demineralised water to a fixed volume was reached. ICP-OES was used for the determination of 17 selected metals including K and P.

The total N in the manures was determined by FIA on the wet manures. A small amount was accurately weighed in an autoclave bottle and digested with potassium peroxodisulphate digestion solution. The solution was digested at 120°C for 30 minutes. After cooling to room temperature the samples were filtrated and diluted with demineralised water to a fixed volume was reached.

7.3 Results and discussion

Some physical and chemical characteristics are represented in *Table 7-1*. The pH of the cattle manure was a bit higher than the pig manure. As expected, the dry matter content was higher in the cattle manure. The nutrient content of N was highest in the cattle manure, while the P and K content was highest in the pig manures.

Table 7-1: Some physical and chemical characteristics of the pig and cattle manure.

	pH	Electric conductivity [µS/cm]	Dry matter (DM) [%]	Total N [mg/kg] Wet	Total P [mg/kg] DM	Total K [mg/kg] DM
Pig manure 1	*NA*	*NA*	*NA*	*NA*	30,182	213,093
Pig manure 2	7.34	461	1.19	1739	19,957	187,604
Cattle manure	7.89	398	2.83	2406	10,799	51,002

The sample of the cattle manure, which was collected at the farmer, was a residual portion taken for nutrient analysis at Eurofins. It is therefore relevant to compare the results obtained in this project by converting it to the same units to these results. The comparison is presented in *Table 7-2* and the test results from Eurofins are seen in *Appendix C - Manure analysis* as well.

Table 7-2: Cattle manure characteristic obtained in this project compared to results from Eurofins.

	Dry matter [%]	Total N [kg/t] Wet	Total P [kg/t] Wet	Total K [kg/t] Wet
Cattle manure	2.83	2.41	0.31	1.44
Cattle manure (Eurofins)	2.5	2.30	0.28	1.6

It is seen that the results obtained in this project is of great consistency with the results received from Eurofins.

The concentrations of 15 selected metals in the dried manure were determined by ICP-OES. The results are presented in *Figure 7-2*. In all three samples, the most dominant elements were Al, Cu, Fe, Mn, and Zn. Of these, the highest concentrations were found in the cattle manure, except for the concentration of zinc, which was highest in the pig manure (sample 2).

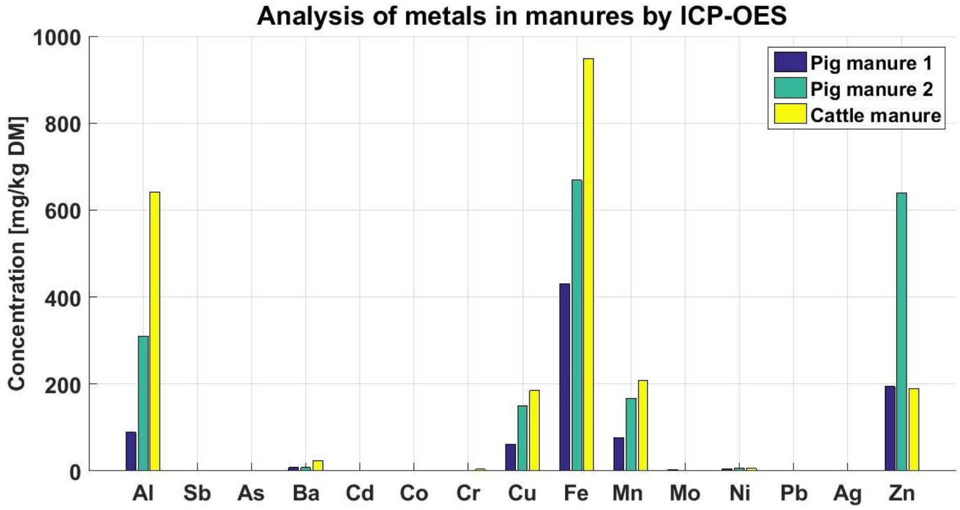

Figure 7-2: Concentration of 15 selected metals in pig manure and cattle manure (mg per kg dry matter). Entries are mean value from triple determination.

The concentrations of the elements copper and zinc are presented in *Figure 7-3* for easier comparison with standard deviation presented on each bar. It is seen that the copper content seems to be highest in the cattle manure (189 mg/kg), while the Pig Manure 2 contains much more zinc (640 mg/kg) than the two other manures (194 and 190 mg/kg respectively).

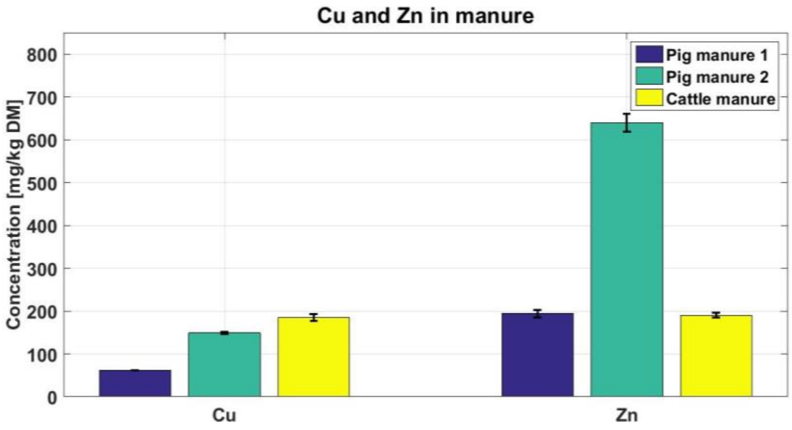

Figure 7-3: Concentration of Cu and Zn in pig and cattle manure (mg per kg dry matter). Entries are mean value from triple determination and the standard deviations are presented on the bars.

The focus in this report are on Cu and Zn and therefore a statistical test are carried out in order to evaluate whether the concentrations are significant different. This is done by a one-factor ANOVA on respectively the Cu and Zn concentrations show in *Table 7-3*.

Table 7-3: Cu and Zn concentrations in the manures.

Cu [mg/kg DM]			Zn [mg/kg DM]		
Pig Manure 1	**Pig Manure 2**	**Cattle Manure**	**Pig Manure 1**	**Pig Manure 2**	**Cattle Manure**
61.2	148	179	194	642	186
61.0	152	181	203	660	187
61.8	147	194	186	619	197

A one-factor ANOVA assumes that all the groups have the same standard deviation (and thus the same variance). This assumption is not very important when all the groups have the same (or almost the same) number of subjects, but is very important when sample sizes differ. In this case, the sample size is equal in all groups; therefore, the test for equality of variance is not carried out. The null hypothesis tested by ANOVA is that the population means for all conditions are the same, expressed as (33):

$$H_0 : \mu_1 = \mu_2 = \ldots = \mu_k,$$
$$H_1 : At\ least\ two\ of\ the\ means\ are\ not\ equal.$$

The ANOVA is performed by using Excel. The data is entered on the spreadsheet, and “ANOVA: single factor” are selected from the data analysis tools. The output table in Excel shows the source of variance as "Between groups" (= between treatments) and "within groups" (= residual). Additionally, the calculated *F* value, the F value that we would need to exceed (*F critical*) in order to have a significant difference between treatments, and the probability (*P-value*) that our calculated F value would be obtained by chance (random error) alone are given. The null hypothesis is rejected when the p value is smaller than the alpha level (which by standard is 0.05). Similar the null hypothesis is rejected if the critical F value is smaller than the F Value (33).

In this case, there are three “treatments”, which gives *k*=3 in the null hypothesis. The ANOVA table of the Cu and Zn concentration in the three manures are seen in *Table 7-4* and *Table 7-5*, respectively. It is seen that the P-value=$1.71\cdot10^{-7}$ for Cu are lower than $\alpha=0.05$ and therefore the null hypothesis is rejected, meaning that the mean Cu values is different.

Table 7-4: ANOVA table of the Cu concentration in the three manures.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	24264	2	12132	538	1.71E-07	5.14
Within Groups	135	6	23			
Total						

In addition, it is seen that the P-value=$1.82\cdot10^{-8}$ for Zn are lower than $\alpha=0.05$ and therefore the null hypothesis is rejected, meaning that the mean Zn values as expected also is different.

Table 7-5: ANOVA table of the Zn concentration in the three manures.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	401765	2	200883	1137	1.82E-08	5.14
Within Groups	1060	6	177			
Total						

The content of Cu and Zn are thus different in the three manures. The zinc content in the pig manure was significant higher than the cattle manure.

In general, the amount and composition of manure depends on numerous factors. This includes the amount of feed and composition, dry matter decomposition, ammonia loss, water needs, water spills, wash water and water evaporation. The characteristic of the manures varies extremely between different livestock. In practice, you will find, for example, nitrogen in pig manure from under 2 kg N per. ton to over 8 kg N per. T (13). The dry matter and other nutrients such as copper and zinc vary accordingly. Stable management and especially feeding strategies have great influence on manure volumes and composition. An optimal feeding strategy will optimize feed utilization and thereby reduce the excretion of nutrients from the pig. Good stable management will also help to optimize feed utilization, but will also minimize for instance water wastage and consumption of washing water in the stables. (13)

Another important factor affecting the characteristic of manures is the sampling. It is important, that the collected sample is representative for the entire manure. The manure characteristic will depend on where the manure is collected: for instance directly from the slurry tank or under the slatted floor etc. and additionally depending on the type of stable. Stirring of the manure before sampling will also influence the obtained characteristic. If the manure just is taken at the top of the tank, it might be thin, since the solid particles will precipitate.

8 Heavy metals in agricultural and forest soils

In this chapter, soil samples from two Danish agricultural fields are screened for the content of metals. The supply of metals to Danish agricultural fields originates from different sources, such as animal manure. When manures containing heavy metals are applied to fields, only small amounts are removed by for instance leaching or by plant uptake. The majority of the applied amounts will therefore accumulate in the soil if the amounts exceed the plants' needs. Thus, prolonged application of manures might cause an accumulation of the metals in the soils.

The purpose of this part is an investigation of the metal levels in soils from two different agricultural soils compared to soil samples collected in a forest. The two farmlands include lands receiving pig manure and cattle manure respectively. The soil from the forest is used as a reference for normal concentrations of metals in a soil with minimal anthropological activity. The soil samples collected in this project represents only a very small part of the agricultural fields in Denmark. Therefore, it should be regarded as s screening of a situation that may be in evidence in agricultural lands all over Denmark.

Reference is made to *Appendix D – Soil analysis* for detailed procedure and results.

8.1 Material and methods

Soil samples were collected from fields receiving pig manure and cattle manure, respectively. In addition, soil samples from a forest were collected as well. All sampling sites were placed in the same area of Denmark and the atmospheric deposition is therefore assumed almost equal. The sampling sites include:

1. Field supplied with cattle manure
2. Field supplied with pig manure
3. A forest

There may be places on the fields that locally have an exceptional high content of certain elements. Therefore, three soil samples were collected from sites distributed at each type of land in order to get a representative characterisation of the field. The area is known to have a very sandy soil and the sites are seen in *Figure 8-1*

Figure 8-1: Sampling sites including field with cattle manure, pig manure and a forest (from left to right).

The three samples from the different sites visually seemed to be identical. One of the samples from the forest was a bit sandier compared to the other two though. In general, all the samples from the forest contained more visible plant material compared to the two other types of soil. The soil samples from the fields were visually almost identical. The fields and the forest are placed in Oksenvad between Jels and Sommersted in Southern Jutland as seen in *Figure 8-2*.

Figure 8-2: Soil sampling sites of fields supplied with 1) cattle manure and 2) pig manure and 3) in a forest. Samples from all three places are analysed with a triple determination. **(www.krak.dk)**

After collection, the samples were brought to the laboratory and small amounts were weighed before drying at 105°C until a constant weight was obtained. The dry matter was calculated then. Some of the dried soils were kept for later analysis while some were ignited a 550°C for the determination of organic matter in the soils.

ICP-OES was used for the determination of 17 selected metals including K and P in the dried soils. The samples were prepared in the following way: A small amount of the dried soils was accurately weighed into an autoclave bottle and added nitric acid (7 M). The solution was digested at 120°C for 30 minutes in an autoclave. After cooling to room temperature the samples were filtrated and diluted with demineralised water to a fixed volume was reached.

Total N in the soils was determined by FIA on the dried soil samples. A small amount was accurately weighed in an autoclave bottle and digested with potassium peroxodisulphate digestion solution. The solution was digested at 120°C for 30 minutes. After cooling to room temperature the samples were filtrated and diluted with demineralised water to a fixed volume was reached.

8.2 Results and discussion

The dry matter (DM) and organic matter (OM) was determined in the different types of the soils. The average DM and OM of the different type of soils at the three sites are presented in *Figure 8-3*.

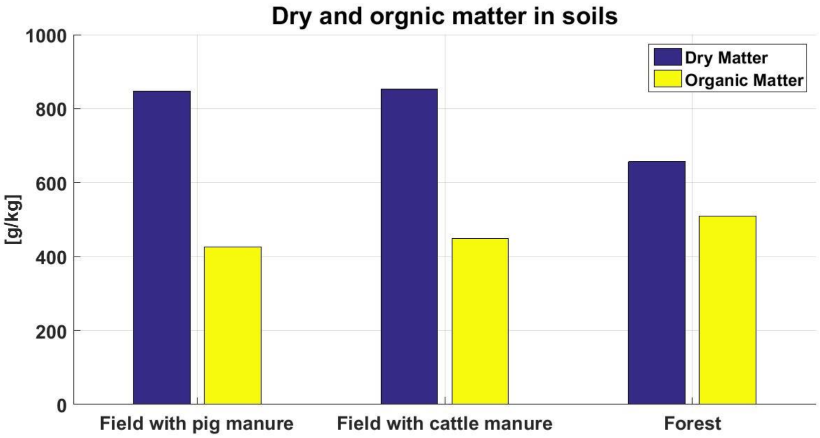

Figure 8-3: Dry matter and organic matter in the different soils. Entries are average on the three sampling sites.

The OM and DM in the fields with pig manure and cattle manure was almost equal. As expected, the OM was a bit higher in the forest soil due to the high content of plant materials and humus. The nutrient content in terms of nitrogen, phosphor and potassium (NPK) in the soils was determined. The average concentrations of the three sites in the different soils are presented in *Figure 8-4*.

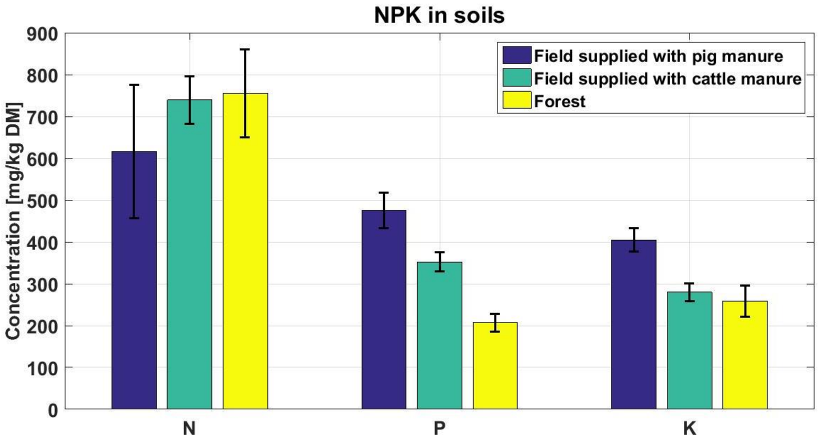

Figure 8-4: Average concentration of N, P and K with standard deviation in the three types of soil.

The nitrogen content was almost equal in the three soils, maybe little lower in the field with pig manure. Contrary, the phosphor and potassium content in the soil applied pig manure was a bit higher. This is consistent with the determined nutrient content in the manures, since the concentration of P and K was higher in the pig manure.

The concentration of metals in the soils obtained by ICP-OES analysis is presented in *Figure 8-6*. Iron, aluminium and manganese are the elements found in the highest concentration of the elements selected in the analysis. The highest concentration of aluminium and iron was found in the field supplied with pig manure, while the concentration of manganese was a bit higher in the field with cattle manure.

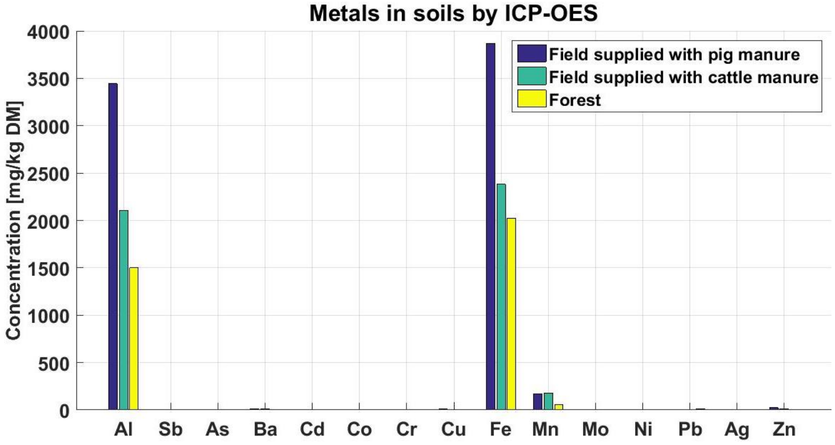

Figure 8-5: Average amount of metals in soils from a forest and fields supplied with pig manure and cattle manure, respectively. Three samples were collected on each type of land and measured with a triple determination.

The concentrations of metals found in the three sites at the different types of land are presented in *Figure 8-6*. Within the different types of soils, the highest deviation was found in the soil from the forest. The sample from site 2 in the forest had a little lower concentration of aluminium and iron than the two other sites. The soil sample from site 2 in the forest was sandier. This might explain the observed results, since leaching of metals is more easily to take place in sandy soils compared to soils with high organic matter.

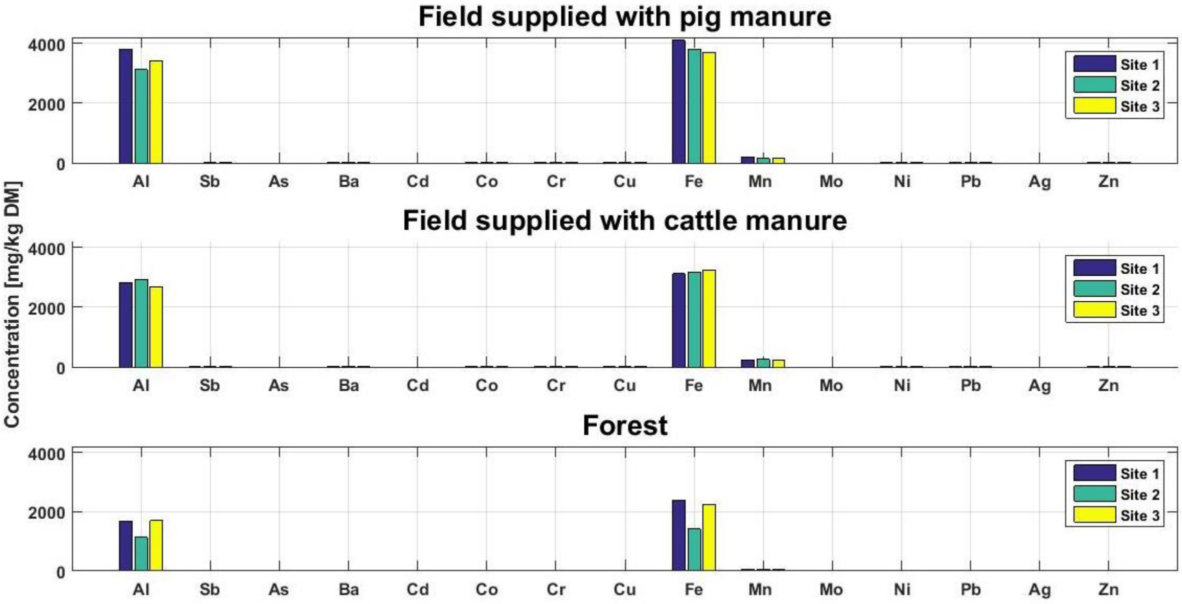

Figure 8-6: Average amount of metals in soils from a forest and fields supplied with pig manure and cattle manure, respectively. The shown values are average on a triple determination of the sites in each type of land.

The metals Cu and Zn were found in the soils too. The concentration was however much lower than aluminium and iron and therefore, they are dwarfed by the concentration of those. For easier comparison, the concentration of Cu and Zn only are seen in *Table 8-1* and the averages are presented in *Figure 8-7*.

Table 8-1: Concentration of Cu and Zn in three sites from a forest and fields supplied with pig manure and cattle manure, respectively

Cu [mg/kg]	Pig field	Cattle field	Forest	Zn [mg/kg]	Pig field	Cattle field	Forest
Site 1	11.9	3.48	2.96	**Site 1**	32.1	15.1	4.42
Site 2	3.85	2.76	3.05	**Site 2**	15.7	14.4	5.34
Site 3	9.21	3.89	2.15	**Site 3**	26.3	15.8	5.43

The concentration of both metals seems to be highest in the field with pig manure, shown with blue bars in *Figure 8-7*.

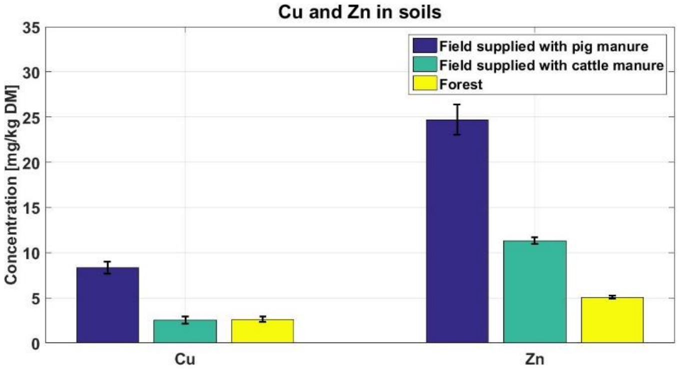

Figure 8-7: Average concentration of Cu and Zn in the three types of soil.

In order to evaluate if the concentration are statistical significant different, a one-factor ANOVA are carried out as previously described. The ANOVA table of the Cu and Zn concentration in the three types of soil are seen in *Table 8-2* and *Table 8-3*, respectively. It is seen that the P-value=0.054 for Cu are higher than α=0.05 and therefore the null hypothesis is accepted, meaning that the mean Cu values are equal.

Table 8-2: ANOVA table of the Cu concentration in the three types of soil.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	57.4	2	28.7	4.95	0.054	5.14
Within Groups	34.8	6	5.80			
Total	92.3	8				

Contrary to this the P-value (=0.0074) in *Table 8-3* for the Zn concentrations in the soils are lower than α=0.05. The null hypothesis is therefore rejected meaning that the mean Zn values are different.

Table 8-3: ANOVA table of the Zn concentration in the three types of soil.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	579	2	290	12.4	0.0074	5.14
Within Groups	140	6	23.4			
Total	719	8				

Therefore, no difference in the content of Cu in the tested types of soil was demonstrated, while the opposite was the case for the concentration of Zn in the soils. The content of Zn in the field supplied with pig manure seems to be significant higher than the two other types of soils.

The obtained data are compared to the soil concentration of Cu and Zn in the surveys discussed in chapter 3 and summarised in *Table 8-4*.

Table 8-4: Comparison of the soil concentrations obtained in this study (marked with bold) to data from the previous surveys described in chapter 3. (14)

		Cu [mg/kg soil]			**Zn [mg/kg soil]**			**Field with cattle manure [mg/kg soil]**		**Forest soil [mg/kg soil]**	
	[cm]	1986	1998	**2015**	1986	1998	**2015**	**Cu**	**Zn**	**Cu**	**Zn**
Field with pig Manure	0-25	8.2	9.6	**8.32**	36.6	32.9	**24.7**	**3.38**	**15.1**	**2.72**	**5.06**
	25-50	6.2	7.4		32.2	28.9					
Field with fertiliser	0-25	7.0	7.7	**NA**	33.0	27.3	**NA**				
	25-50	4.9	6.7		31.2	25.3					

It is seen, that the Cu concentration in the soil with pig manure in this study almost equals the concentrations in the studies from 1986 and 1998. Contrary to this, the concentration of Zn is a bit lower. Both the concentration of Cu and Zn from soils at the field with cattle manure and the forest in this study are generally lower than the data of the fields with pig manure. It is hereby indicated, that the application of pig manure as fertiliser causes an accumulation of Cu and Zn on agricultural land. The data obtained in this study, does not demonstrate an increase since the survey in 1998 though.

This study evaluates the impact of the application of manures as fertiliser based on three samples from only two agricultural fields placed in Denmark due to the time constraint in the project. Seen in that light, no thorough conclusions should be stated. The detected metal content in soils applied manures will depends on numerous factors including the application rate of manure and the corresponding concentration in the manure as well as the application method and the following soil preparation. According to this, the concentration in the soil layers in different depth will differ. This will moreover depend on the mobility of the metals, which will be affected by the soil characteristics.

9 Preparation of plant experiments

Some pot experiments were carried out in this project and the detailed experimental design are described in the following chapters. This chapter covers a general description of the material used in all experiment, such as the soil and water used for the pot experiments. Planting soil bought at the local garden centre was used in all plant experiments. Tap water was used for the watering of the plants. The plant experiments were carried out in plastic pots, which are not expected to emit metals. The concentrations obtained in this chapter will be an expression of the background concentrations in the following plant experiments.

Reference is made to *Appendix E – Preparation of plant experiments* for details about the procedure of the analyses as well as raw data results.

9.1 Material and methods

Pot experiments

In the plant experiments presented in the following chapters, plants are grown in small pots in planting soil. By carrying out the experiment in small pots instead of a field, the soil variations are minimised. Planting soil from a sack is used to fill up the pots. By mixing the soil in the sack and filling up the pots after a randomised plan, effects from local variations in the sack are minimised as well. However, some variations in the soils might exist and might affect the results.

Metal application to soil in pot experiments

Different concentrations of Cu and Zn are applied to the planting soil used in the pot experiments. Plants are sown in the pots and after growing for a number of weeks, they are harvest and analysed for the content of Cu and Zn as an expression for the uptake of those metals from the soil. The concentration of metals in the plants and soils are analysed by ICP-OES, which is the most widely used methods for environmental trace metal analysis (34). The sample preparations of the soil and plant samples are illustrated in *Figure 9-1*.

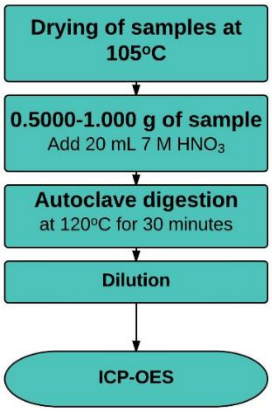

Figure 9-1: Sample preparation for metals in soils and plants.

The device is available in the university laboratory and is the same as previously used in this project. The advantage of this method is that all atoms in the samples are excited and therefore they can be detected simultaneously (34). In addition, the connected auto sampler ensures analysis of all the prepared samples according to the sample list. The pot experiments are carried out in a sunroom and/or a greenhouse. Radish and cress were used, since they are fast growing plants, which is an advantage due to the time limit of the project.

Experimental design

In the industry, processes typically are optimised by empirical methods, i.e. by trial and error and from observations and experiences. This empirical modeling can in a way be good enough for specific problems where there are no interactions between the constituent factors, but extrapolation should always be done with care. Additionally, the factors are varied individually and not simultaneously, thus not taken into account any interactions. Finally, it is inevitable that the empirical method arises systematic experimental work, and thus the results may depend on external factors such as ambient temperature and so on. Factorial experimental design in which all factors are varied and investigated simultaneously includes interactions. In addition, randomisation is used here contrary to the aforementioned model, which means that the results are much more independent of the test conditions (35). In the following plant experiments, factorial design is used in two of the three experiments. In the following section, the planting soil and watering water are analysed.

9.2 Metals in planting soil and watering water

The main constituents of the planting soil are sphagnum, sand and clay minerals. The content of organic matter was determined to 55.6 %, which was a little higher than the agricultural and forest soils described previously. This might be explained by the content of sphagnum. The average concentration of metals in the planting soil used in the following pot experiments are presented in *Figure 9-2*. As seen in the previous chapter concerning soil characterisation, the most dominant elements were aluminium and iron.

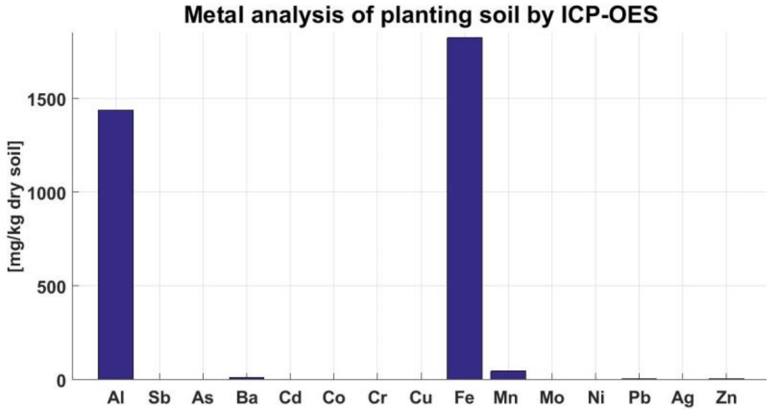

Figure 9-2: Average content of a triple determination of metals in three samples of planting soil determined by ICP-OES.

Some other metals were detected as well. Of those Cu and Zn are the most interesting metals, since they were used in the following plant experiments. Therefore, their concentrations are presented in *Figure 9-3*. The average concentration of Cu was 2.45 mg per kg dry soil and the average Zn concentration was 6.37 mg per kg dry soil.

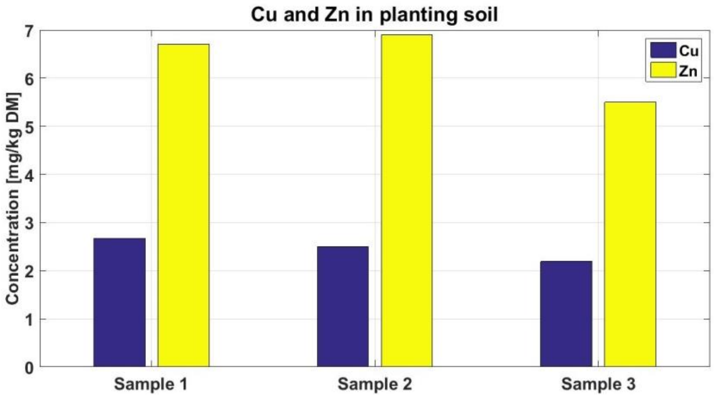

Figure 9-3: Cu and Zn in three samples of planting soil.

Since the planting soil was used in all experiments, the background concentration of Cu and Zn were equal in all experiments. The NPK content in the planting soil was determined as well. The results are presented in *Figure 9-4*.

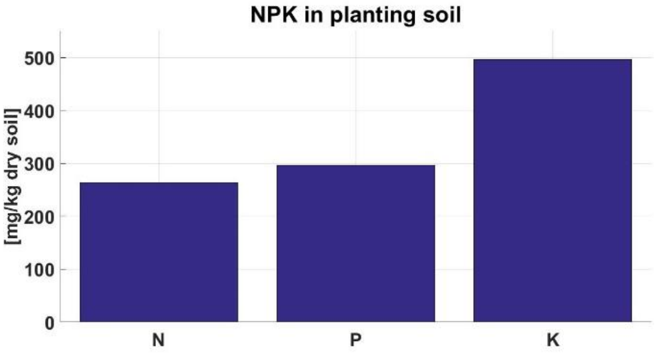

Figure 9-4: NPK in planting soil. N was determined by FIA while P and K were determined by ICP-OES. Average values of a triple determination are shown.

Nitrogen (N), phosphor (P) and potassium (K) are all micronutrients needed to build the complex molecules that plants need to survive and grow. This includes for instance proteins, enzymes and DNA (30). The amount required for optimal growth of plants will differ along with the plant type and desired plant yield. This soil is made for growing of vegetables and the like; therefore, extra nutrition is not supplied in all the following experiments.

The watering water used in the plant experiments was ordinary tap water. The metal content in the water was analysed by ICP-OES and the results are presented in *Figure 9-5*.

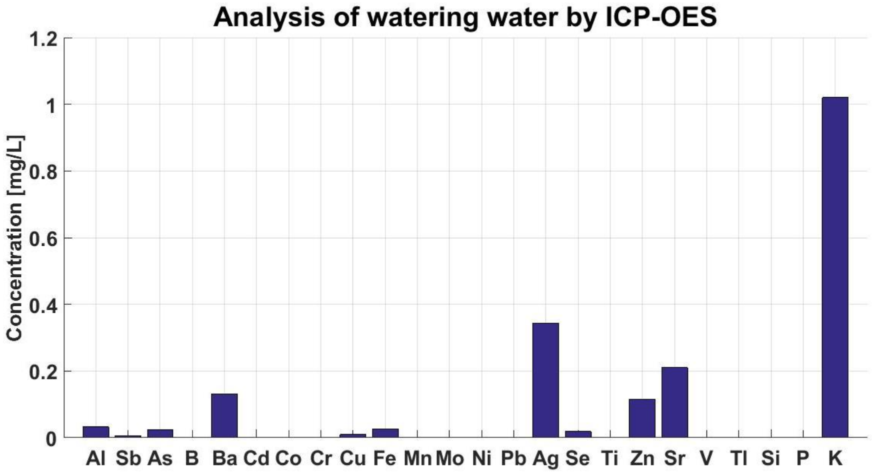

Figure 9-5: Metal analysis of watering water (tap water). Average values of a triple determination are shown.

In general the concentrations of metals found in the watering water were low. The plants in the plant experiments are watered with the same amount of water. The concentration of metals in the watering water thus will be an expression of a background concentration that will be the same in all experiments.

10 Accumulation of Cu and Zn in radish

When soils are supplied with manure, the metals from the manure are accumulated in the soil as previously described. Increasing concentrations of metals in the soil may lead to their increased availability to plants. The plants growing in the soils will take up some of the metals dependent on the soil characteristic and type of plant. In this experiment, radishes were grown in pots, which were added different known concentrations of Cu and Zn to simulate soil metal accumulation by manure application. Thus, the aim of this study was to evaluate the uptake of Cu and Zn in plants from a contaminated soil as well as the effect of Cu and Zn on biomass production. The application rates of Cu and Zn were 60 and 150 mg/kg respectively. The manure containing Cu and Zn was not used in this experiment. This is because it probably would have affected the soil properties in pots receiving manure and the results would be difficult to compare by pots that were not supplied with manure.

In the case of multi-element contamination some interactions between the metals may occur, both at the root surface, affecting uptake and within the plant. These interactions are described as antagonisms or synergisms. In order to evaluate possible interactions, a full factorial experimental design was used.

Reference is made to *Appendix F – Radish experiment* for details about the procedure of the experiment as well as raw data results.

10.1 Design of experiment

A two-factor two-level (2^2) full factorial design was used to study the effect on growth and the uptake of Cu and Zn in radishes. When an experiment consists of two or more factors, the factors can influence the response individually or jointly. Thus, the effect of each factor was studied as well the effects of interactions between the factors on the response variables. The two factors were Cu and Zn and the response variables were biomass (weight of radish plants) and uptake (concentration of Cu and Zn in radish plants). In the majority of full factorial experiments, each factor consists of two levels only. This was also the case in this experiment in which the factors was designated low (-) and high (+) as seen in *Table 10-1*.

Table 10-1: The two factors were application of Cu and Zn to the soil with two levels each.

Factors	**Levels [mg/kg]**	
	Low (-)	High (+)
A: Cu	0	60
B: Zn	0	150

The low level was zero and the high level was some appropriate value. The high levels were selected on the basis of similar experiments (36,37) (using maximum 50 mg Cu and 140 mg Zn per kg soil) and from the upper critical soil concentration of Cu and Zn (318 mg Zn and 105 mg Cu per kg soil) on the growth of ryegrass (38), since it was not possible to find it for radish. The concentration of metal added in the treatments, were based on this, intended to be large enough to cause an accumulation of Cu and Zn but assumable no possibility of casing phyto-toxicity.

A combination of the two factors and their levels is called a *trial*. When a combination is tested, it is called a *run*. The number of trials in a 2^2 design is 4. In order to test all combinations of factors and their levels four runs therefore were needed, as seen in *Table 10-2*. The first is a control in which both factors are at low levels, in the next run the interaction effects of Cu and Zn on the response variable is tested, in run number three the effect of factor A (Cu) is tested and in run number four, factor B (Zn) is tested.

Table 10-2: Design matrix for radish experiment.

Run	Factor A	Factor B	Test of factor
1	-	-	0
2	+	+	AB
3	+	-	A
4	-	+	B

A triple determination was carried out resulting in 12 runs totally. The four trials with three replicates were tested in a completely randomised design by running the treatment combinations in a random order, as seen in *Table 10-3*.

Table 10-3: The four trials were tested with triple determination in a randomised sequence.

Replicate	Run	Sequence	Factor A	Factor B	Test of factor
1	1-1	10	-	-	0
	2-1	4	+	+	AB
	3-1	5	+	-	A
	4-1	2	-	+	B
2	1-2	9	-	-	0
	2-2	7	+	+	AB
	3-2	3	+	-	A
	4-2	11	-	+	B
3	1-3	6	-	-	0
	2-3	12	+	+	AB
	3-3	1	+	-	A
	4-3	8	-	+	B

Randomisation is an objective method of random allocation of the experimental material or treatments in an experiment to the experimental units. The randomisation ensures that the orders in which the trials are performed are random, including for instance the preparation of the pot experiments as well as the order of analysis. Thus, randomisation subjects all treatments to as nearly equal conditions as possible. Hereby the randomisation affords protection against possible bias effects or systematic error due to unexpected causes during the course of an experiment (39).

10.2 Material and methods

Pot experiments were carried out to examine the uptake of Cu and Zn in radish plants. The planting soil described in the previous chapter was used for pot experiments. Samples (1 kg air-dried) of the soil were filled into plastic pots and the metal addition were made by adding appropriate amounts of Cu sulphate and Zn sulphate as powdered solids. A basal application of fertiliser (1.00 g NH_4NO_3 and 1.75 g KH_2PO_4) was also made at that time. In each pot the soil was thoroughly mixed with the chemicals and watered with tap water. Radish (*Raphanus sativus*) was sown at a rate of 15 seeds per pot. The pots were placed randomly in a sunroom with no controlled climate. After 10 days, the seedlings were thinned to nine seedlings per pot. The pots were watered regularly with tap water to keep the soil humid.

The experiment was terminated 32 days after sowing. The radishes were harvested and the soil was sampled at the same time. The radish were divided into two parts as the above-ground part and root, washed with tap water to remove any attached soil particles, and rinsed twice with deionized water. The fresh plants were weighed. The plant samples were then dried in a stove at 105°C to a constant weight. The Cu and Zn in the plant material were analysed as follows: First, the dry material was grinded and about 0.900 g was weighed and digested with 7 M nitric acid. The digestion was carried out in an autoclave at 120°C for 30 minutes. The digestion solution was transferred to a volumetric flask and demineralised water was added to reach a fixed volume. The concentrations of Cu and Zn were determined with ICP-OES.

The soil samples were dried at 105°C to a constant weight was obtained. The concentration of Cu and Zn in the dried soil samples were determined with ICP-OES as previous described. The plant-available concentrations of Cu and Zn in the soils were determined in the following way as described in (40). The dried soil samples were suspended in $CaCl_2$ and heated at 90°C for 30 minutes. The suspension was filtered through Whatman 42 filter paper and 2 drops of 1 M HNO_3 were added to prevent metal precipitation. The filtered solutions were analysed by ICP-OES as previous described.

10.3 Results and discussion

The plants were harvested 32 days after sowing. The plants in run 1 and 4 were distinctly smaller than the plants in run 2 and 3 as seen in *Figure 10-1*.

Figure 10-1: Radish plants were harvested after 32 days of growth. The plants in run 1 and 4 were distinctly smaller than the plants in run 2 and 3.

The plants and soil were analysed as described in *Appendix F – Radish experiment*. Each treatment was carried out in triple determination and each sample of the triple were analysed in duplicate, giving a total of six samples for each treatment. The results are presented in the following.

10.3.1Biomass of radish as affected by Cu and Zn

The biomasses (fresh weight) of the radish plants after harvest are presented in *Figure 10-2* in the four different runs. The total biomass as well as the aboveground and root biomass are shown.

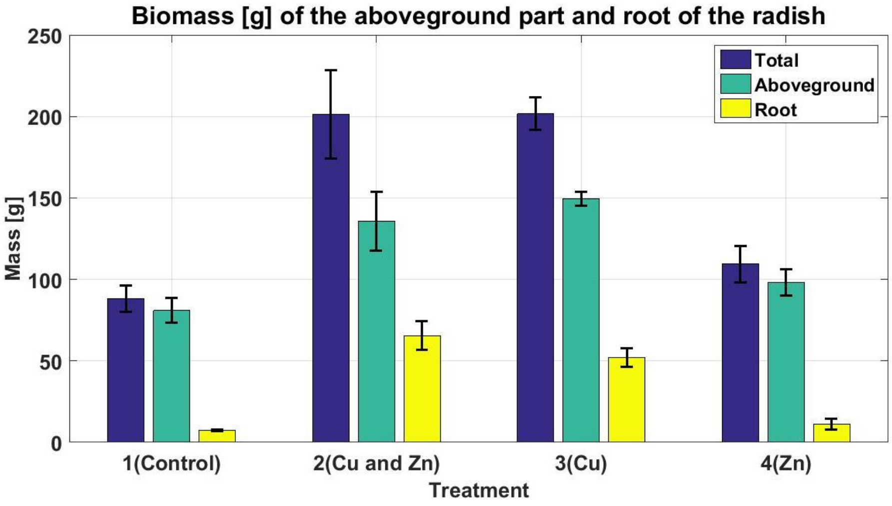

Figure 10-2: Biomass of the total, aboveground and root of the radish.

The biomasses of the aboveground part of the radishes were highest in treatment 2 and 3. Likewise, the root biomass was significant higher in treatment 2 and 3 compared to treatment 1 and 4. The root biomass of treatment 2 was approximately nine times higher than of treatment 2. This indicates that the treatment with Cu and Zn (treatment 2) has a positive effect on the biomass production of radish compared to control treatment 1, which was not supplied with Cu or Zn. The biomass of treatment 3 was higher than treatment 4. This indicates that especially Cu had a positive effect on the biomass production of radish growth in the experiment. It was hereby indicated, that either Cu is the constraining factor in this experiment or that Cu causes some positive interactions with other nutrient that increases the growth of the radish plants.

The obtained response values (biomass) are evaluated according the full factorial design. MATLAB is used for this and reference is made to *Appendix F – Radish experiment* for MATLAB operations. At first, the *effects* are calculated. The effects are a change in the response caused by a change in one or more factors. The main effects are an effect caused by a change in one of the main factors, in this case Cu or Zn. The calculated values are seen in *Table 10-4*. The main effects are calculated with total, aboveground part and root biomass, respectively as response

Table 10-4: Main effects of Cu and Zn on total, aboveground part and root biomass of radish.

	Total biomass		Aboveground part		Root	
Factor	**A: Cu**	**B: Zn**	**A: Cu**	**B: Zn**	**A: Cu**	**B: Zn**
Main effect	102.6	10.41	53.10	1.800	49.52	8.61

It is seen, that the total biomass is affected of both Cu and Zn, with Cu as the most dominating factor, i.e. the factor of which a change in the level will have the greatest effect on the response value (biomass). In the biomass of the aboveground part of the radish, Cu is the dominating factor. This is also the case for the root biomass; here Cu is the most dominating factor as well. All effects are positive, which means that changing the levels from low to high will cause a higher biomass of the radishes.

A visual evaluation of the main effects is made by making plots of the main effects as presented in *Figure 10-3*. This gives an easier comparison of the effects. The main effects, which have a steep slope, will have larger effect and thus larger impact on the biomass. In all three cases the slope of Cu are steepest and Cu thus have the highest effect on the biomass. Since the sign of the slope of all lines are positive, an increase in both factors Cu and Zn will increase the biomass of the radish plants, including both the aboveground part and root biomass. If the line are horizontal, there are no effect from the factor. This means that increasing the concentration of Zn almost does not increase the biomass of the aboveground part of the radish, since the line is almost horizontal.

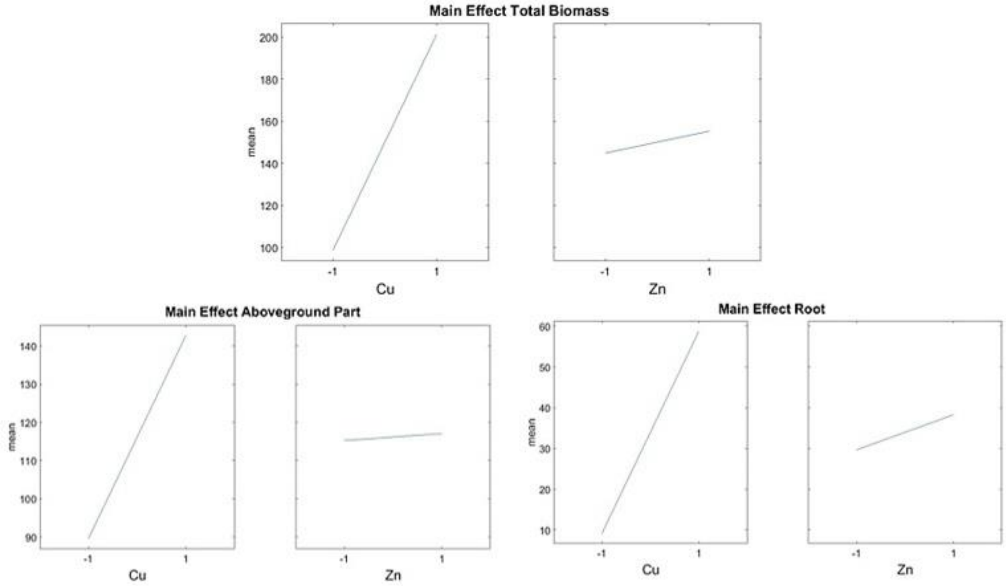

Figure 10-3: Main effect plots of Cu and Zn in soil on total, aboveground part, and root biomass of radish.

It is hereby confirmed that the application of Cu to soil increases the biomass of radish plants. There may be several factors explaining these observations. Cu is involved in plants growth as an enzyme activator and deficiency symptoms includes reduced growth (41). If Cu is not found in sufficient quantities, it might be a constraining factor by which the growth is reduced.

The interaction effects are the effect caused by an interaction between two or more factors. In this case, there are two main factors (A and B), which as previous described gives one possible interaction (AB). An interaction is present when the simple effects of one independent variable are not the same at all levels of the other independent variable. In other words, an interaction occurs when one factor affects the results differently depending on a second factor. The calculated interaction effect for total, aboveground and root biomass respectively, are seen in *Table 10-5*.

Table 10-5: Interaction effects for the three responses: total biomass, aboveground and root biomass.

	Total biomass	Aboveground part	Root
Interaction	**AB**	**AB**	**AB**
Effect	-10.84	-15.60	4.760

Negative interaction effects are seen in two of the three different responses. This means that some interaction between soil Cu and Zn decreases the effect of these on the total and aboveground part of radish biomass. Conversely, an AB interaction increases the effect of soil Cu and Zn on radish root biomass.

Interaction effects are typically visualised in interaction plots. The slope of the lines in these plots is used to tell about the interaction. If the lines are parallel, interaction effects are zero and there is no interaction between factors. The more different the slopes of the lines are, the more influence the interaction effect has on the response. If lines are not parallel and sign of slope of the lines is different, the interaction is antagonistic. If lines are not parallel and sign of slope of the lines is the same, the interaction is synergistic.

The interaction effects are visualised in *Figure 10-4*. In all interaction plots, the lines are not parallel and therefore there are interactions between the factors. In the interaction plot for *Total Biomass* it is seen that if the Cu are at high level, the increase of Zn from low to high will have no effect on the total biomass. If Cu is at low level, a change of Zn from low to high will increase the total biomass a little. Changing Cu from low to high will increase the total biomass a little more if Zn are at low level, because the effect are more steep than Zn at high level.

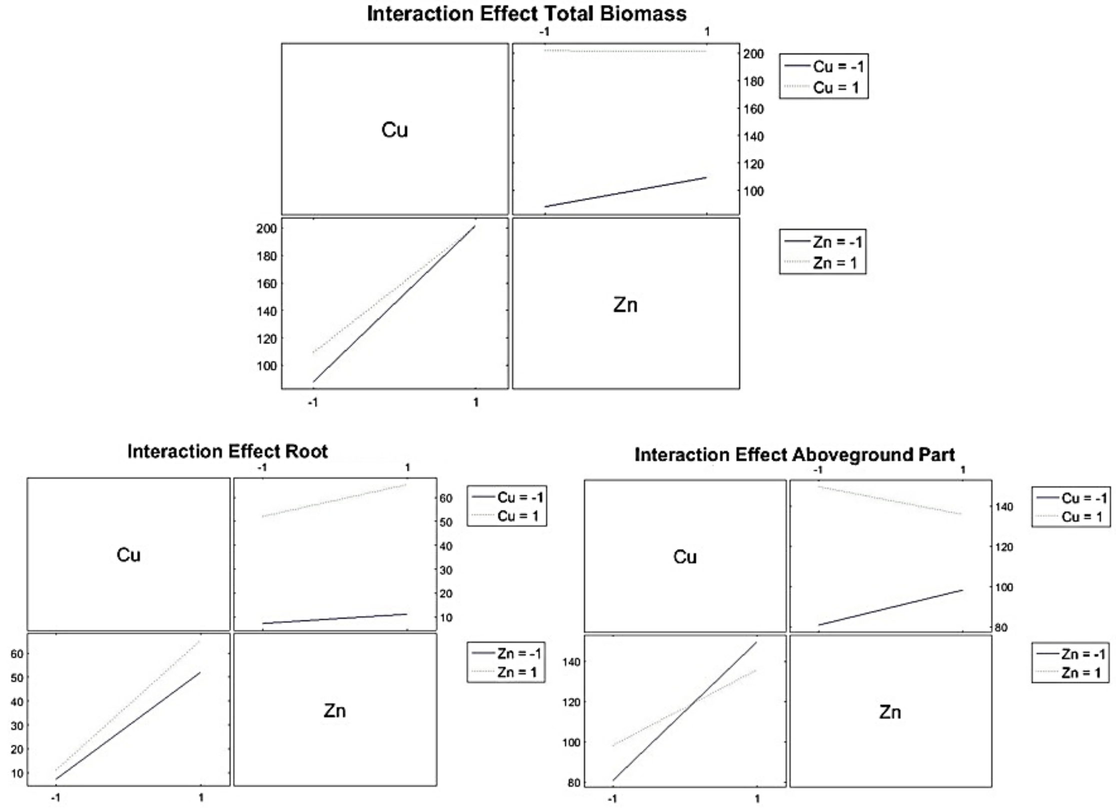

Figure 10-4: Interaction effects between Cu and Zn on total, aboveground part and root biomass of radish. The less parallel the lines are, the more likely there is to be a significant interaction

The plot of the interaction effect on root biomass of the radish shows that changing Cu from low to high has a greater effect when Zn is at high level. This is also the case when increasing Zn the effect is a little greater when Cu is at high level. The interaction plot of the biomass of the aboveground part of radish shows clearly interaction. Changing Cu from low to high has greater effect on the biomass when Zn is at low level. Changing Zn from low to high will increase the biomass if Cu is at low level, but decrease the biomass if Cu is at high level.

10.3.2 Cu and Zn in soil before seeding and after harvest

For the plant growth, the soil is a medium, which provides nutrients to the plants. The plants growing in the soil therefore take up some nutrients. The concentration of *Cu* and *Zn* in the soil is of most interest in this experiment since it is the controlled factors. The soil concentration of Cu and Zn are therefore analysed before seeding and after harvest. The measured soil concentrations of Cu and Zn in the different treatments are presented in *Figure 10-5*. The represented values are average on the three replicates of each treatment. As expected, there is a tendency that the Cu and Zn concentrations after harvest are slightly lower than before sowing. This is due to some metal uptake by the radish plants during growth.

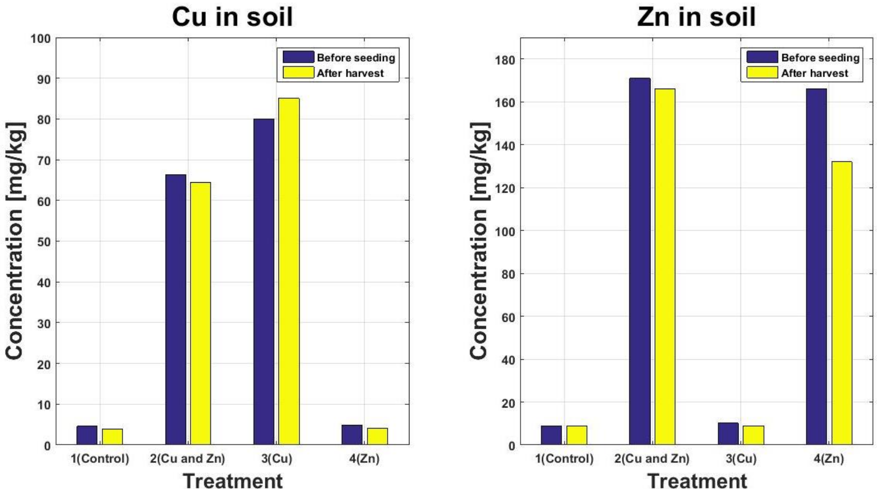

Figure 10-5: Concentration of Cu and Zn, respectively in soil before seeding and after harvest in the four treatments.

The measured soil Cu concentration in treatment 3 however seems to be higher after harvest of radish plants. This is however unlikely, since the concentration of Cu suddenly cannot increase. Instead, some measurement uncertainties as well as the soil sampling probably explain this. It is probably due to the soil preparation before seeding concerning the metal application and mixing as well as the sampling of the soil. It is not certain that the metals have been completely evenly distributed in the soil and, therefore, there are likely to be local high/low concentrations in the soil in the pots. This means that when the samples are collected, they will not be representative of the average concentration in the soil, but rather represent a local high/low concentration. In addition, the measured concentration depends on where the soil sample is collected compared to where the plant roots have taken up the nutrients.

Plant-available concentration of Cu and Zn

The amount of metals taken up from the soil by plants will depend upon a variety of factors affecting the plant-availability of metals. These factors include the soil properties, such as pH, clay and organic matter content, phosphorus content, cation exchange capacity (CEC), redox potential, microorganisms and plant type. Soil properties control CEC, specific adsorption, precipitation, and complexation, which are the main processes governing the partition of metals between the solid and solution phases of soil. (42,43)

The plant-available metals are mostly located on mineral surfaces and can be displaced by other cations. Therefore, a neutral salt extraction method can be used as a useful indication of metal plant-availability (40,44). This includes salt extractions such as 0.01 $CaCl_2$ or 0.1 M $NaNO_3$. The plant-available concentration of Cu and Zn in this experiment was determined by the 0.01 M $CaCl_2$-extraction method (40). The results are presented in *Figure 10-6*.

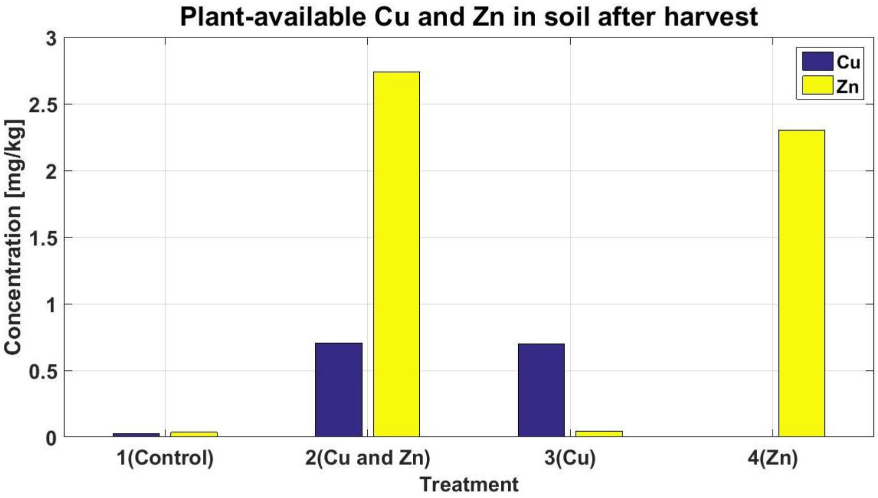

Figure 10-6: Plant-available concentration of Cu and Zn after harvest of radish in pot experiments.

The plant-available Cu and Zn concentrations where much lower than the total concentrations seen in *Figure 10-5*. The plant-available concentration however shows levels depending and corresponding the different metal treatments. The plant-available concentration of Cu in treatment 2 and 3 was almost identical. Contrary, the plant-available concentration of Zn in treatment 2 and 3 was a bit different. This might be explained by an interactive effect with added Cu, such that additional Cu increases the amount of plant-available Zn. Similar observations were made in (37).

The uptake of Cu and Zn is expected to correlate the concerned metal treatments. It is expected that the higher the soil concentration of Cu and Zn are, the higher the uptake of Cu and Zn in radish will be.

10.3.3 Uptake of Cu and Zn in radish

The uptake of Cu and Zn in radish plant as affected by different soil metal treatment with Cu and Zn were investigated in this study. The measured average concentrations in the radish root and aboveground part of the radish in the four different treatments are seen in *Figure 10-7*. In general, the Cu and Zn concentration in radish is higher in treatments with Cu and Zn, respectively. This indicates that increasing soil Cu and Zn concentrations, increases the uptake of Cu and Zn. It is seen, that the radish concentration of Zn in treatment 3 (Cu) seems to be lower than in the controls for both the root and aboveground part of the radish. This might indicate some interaction between Cu and Zn, in which Cu decreases the uptake of Zn.

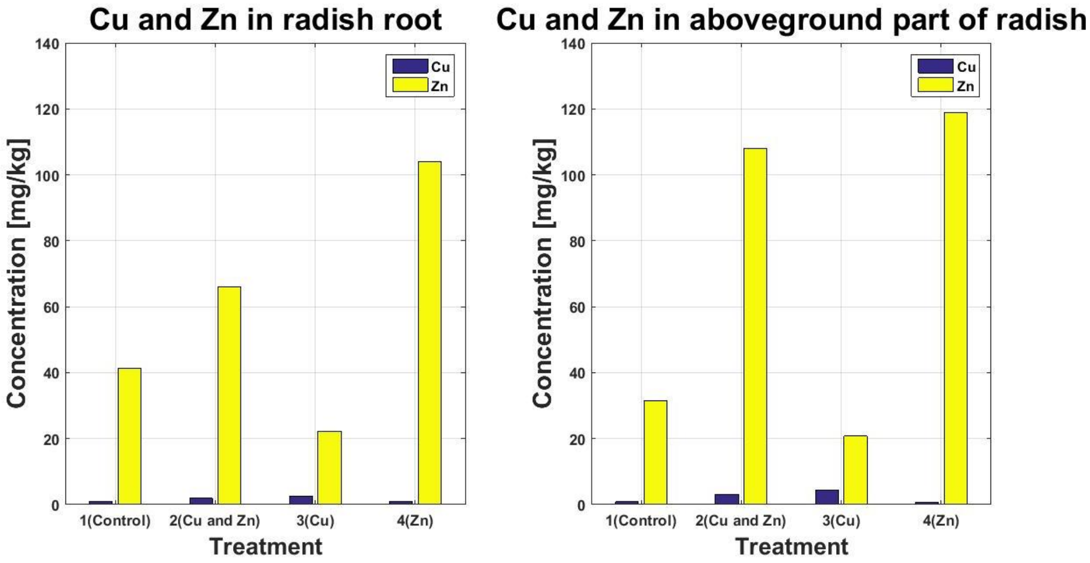

Figure 10-7: Cu and Zn concentrations in radish root and aboveground part of radish.

Similar to the section including the biomass, the response values (in this case the Cu and Zn concentration in radish) are investigated from a statistical point of view, by calculating the effects as seen in *Table 10-6*.

Table 10-6: Main effects of Cu and Zn in soil on Cu and Zn uptake in aboveground part and root biomass of radish.

	Cu uptake aboveground part		Cu uptake root		Zn uptake aboveground part		Zn uptake root	
Factor	**A: Cu**	**B: Zn**	**A: Cu**	**B: Zn**	**A: Cu**	**B: Zn**	**A: Cu**	**B: Zn**
Main Effect	3.05	-0.731	1.183	-0.257	-10.9	87.3	-28.4	53.3

It is seen, that the dominating factor on the Cu uptake in the aboveground part of the radish is factor A (the Cu concentration in the soil). Likewise the main effect on the Cu uptake in the radish root is Cu, however this effect is lower than the effect on the aboveground part of the radish. The Zn effect is negative, but very small. The dominating factor on the Zn uptake in both the aboveground part and root is by factor B (the Zn concentration in soil). The effect of Cu is of some importance as well, however the effect is negatively. These interactions are studied later in this section.

These main effects are illustrated in *Figure 10-8* as well. Here it is clearly seen that by increasing the concentration of Cu and Zn in the soil, the uptake of Cu and Zn, respectively in the radish are increased as well. In addition, it is seen that by increasing the soil concentration of Zn from low to high, the uptake of Cu decreases and vice versa.

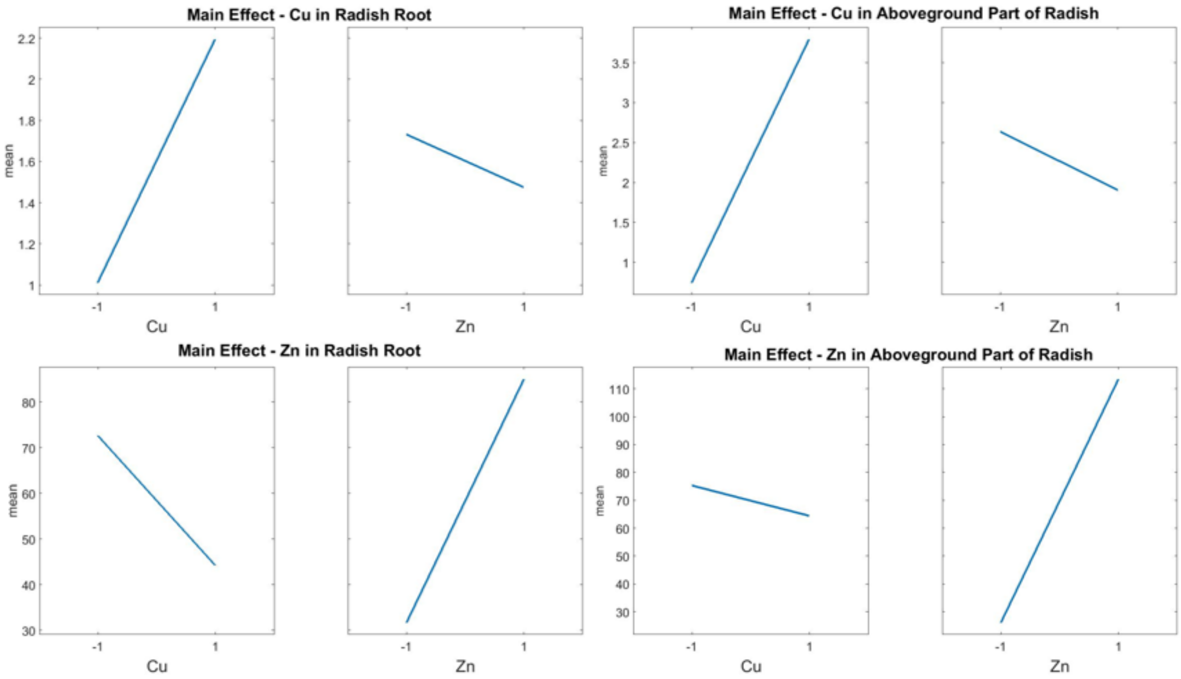

Figure 10-8: Main effect of Cu and Zn on uptake of Cu and Zn in the aboveground part and root of radish, respectively.

The interaction effects are seen in *Table 10-7* and the interaction plots are seen in *Figure 10-9*.

Table 10-7: Interaction effects of factor AB on the uptake of Cu and Zn in the aboveground part and root of radish, respectively.

	Cu uptake aboveground part	Cu uptake root	Zn uptake aboveground part	Zn uptake root
Interaction	**AB**	**AB**	**AB**	**AB**
Effect	-0.5195	-0.313	-0.100	-9.50

In general, the interaction effects are low and negative. The most dominating interaction is on the Zn uptake in the root. This effect is also negative, which means that some interactions between soil Cu and Zn decreases the uptake of Zn in the radish root. This means that changing the soil concentration of Cu from zero to high in some way decreases the uptake of Zn in radish plants. In general, some interactions are seen in the interaction plots in *Figure 10-9*.

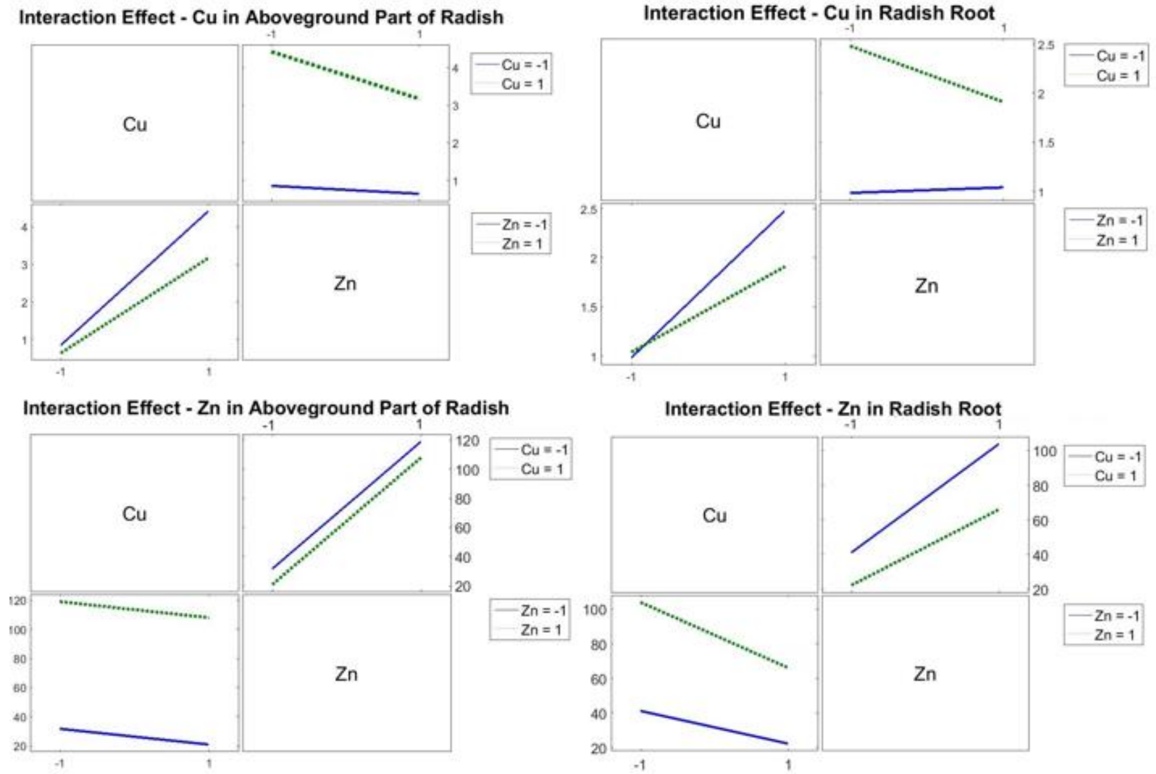

Figure 10-9: Interaction effects between Cu and Zn in soil on uptake of Cu and Zn in aboveground part and root of radish.

Interaction Effect – Cu in aboveground part of radish

There are some interaction since lines are not parallel. The effect of changing soil concentration of Cu from low to high is steeper with Zn at low level compared to high Zn level. This means that increasing soil concentrations of Zn decreases the uptake of Cu in radish**.** The decreasing effect of changing soil concentration of Zn on Cu uptake is more dominating at the high level of soil Cu seen by the steeper slope compared to low Cu level.

Interaction Effect – Cu in radish root

There are some interactions since lines are not parallel. The same interaction effects as on the Cu uptake in the *aboveground part* of the radish is observed here in the radish *root* concentration of Cu.

Interaction Effect – Zn in aboveground part of radish

No interactions are observed, since the lines are parallel. In this plot is it also seen, that Cu is not a main factor since the lines are almost horizontal and very close, respectively.

Interaction Effect – Zn in radish root

There are some interactions since lines are not parallel. The effect on Zn uptake of changing soil concentration of Cu from low to high is a little greater at high level of Zn than at low level of Zinc. The effect of changing Zn from low to high level has the greatest effect on the Zn uptake in

Cu at low level, since this line is a bit steeper than Cu at high level. The interactions are negative, this mean that the observed tendency in *Figure 10-7* is confirmed: Cu decreases the uptake of Zn in radish.

There are some uncertainties associated with the determination of the uptake of Cu and Zn in radish plants in terms of the concentration of Cu and Zn:

- If the radish plants are not washed carefully, Cu and Zn from the soil will affect the detected concentrations and hereby overestimate the uptake of Cu and Zn in the radish plants.
- If the digestion of the plant material is not complete, all metals from the plants are not dissolved in the solution and the uptake of Cu and Zn is underestimated. This is not expected to be the case though, since all plant material was destroyed after the utilised digestion procedure.

11 Accumulation of Cu and Zn in cress by application of pig manure

The aim of this study is to evaluate the uptake of Cu and Zn from a contaminated soil as well as the effect on this due to application of pig manure. The uptake of metals form a contaminated soil depends on the concentration and plant-availability of the metals as previously described. The bioavailability and the vertical movement of the metals in the soil profile are controlled by the soil properties, such as pH, organic matter, cation exchange capacity and many more. Application of manures to agricultural soils may affect the soil properties and thus the bioavailability of the metals. In this experiment an application of pig manure, containing Cu and Zn, on agricultural lands were simulated and the uptake of Cu and Zn was investigated. This time cress was used because it is a fast growing plant. Cu and Zn were used once again as factors along with pig manure in a three-factor full factorial experiment. Possible interactions between the uptake of metals and application of pig manure were hereby evaluated.

Reference is made to *Appendix G –Cu and Zn in cress by application of pig manure* for details about the procedure of the experiment as well as raw data results.

11.1 Design of experiment

A three-factor two-level (2^3) full factorial design was used to examine the growth and metal uptake of cress in a garden soil by application of pig manure, Cu and Zn. The factors and appertaining levels are seen in *Table 11-1*. Each factor consisted of two levels; zero as the low level and some appropriate value as the high level.

Table 11-1: The factors were application of pig manure, Cu and Zn with two levels each.

Factors	Levels	
	Low (-)	High (+)
A: Pig manure	0	172 g/pot
B: Cu	0	60 mg/kg
C: Zn	0	150 mg/kg

The same levels of Cu and Zn as in the radish experiment were used in this experiment too. In order to evaluate how much pig manure to be used in this experiment, the harmony rule is used since the maximum permitted amount of manure application is used. The harmony rule provided the maximum permitted amount of manures applied to fields. As previously described, the harmony rule states that maximum 140 kg nitrogen from a pig production may be applied per hectare. The content of nitrogen in the pig manure used in this experiment was determined to 1.739 kg/t. The maximum permitted amount applied to fields of this manure is therefore calculated in the following:

$$Maximum\ ton\ per\ hectare\colon \frac{140\ kg\ N\ per\ ha}{1.739\ kg/t} = 80.5\ t/ha$$

In order to calculate the amount of manure, which gives a realistic demonstration of the conditions on a real farmland reflected in the pot experiments the area of the soil surface in the pots are calculated to 214 cm^2. Reference is made to *Appendix G – Cu and Zn in cress by application of pig manure* for detailed calculations. In order to convert 80.5 ton/ha to units suited for the pot experiment the following calculation are made:

$$214\ cm^2 = 214\ cm^2 \cdot 10^{-8}\frac{ha}{cm^2} = 0.00000214\ ha$$

$$Amount\ per\ pot\ [t]{:}\ 80.5\frac{t}{ha} \cdot 0.00000214\ ha = 0.000172\ t$$

$$Amount\ per\ pot\ [g]{:}\ 0.000172\ t \cdot 10^6\frac{g}{t} = \underline{172\ g\ per\ pot}$$

Therefore the high level of factor A: Pig manure is 172 g, demonstrating the highest permitted amount of the utilised pig manure applied to agricultural fields. The pots receiving manure will be supplied with some Cu and Zn from the manure as well. In order to evaluate the effect of this, the average applied amounts are calculated. Pig manure was applied in the rate of 172 g with a dry matter content on 1.19 % and a Cu and Zn content on 149 and 640 mg/kg DM, respectively. The applied amounts per kg soil are therefore:

$$Applied\ DM\ [kg] = 1.19\% \cdot 172g \cdot 10^{-3}\frac{g}{kg} = 2.05 \cdot 10^{-3}\ kg\ DM$$

$$Applied\ Cu\ [mg] = 149\frac{mg\ Cu}{kg\ DM} \cdot 2.05 \cdot 10^{-3} kg\ DM = 0.305\ mg\ Cu$$

$$Applied\ Cu\ \left[\frac{mg}{kg\ soil}\right] = 0.305\ mg\ Cu \cdot 0.6\frac{kg\ soil}{pot} = 0.183\frac{mg\ Cu}{kg\ soil}$$

Similar the amount of applied Zn with pig manure are calculated to 0.786 mg Zn per kg soil. The individually effect of each factor and interaction effects on the response variable was tested in the full factorial design by combining the factors and levels as seen in *Table 11-2*. The experiment was carried out with a duplicate determination giving a total of 16 runs.

Table 11-2: Design matrix with two replicates for cress experiment with test of individually and interaction effects on the response variable.

Replicate	Run	Sequence	Factor A	Factor B	Factor C	Test of factor
1	1-1	2	–	–	–	0
	2-1	16	+	–	–	A
	3-1	4	–	+	–	B
	4-1	10	+	+	–	AB
	5-1	8	–	–	+	C
	6-1	5	+	–	+	AC
	7-1	13	–	+	+	BC
	8-1	6	+	+	+	ABC
2	1-2	12	–	–	–	0
	2-2	15	+	–	–	A
	3-2	14	–	+	–	B
	4-2	11	+	+	–	AB
	5-2	3	–	–	+	C
	6-2	1	+	–	+	AC
	7-2	7	–	+	+	BC
	8-2	9	+	+	+	ABC

The runs were tested in a completely randomised design by running the treatment combinations in a random order as previously described.

11.2 Material and methods

Pot experiments were carried out to examine the uptake of Cu and Zn in cress affected by the application of pig manure. As in the radish experiment the planting soil was used in this experiment too. Samples (600 g air-dried) of the soil were filled into plastic pots and the metal addition were made by adding appropriate amounts of Cu sulphate and Zn sulphate as powdered solids. In each pot, the soil was thoroughly mixed with the chemicals. The pig manure was added without any preparation as liquid by spreading on the soil surface. The soil was watered with tap water. Cress (*Lepidium sativum*) was sown at a rate of 5 g per pot. The pots were placed randomly in a greenhouse with no controlled climate. The pots were watered regularly with tap water to keep the soil humid.

The experiment was terminated 25 days after sowing. The cress was harvested and the soil was sampled at the same time. The plants were washed with tap water to remove any attached soil particles, and rinsed twice with deionized water. The plant samples were dried in a stove at 105°C to a constant weight. The Cu and Zn in the plant material were analysed as previously described in the preceding experiment.

The soil samples were dried at 105°C to a constant weight was obtained. The concentration of Cu and Zn in the dried soil samples were determined with ICP-OES as previous described.

11.3 Results and discussion

The cress were harvested 25 days after sowing. The plants in the four (eight in total) treatments with manure were as expected much larger than the treatments with no manure as seen in *Figure 11-1* due to the nutrient content in the manures.

Figure 11-1: Pot experiment with cress 25 days after sowing. The cress in the four pots not supplied with pig manure were distinctly smaller than the cress treated with pig manure in both replicates.

The biomass was not determined in this experiment, since the manures as expected had a positive effect on the growth of the cress. The most interesting thing in this experiment was instead the Cu and Zn concentrations in the soil and the corresponding uptake of those metals in the cress, which is presented in the following.

11.3.1Cu and Zn in soil

The concentrations of Cu and Zn in the soil before pot experiment were measured after application of those metals according to the experimental plan. The measured concentrations are seen in *Figure 11-2*.

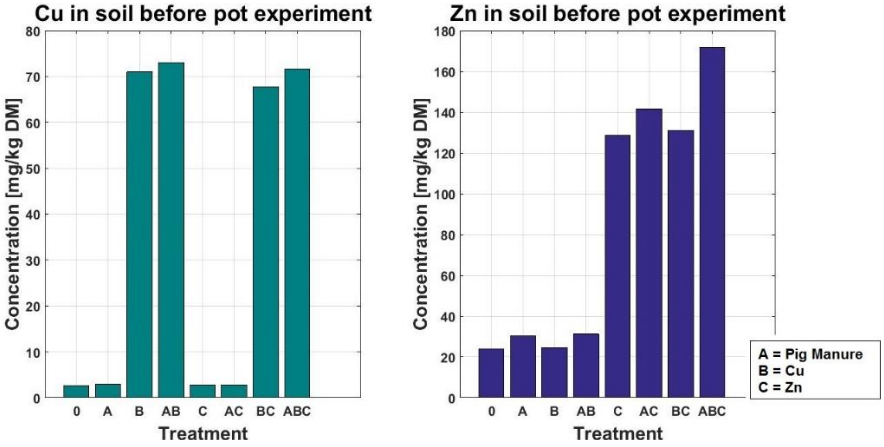

Figure 11-2: Concentration of Cu and Zn in soil before pot experiment with cress.

The measured concentration of Cu and Zn in the soils is consistent with the applied concentrations according to the experimental plan of the full factorial design. However, some variations exist. This might as discussed in the radish experiment, be explained by the sampling and preparation of the soils.

11.3.2 Uptake of Cu and Zn in cress

The uptake of Cu and Zn in cress after pot experiments are presented in *Figure 11-3* as the Cu and Zn concentration in the cress. The y-axis is different in the figure since the Cu content are lower than Zn.

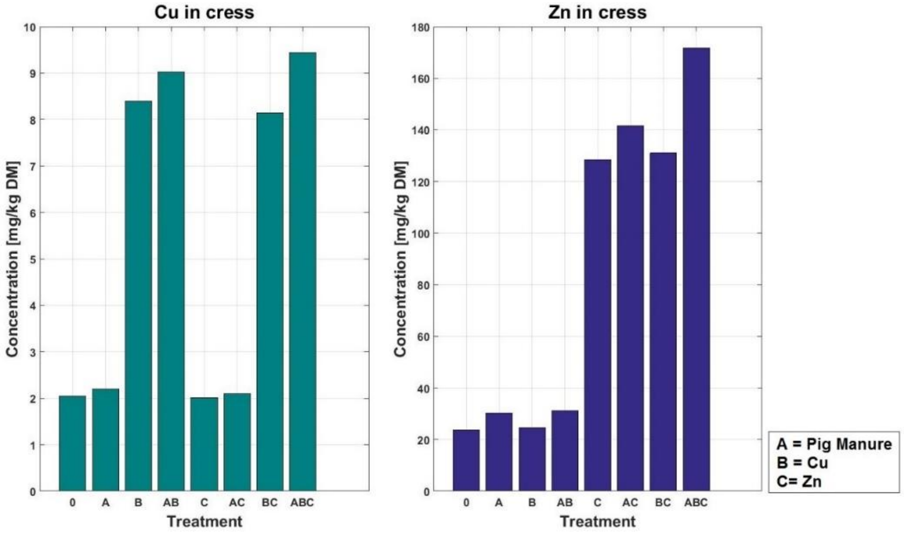

Figure 11-3: Cu and Zn concentration in cress after pot experiment with different treatments. NB the y-axis are different since the Cu content are lower than Zn.

In the treatments with factor B (Cu) the Cu concentration are highest. It seems like factor B (Pig manure) in the treatments with both factor A and B increases the Cu uptake, since the Cu content are higher in treatment AB and ABC compared to treatment B and BC. The higher Cu content in treatment ABC might be explained by factor C (Zn) also. Similar, the concentration of Zn are highest in the treatments with factor C (Zn). Here the same tendency is observed; the Zn content in cress seems to be higher in treatments with both factor A and C.

The uptakes of Cu and Zn in the cress are evaluated in the factorial design with the concentration of Cu and Zn in the cress as the response. The calculated main effects of the three factors are seen in *Table 11-3*. It is seen that the dominating factor on the Cu and Zn uptake as expected are Cu and Zn application to the soil respectively. In addition, it seems that the application of pig manure increases the uptake of Zn in cress as well.

Table 11-3: Main effect of application of pig manure, Cu and Zn to the soil on Cu and Zn uptake in cress.

	Cu uptake in cress			Zn uptake in cress		
Factor	**A: Pig Manure**	**B: Cu**	**C: Zn**	**A: Pig Manure**	**B: Cu**	**C: Zn**
Main Effect	0.550	6.66	-0.005	16.7	8.65	115

These facts are observed in the visual presentation of the main effect in the main effect plots seen in *Figure 11-4*. Here a steep slope on the lines for the Cu and Zn are observed indicating that these factors increase the Cu and Zn uptake respectively in the cress significant. In addition the application of pig manure affects the Cu and mostly the Zn uptake in some may.

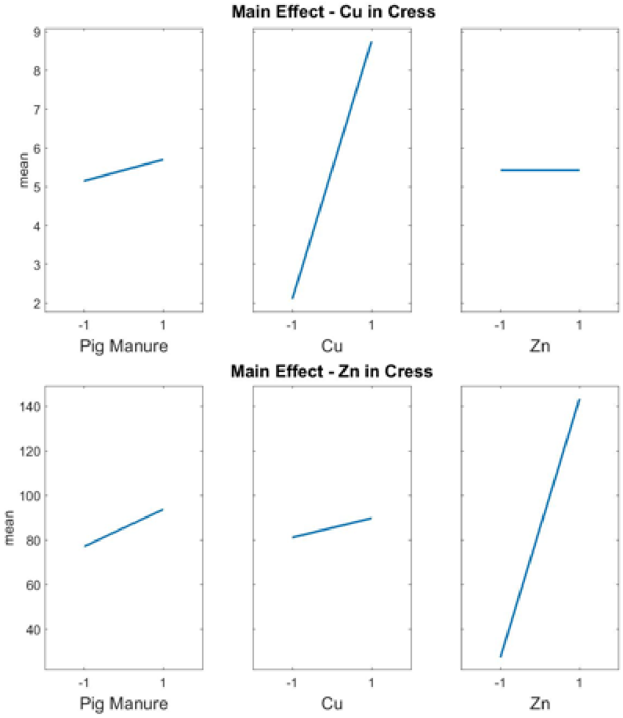

Figure 11-4: Main effect of application of pig manure, Cu and Zn to soil on Cu and Zn uptake in cress.

The interaction effects are calculated and seen in *Table 11-4*. This time there is four possible interactions; three two-factor interactions AB, AC, BC and one three-factor interaction ABC.

Table 11-4: Interaction effect of application of pig manure, Cu and Zn to soil on Cu and Zn uptake in cress.

	Cu uptake in cress				Zn uptake in cress			
Interaction	**AB**	**AC**	**BC**	**ABC**	**AB**	**AC**	**BC**	**ABC**
Effect	0.415	0.145	0.080	0.190	6.90	10.2	7.75	6.90

The interactions plots are presented in *Figure 11-5* and *Figure 11-6*. Again, the less parallel the lines are, the more likely there is to be a significant interaction. In the interaction plots of the Cu uptake in cress, several of the lines are parallel though and thus there is no interaction. This is consistent with the calculated low values in *Table 11-4*. However, some interactions are observed between Pig manure and Cu since these lines are not parallel. The effect on the Cu uptake in cress by increasing soil Cu to high level is more dominating by application of pig manure (high level).

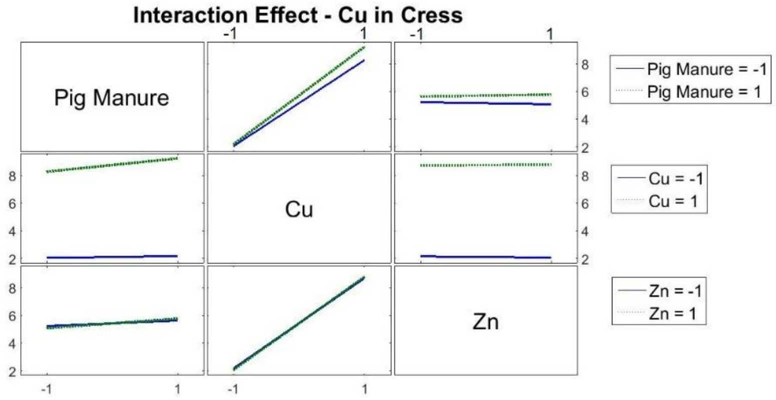

Figure 11-5: Interaction effects by application of pig manure, Cu and Zn to soil on Cu uptake in cress.

The calculated interactions effects on the Zn uptake in Cress in *Table 11-4* are in general higher than the effect on the Cu uptake. This is confirmed by the less parallel lines seen in *Figure 11-6* compared to *Figure 11-5*.

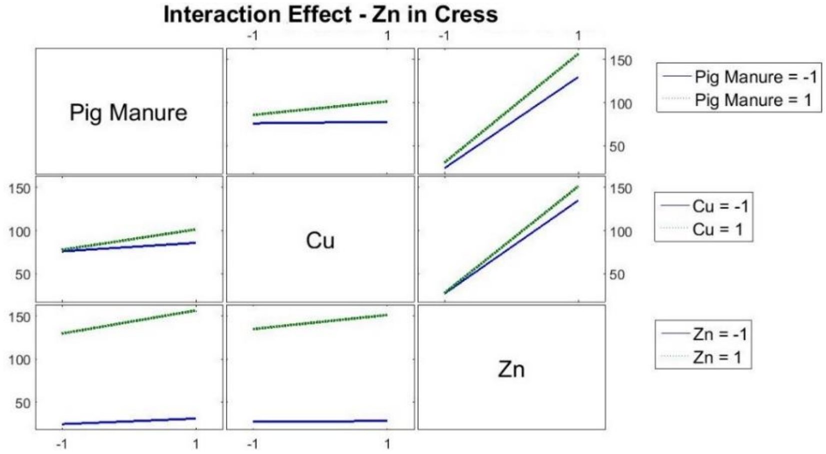

Figure 11-6: Interaction effects by application of pig manure, Cu and Zn to soil on Zn uptake in cress.

Some positive interaction are seen between soil Zn and both Cu and pig manure, since the increasing effect of soil application of Zn on the Zn uptake in cress is increased at the high levels of Cu and pig manure, respectively.

This pot experiment thus have shown some interactions by the application of pig manure to Cu and Zn containing soils that increases the uptake of those metals in cress compared to control treatments with no manure. This indicates that the manures influences soil properties responsible for the plant-availability of the metals and hereby increases the uptake of metals from the soil.

The effects of manure application on the functionality of the soil are investigated in previous studies as well (45). They have shown that the application of Cu-poor pig manure to a Cu-containing soil caused a dramatic increase of the plant-availability of Cu. Interrelated chemical and biological analyses proved that this effect was due to both an increase in the amount of soluble Cu, as well as an increased bioavailability of the dissolved Cu. The results suggest that the easily degradable organic material from the slurry forms soluble complexes with Cu, and that these complexes are plant-available and consequently toxic. Cu being applied to the soil together with the manure can therefore be expected to be more toxic than Cu supplied as a simple Cu-salt.

The same factors as discussed in the radish experiment might affect the results and over or underestimate the effects of application of Cu, Zn and pig manure.

12 Accumulation of Cu and Zn in cress by application of pig vs. cattle manure

Prolonged and/or excessive use of animal manure as organic fertiliser can result in the accumulation of Cu and Zn in the soil as previously described. Consequently, the soil properties might change due to changed microbial activity and the phyto-toxicity to plants may increase. Additionally, the uptake of Cu and Zn may increase as well. Therefore, the aim of this study was to simulate accumulation of Cu and Zn in soil due to prolonged use of animal manures and as a consequence the uptake of Cu and Zn in plants. The effect on the uptake of Cu and Zn in cress due to the application of pig manure was compared to the effect from application of cattle manure, both compared to a control treatment with no manures.

Pot experiments were carried out to examine the uptake of Cu and Zn in cress from contaminated soils. Soils with increasing concentrations of Cu and Zn were made to simulate the accumulation due to prolonged use of animal manure. The contaminated soils were supplied with pig and cattle manure, respectively compared with a control not receiving manure. Hereby the effect from the manures on the uptake of Cu and Zn was evaluated.

Reference is made to *Appendix H – Pig manure vs. cattle manure* for details about the procedure of the experiment as well as raw data results.

12.1 Experimental plan

The concentrations of Cu and Zn used for the experiment are seen in *Table 12-1*. Increasing concentrations were used in order to simulate metal accumulation in soil due to prolonged application of pig manure and cattle manure. Pots with a control treatment not added any manures but with the same amount of metals in the soil was compared to pots supplied with pig manure and cattle manure, respectively.

Table 12-1: Cu and Zn concentrations mixed in the soils for pot experiments.

Pot	Cu [mg/kg]	Zn [mg/kg]
Control 1	25	75
Control 2	75	175
Control 3	100	225
Pig manure 1	25	75
Pig manure 2	75	175
Pig manure 3	100	225
Cattle manure 1	25	75
Cattle manure 2	75	175
Cattle manure 3	100	225

The experiment was carried out with duplicate determination, giving 18 pots in total. The background concentration of Cu and Zn from the planting soil and the watering water will be the same in all pots. The application rate of pig manure was the same as in the previous experiment: 172 g per pot. The application rate of cattle manure was calculated in the same way from the content of nitrogen and the harmony rule to 125 g per pot. Reference is made to *Appendix H – Pig manure vs. cattle manure* for detailed calculations.

The pots receiving manure will be supplied with some Cu and Zn from the manures as well, as described in the previous experiment. The applied amounts of Cu and Zn from the pig manure were in the previous experiment calculated to 0.183 mg Cu and 0.786 mg Zn per kg soil, respectively. The amounts of applied Cu and Zn with the cattle manure are calculated to 0.393 mg Cu and 0.403 mg Zn per kg soil, respectively. The largest percentage extra applied Cu and Zn in treatments with manure compared to the control are therefore; In treatment *Pig manure 1* the soil concentration of Zn are increased with 1.0 % compared to *Control 1*. Likewise, in treatment *Cattle manure 1* the soil concentration of Cu are increased with 1.6 % compared to *Control 1*. Based on these calculations it is assumed that a possible increased uptake of Cu and Zn in the manure treatments will not be due to the extra added amount of Cu and Zn from the manures, but instead effects of the slurry on the soil properties.

12.2 Material and methods

Samples (600 g air-dried) of the soil were filled into plastic pots and the metal addition were made by adding appropriate amounts of Cu sulphate and Zn sulphate as powdered solids. In each pot the soil was thoroughly mixed with the chemicals. The manures were added without any preparation as liquid by spreading on the soil surface. The soil was watered with tap water. Cress (*Lepidium sativum*) was sown at a rate of 5 g per pot. The pots were placed randomly in a greenhouse with no controlled climate. The pots were watered regularly with tap water to keep the soil humid. The experiment was terminated 25 days after sowing. The cress was harvested and the soil was sampled at the same time. The plants were washed with tap water to remove any attached soil particles, and rinsed twice with deionized water. The plant samples were dried in a stove at 105°C to a constant weight. The Cu and Zn in the plant material were analysed as previously described by ICP-OES. The soil samples were dried at 105°C to a constant weight was obtained. The concentration of Cu and Zn in the dried soil samples were determined with ICP-OES as previous described.

12.3 Results and discussion

The pot experiment with cress 25 days after sowing are seen in *Figure 12-1*.

Figure 12-1: Pot experiment with cress 25 days after sowing. Three controls not supplied with manure and three pots supplied with pig manure and cattle manure, respectively made in duplicate are seen.

The plants with control treatment were as expected distinctly smaller than the pig manure and cattle manure treatments due to the nutrient content in the manures. Significant differences between the treatment with pig and cattle manures were not observed.

12.3.1 Cu and Zn in soil after harvest

The concentrations of Cu and Zn in the soils are determined after the pot experiment and after harvest of the cress. The results are presented in *Figure 12-2*.

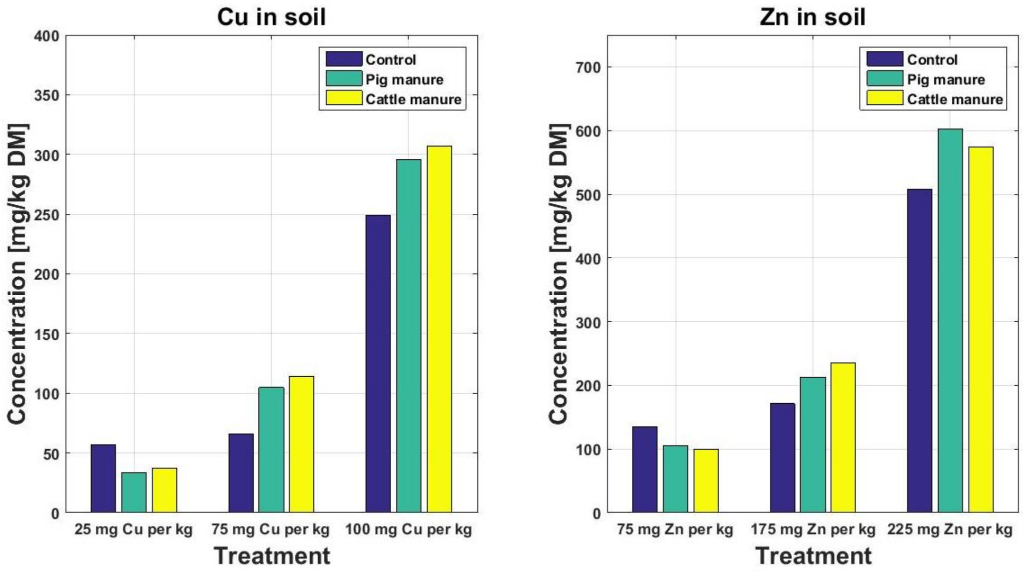

Figure 12-2: Concentration of Cu and Zn in soil after pot experiment with cress.

The different application of Cu and Zn was reflected clearly in the results. In general, the measured concentrations of Cu and Zn in the soil after pot experiments however were a bit high in relation to the applied concentration. In all treatments, abundant amounts of Cu and Zn were left in the soils after harvest of the cress. As previously mentioned the amount of metals taken up by plants will depend on numerous factors. It will certainly depend on the amount of plant-available metals, which is depended on the soil properties mentioned earlier. In addition, the uptake depend on the type of plants, since some plants are known to take up high concentrations of some metals, while others only accumulates small amounts.

12.3.2 Uptake of Cu and Zn in cress

The concentrations of Cu and Zn in the cress are seen in *Figure 12-3*. In general, it is seen that the uptake of Cu and Zn as expected are increased with increasing soil concentration. Especially for Zn there is not observed great differences in treatment 1 and 2, though. This may however be due to some measurement uncertainty or errors in the preparation of the soil.

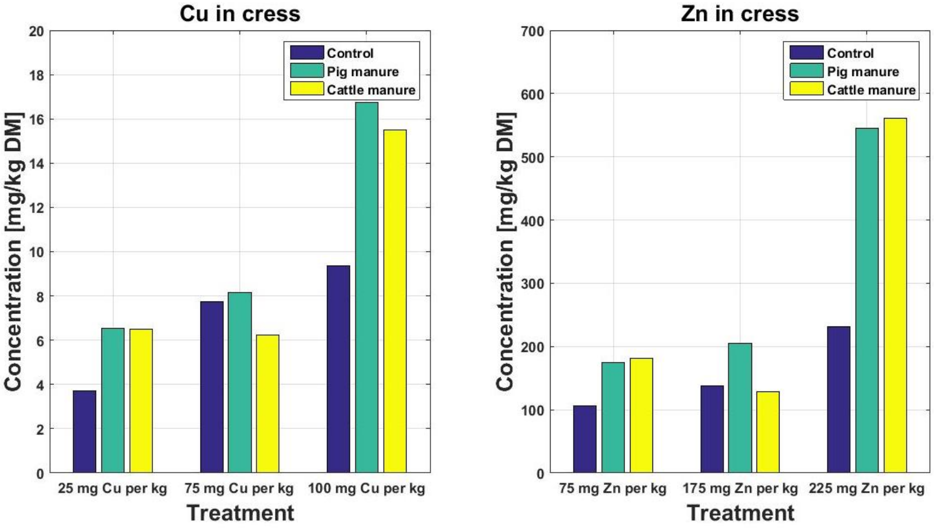

Figure 12-3: Concentration of Cu and Zn in the different treatments.

It is seen, that the uptake of both Cu and Zn are increased significantly by the application of manures especially in treatment 3. The Cu concentration in treatment 3 with pig manure and cattle manure was 79 and 66% higher than the control treatment, respectively. Similar, the Zn concentrations was 136 and 143% higher in treatments with pig manure and cattle manure, respectively. This clearly indicates that the manures influences soil properties responsible for the plant-availability of the metals and hereby increases the uptake of metals from the soil. Thus, observations from the previous experiment and previous studies were clearly confirmed.

A total characterisation of the soil properties by chemical and biological analyses should have been carried out in order to evaluate eventual changes in the soil corresponding changes in the availability of the metals as affected by the application of manures. However, this is very extensive, and was therefore not carried out in this project. One of the factors that might be affected by the manure application and is crucial for the plant-availability of the metals is the cation exchange capacity. This soil property is described and determined in the soils used in this project in the following chapter.

13 Cation Exchange Capacity (CEC) in soil

CEC is a very important soil property influencing the nutrient availability and it is therefore a useful indicator of soil fertility because it shows the soil's ability to supply three important plant nutrients: calcium (Ca^{2+}), magnesium (Mg^{2+}) and potassium (K^{+}). In a soil, clay minerals and organic particles typically will have a negatively charge. Hereby positively charged ions (cations) are attracted on their surfaces by ion adsorption and the negatively charge are hereby neutralised. As a result, the cations remain within the soil root zone and are not easily lost by leaching which is the case in sandy soils. The adsorbed cations may easily exchange with other cations in the soil solution, hence the term "cation exchange." As plant roots take up adsorbed cations, other cations in the soil water replace them. (46,47)

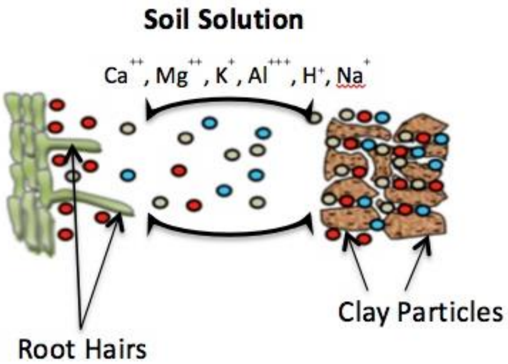

Figure 13-1: Exchange of cations between the soil surfaces and the soil solution, and the movement of these cations from soil solution to roots (rhizosphere) for uptake.(46)

Figure 13-1 illustrates cations retained on soil clay minerals that can exchange with those in the soil solution. The nutrients are moving away from the clay particles as plant roots remove nutrients from the soil solution. Addition of fertiliser/manure to soil causes an initial increase in nutrient concentration in the soil solution, which results in nutrients moving toward clay particles. Other cations adsorbed on exchange sites are sodium (Na^{+}), aluminium (Al^{3+}) and hydrogen (H^{+}). Heavy metals such as copper (Cu^{2+}) and Zn (Zn^{2+}) might be absorbed as well, and the CEC also influences the metal uptake. Soils with higher CEC's have greater ability to adsorb cationic heavy metals (46,47). Opinion is divided on whether a high CEC of the soil increases the root CEC and thus the uptake of heavy metals as well (48), or contrary causes a decrease in the uptake of heavy metals by plants (49).

Reference is made to *Appendix I – Cation Exchange Capacity* for details about the procedure as well as raw data results.

13.1 Material and methods

The cation exchange capacity is normally determined by leaching the soil with a neutral salt solution (47). In this case, the soils samples are saturated with barium by treatment with 0.5 M barium chloride solution by shaking and stand overnight. The samples are centrifuged and the supernatant are discarded. The washing procedure includes washing with water by shaking for 2-3 minutes followed by centrifuging and discards of the supernatant. The displacement procedure includes addition of 0.025 M magnesium sulphate solution and shaking for 2 hours. The excess (i.e. non-adsorbed) magnesium is determined by ICP-OES and related to the CEC. The efficiency of displacement of barium by magnesium in the magnesium sulphate treatment is increased by the precipitation of barium sulphate so achieving a *compulsive exchange*.

13.2 Results and discussion

The concentration of magnesium in the residual solution from the displacement procedure was determined by ICP-OES and converted to milliequivalents per unit mass (meq/kg soil) as described in *Appendix I – Cation Exchange Capacity*. The CEC was determined in soil samples from the three sites at the two agricultural soils, as well as in the forest and planting soil. The results are presented in *Figure 13-2*.

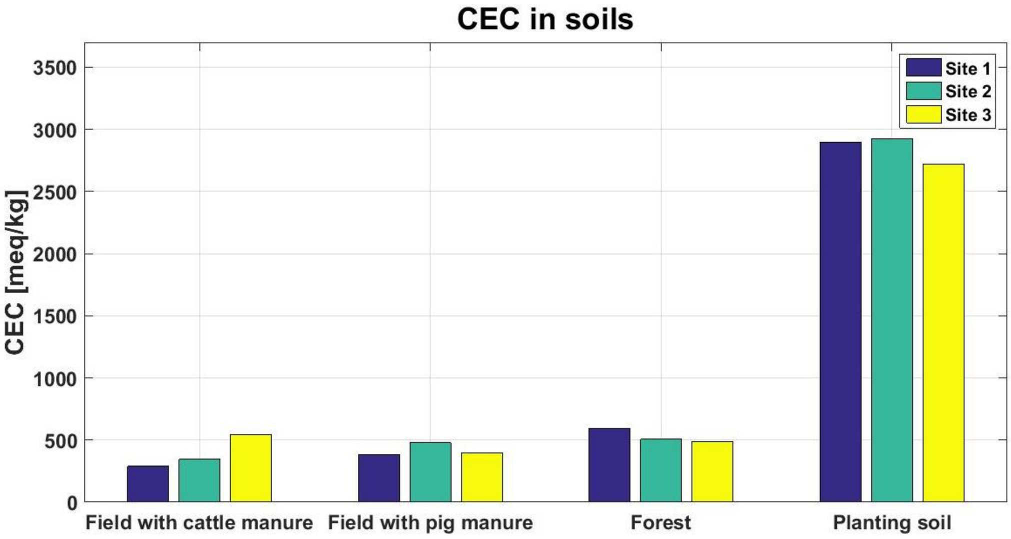

Figure 13-2: Cation Exchange Capacity [meq/kg] in the different soils investigated in this project.

From the figure, it is clearly seen that the CEC in the planting soil was much higher than the agricultural soils and the forest soil. The determined CEC in the agricultural soils and the forest soils looks very similar. The high CEC in the planting soil means that more heavy metals are adsorbed in this soil. This is probably due to the high content of organic materials, since it increases the CEC through an increase in available negative charges.

If the plant experiments had been performed with soil from the agricultural fields instead of planting soil, the results (uptake of Cu and Zn) probably would have been different. In order to extrapolate the results obtained in the planting soil, the fact of whether a high soil CEC increases the uptake of metals or not should be considered further. A soil with high CEC is likely to increase the plant uptake of metals in the case that rainwater seeps through the soil (which might be the case in all fields in the nature) and in a soil with low CEC would cause a leaching of the metals. Conversely, it is debatable whether soils with high CEC will keep the metals so strongly adsorbed that plants will not be able to absorb them. Generally seen, the uptake of metals form the soil will depend on the concentration of the metals as well as the plant-availability, which as previously described will depend on several other factors.

In the previous plant experiments, the plants were grown in closed pots (with a bottom). The soil layer has probably not been high enough, so that the applied watering water might be thought to make the metals inaccessible by leaching. If it is assumed that a high CEC will keep metals stronger bound, the obtained results (uptake of cu and Zn) might be higher if the plant experiments were carried out with the agricultural soils, in which the CEC is lower and metals therefore are not adsorb so strongly. If the plant experiment had been carried out in an open countryside, Cu and Zn in the agricultural soils with low CEC probably in a greater extent would have been removed by leaching and hereby causing a lower uptake of Cu and Cn compared to the uptake observed in the planting soil.

Evaluating the effect of a high CEC in a wider perspective according contaminated soil depends on the location of the field and if the field are cultivated for animal/human consuming. A high CEC will be desirable if the contaminated soils are located nearby places where the ground water is recovered. The high soil CEC will minimise the leaching of metals to the ground water. The metal concentration will therefore be higher in soils with high CEC. This might be a problem if the fields are cultivated with crops for consumption, since they might take up high concentrations of the metals, which hereby ends up in the food chain. If the plants is not be used for human consumption, certain plants able to absorb large amounts of metals could be used to decontaminate contaminated sites (phytoremediation). As previously mentioned several factors affect the CEC and the metals uptake. For instance soil pH is important for CEC because as pH increases, the number of negative charges on the colloids increase, thereby increasing CEC. Therefore, these factors should also be included in the discussion above.

14 General discussion

The well-document positive effects of high levels of dietary Cu and Zn in post weaning diets on weight gain, feed intake and diarrhoea entails a high consumption of these heavy metals in the pig industry. The use is regulated by norms and legislation provides a limit on the use. Not all farmers follow these rules though. An investigation by the Food Administration in 2007-2011 aimed to investigate the extent of exceedance of the maximum permitted content of Cu and Zn in feed. The study showed an excess of allowable maximum levels of copper and zinc in the complete feed at 15 and 20%, respectively (50). It is therefore debatable whether there should be restricted control of this.

Due to the use of Cu and Zn as growth-promoters, some pig manures contains very high concentrations of heavy metals and accordingly because the positive effect of Cu and Zn mainly takes place in the intestine, only a small part is absorbed in the pig body. Consequently large amounts of Cu and Zn pass through the pig's body and ends up in the manure. By the application of manure as organic fertilisers, Cu and Zn thus are accumulated in the agricultural soils.

Plants have an essential need for certain metals (such as Cu and Zn) and therefore these metals are applied for optimal growth of crops on agricultural fields. The problem arises as the application exceeds the plants' need and thus accumulates in the soil. In order to demonstrate what happens when metals are applied to soils in concentrations that exceed the needs of the plants some plant experiment was carried out. The experiments showed that the metal content in the plants clearly increased when the plants were growing in Cu- and Zn-containing soils compared to control treatments with low metal concentrations. This demonstration was carried out in small pots and therefore the results of the pot experiments probably might be different from field trials, and further study in field (open nature) should be made in order to evaluate the environmental impact of manure application to agricultural fields further. In open nature fields, the mobilisation of metals by leaching will occur and thus affect the uptake.

Heavy metals' tendency to accumulate in plants also depends on the type of plant. In the plant experiments, radishes and cress were used. The Cu and Zn concentration in radish plants after pot experiment were 4.41 and 119 mg per kg dry matter, respectively, while the Cu and Zn concentration in cress were as high as 8.14 and 131 mg per kg dry matter, respectively. The Cu uptake was clearly different since it was 85% higher in the cress even though it was growing in the same type of soil. In order to estimate the uptake of heavy metals in agricultural crops such as grain and corn from contaminated soils, further investigation must be carried out.

Further experiments were made in this project, investigating the effect on the uptake of heavy metals by application of manures. Here, it was demonstrated that the slurry clearly affected the soil properties and hereby increased the uptake of Cu and Cn compared to control treatments. In order to evaluate how the manure affects the soil properties several chemicals and biological investigations of the soil characteristic should have been made. However, it is well known that factors such as pH, CEC and content of organic matter affects the plant availability by changing the mobility of metals in the soil. The total concentration of metals in the soil is not an expression of the impact. Knowing the dynamics of the metals in soils is therefore essential for assessing the environmental impact caused by the use of manure, since the extent of this impact is directly related to the ability of soils to retain these metals. Moreover, it is conceivable that the microbiological processes in the soil will be affected. This might affect the plant growth, as several of these processes affect the availability of nutrients in the soil.

In scientific studies a certain amount of samples are required in order to conclude that something is of statistical confidence. In addition, all analyses should have been carried out in triple determination in order to identify uncertainties and errors occurred during the analysis. In some parts of this project, conclusions are based on only a few samples and duplicates only, otherwise it would have been very time consuming. ICP-OES is used for a large part of the analyses, and it was seen that some uncertainties were associated with some of the results. To validate the methods used in the project for instance, some samples could have been spiked with a known concentration to estimate the recovery percentage. In order to evaluate the digestion procedure of the different matrices a certified reference materials with known metal content could have been used to check whether the method were sufficient of dissolving all metals.

It is not only the above-mentioned effects, which can cause problems due to the high consumption of particularly Cu. The whole concept is to replace the antibiotic growth-promoters in the struggle to reduce multi-resistant bacteria. However, studies have shown that increasing concentrations of Cu in soils increases the resistance of Cu in the soil bacteria, and at the same time, these bacteria were more resistant to a variety of antibiotics as well (45). Thus, the increase of antibiotic-resistant bacteria is promoted by the application of heavy metals.

In addition, it should be pointed out that there is a need for studies of the pigs' physiological needs for Cu and Zn and bio-availability of nutrients in the feed in order to reduce the content of the manure. The animal welfare should also be included in the evaluation of this problem. Maybe the whole problem is solved by letting the piglets die longer time. However, it is constantly a question of money and profits to the farmer. This makes the finding of the solution on the problem mush more difficult to find than it seems.

15 Conclusion

In order to evaluate the environmental impact by the use of Cu and Zn as growth-promoters some laboratory work was carried out in which the following results was obtained:

Heavy metals in human urine
According some previously studies, heavy metal concentration of toxic heavy metals such as cadmium in human urine was measured as high as 1.2 mg/L. In this study urine from a limited group of persons at different ages were analysed by ICP-OES. However, no remarkable concentrations were detected.

Heavy metals in foodstuff
A screening of the metal content in some foodstuffs such as fish and meats were carried out in this study. The essential metals iron and zinc, on average, were found at concentrations of 44 and 50 mg per kg. The toxic metals, aluminum, lead and nickel were detected too. Of these, aluminum was detected in the highest concentration of 9 mg per kg in canned tuna fish.

Characterisation of manure
A limited number of some samples of pig and cattle manure was collected from local farmers in Southern Jutland. The NPK nutrient content in the cattle manure was determined to 2.41, 0.31 and 1.44 kg per tonne, respectively. Similar the NPK content in the pig manure was determined to 1.74, 0.24 and 2.23 kg per tonne, respectively. The copper and zinc concentration in the cattle manure was 185 and 190 mg per kg dry manure, respectively and 149 mg Cu and 640 mg Zn per kg was found in the dried pig manure.

Characterisation of agricultural and forest soils
Some agricultural soils were screened for the content of heavy metals with special emphasis on copper and zinc. The concentration of copper and zinc in fields applied pig and cattle manure was determined to 8.3 mg Cu and 25 mg Zn per kg soil, and 2.5 mg Cu and 11 mg Zn per kg soil, respectively. In addition, the concentration in a reference soil in terms of a forest soil was determined to 2.6 mg Cu and 5.1 mg Zn per kg soil. The surveys evaluated in the theoretical study, claiming that the use of manures as fertilisers increases the concentration of copper and zinc in the soils was by the limited screening in this study hereby confirmed.

Radish experiment
Increasing the soil concentration of Cu and Zn increases the uptake of Cu and Zn in radish plants. The highest concentration of Cu in radish plants grown in soils applied Cu at a rate of 60 mg per kg soil was determined to7.37 mg per kg in aboveground part of radish. Similar the highest concentration of Zn in soils applied Zn at a rate of 150 mg per kg soil was 79.3 mg per kg in aboveground part of radish too. In addition, the full factorial experiment indicated that increasing soil concentrations of Zn decrease the uptake of Cu in radish plants, and vice versa: Cu decreases the uptake of Zn.

Accumulation of Cu and Zn in cress by application of pig manure

Some positive interactions were demonstrated in the experiment by applying pig manure to Cu- and Zn-containing soils compared to control treatments with no manure. This indicated that the manures influences soil properties responsible for the plant-availability of the metals and hereby increases the uptake of metals from the soil.

Accumulation of Cu and Zn in cress by application of pig vs. cattle manure

In comparison with controls, treatments with pig and cattle manures in Cu and Zn containing soils clearly increased the concentration of Cu and Zn in cress plant tissue. The experiment demonstrated an increased Cu and Zn uptake on 79 and 136% respectively by application of pig manure. Similar the Cu and Zn uptake was increased with 66 and 143 % respectively by application of cattle manure.

16 Further work

This study and laboratory work only covers a small part of the whole problem concerning the high consumption of copper and zinc in the pig industry. Only a limiting amount of research and experiments has been carried out during the time limiting project period. As a continuation of this project, several aspects could be investigated in more details or from an extended point of view of which a few is discussed in the following. Based on this thesis, it is suggested that future research, including both scientific as well as more practical studies should focus on the following:

- The studies from 1986 and 1998 should be followed-up. More soil samples than the few that has been studied in this project should be collected and analysed for the content of heavy metals in all across the country, like the previous studies. The consumption of copper and zinc until today should be compared with the 12-year period to see whether the consumption has increased significantly.

- Similar an additional number of manure samples should be analysed in order to evaluate the present concentrations of heavy metals in different livestock across the entire country. The farmer's feeding habits and the like should be involved in the investigation, because as mentioned earlier, it is of great importance for the composition of the manures.

- Some copper and zinc were detected in the cattle manure as well. Therefore, feed crops that are grown in fields, which have been applied manure, should be investigated for the content of those metals. In this way, the actual uptake of heavy metals in crops in open nature is evaluated as well.

- This thesis strongly suggest that the uptake of heavy metals is highly increased by the manure application. Therefore, soils should be characterised before and after the application of manures in order to evaluate which properties that are affected by the manure application. In addition, experiments in which the soil-characteristics as pH, redox potential, organic matter content and similar should be controlled and modified to determine the effect of these factors on the plant-availability of metals.

- In this project, an adverse effect on the plant growth was not demonstrated at the used application rate of the heavy metals to the soil. Experiments regarding phyto-toxicity can be made in order to evaluate when soil concentrations of Cu and Zn is too high so that the plant growth is inhibited and phyto-toxicity is shown.

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Appendices

A Heavy metals in urine by ICP-OES

Urine from four different people was analysed in this project. The samples were analysed by use of Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). A method including analysis of 27 metals was developed by the laboratory staff and used in this project. The method was developed based on the standard *DS/EN ISO 11885: Water quality – Determination of selected elements by inductively coupled plasma optical emission spectrometry (ICP-OES)*. A calibration was made using multi element standards with the following concentrations:

0.1; 0.5; 1.0; 5.0; 10.0 mg/L.

The calibration made during the method development was recalled and used in this project. Prior to analysis, some standards with known concentrations were always analysed in order to check the method. The results for some measured standards and the deviation from the real concentration are seen in *Table A-1*.

Table A-1: Control of the method with some standards with known concentrations.

	Standard 0.05 mg/L		Standard 0.20 mg/L		Standard 1.0 mg/L	
	Measured [mg/L]	Deviation [%]	Measured [mg/L]	Deviation [%]	Measured [mg/L]	Deviation [%]
Aluminium	0.081	62	0.223	12	1.027	2.7
Antimony	0.019	-62	0.189	-5.5	0.980	-2.0
Arsenic	0.025	-50	0.183	-8.5	1.029	2.9
Boron	0.000	-100	0.261	31	1.781	78
Barium	0.028	-44	0.199	-0.5	1.092	9.2
Cadmium	0.030	-40	0.200	0.0	1.076	7.6
Cobalt	0.044	-12	0.214	7.0	1.081	8.1
Chrome	0.028	-44	0.199	-0.5	1.052	5.2
Copper	0.068	36	0.211	5.5	0.953	-4.7
Iron	0.003	-94	0.172	-14	1.015	1.5
Manganese	0.049	-2.0	0.218	9.0	1.046	4.6
Molybdenum	0.045	-10	0.201	0.5	0.947	-5.3
Nickel	0.044	-12	0.217	8.5	1.073	7.3
Lead	0.032	-36	0.199	-0.5	1.047	4.7
Silver	0.065	30	0.220	10	0.984	-1.6
Selenium	0.003	-94	0.179	-11	1.018	1.8
Titanium	0.022	-56	0.185	-7.5	0.972	-2.8
Zinc	0.025	-50	0.202	1.0	1.070	7.0
Strontium	0.052	4.0	0.205	2.5	0.996	-0.4
Vanadium	0.000	-100	0.148	-26	0.848	-15
Thallium	0.081	62	0.224	12	0.985	-1.5
Silicon	0.021	-58	0.523	162	1.754	75

It is seen that the deviation is very high for the standard with the low concentration of 0.05 mg/L. It means that such low concentrations are considered to be below the detection limit. The lowest concentration of the calibration standards was 0.1 mg/L. The detection limit is therefore as least 0.1 mg/L. The deviation of standard with concentration 0.2 mg/L was therefore much lower. However, some elements, such as silicon and boron have a very high deviation from the real concentration. Therefore, for some of the obtained results in the following caution should be taken.

A.1 Sample preparation

In order to examine whether digestion of the urine samples was needed or not, three samples from person A were analysed directly while three samples were digested prior to analysis. The digestion was carried by taking 40 mL urine and mixing it with 10 mL concentrated HNO_3. The solutions were autoclaved at 121°C in 30 min and filtered before analysis, see *Figure A-1*.

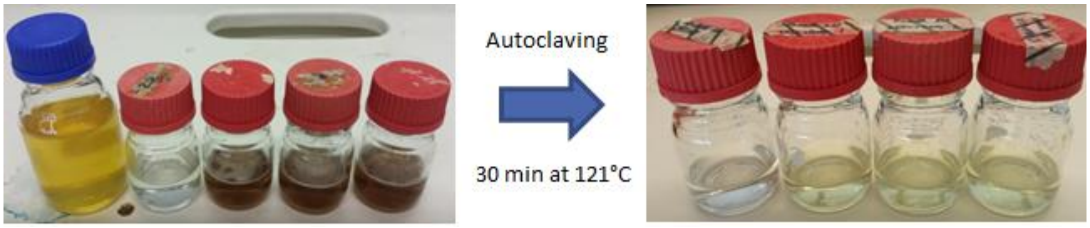


Figure A-1: Digestion of three sample of urine with HNO_3 and a blank made with demineralised water and HNO_3.

A triple determination was carried out. Three blank were prepared in the same manner with demineralised water instead of urine and concentrated HNO_3. After autoclaving, the samples were filtered after cooling to room temperature and stored cool until analysis.

Figure A-2: ICP-OES used for metal analysis with auto sampler seen to the left.

The samples were analysed by ICP-OES as seen in *Figure A-2*.

A.2 Results

Elements such as sodium, potassium, calcium and magnesium are included in the method. Since these elements are naturally present in relatively high concentrations in the urine, a dilution of the samples would have been required in order to be within the calibration. Since these elements are beyond the interest of this project, the results of these elements are not included in the results.

Blanks with demineralised water instead of urine were treated as the samples and analysed by ICP-OES. The results are seen in *Table A-2*.

Table A-2: Element concentration in three identical prepared blank measured with ICP-OES.

Element	Blank 1 [mg/L]	Blank 2 [mg/L]	Blank 3 [mg/L]	Mean [mg/L]	SD [mg/L]
Aluminium	0.038	0.029	0.031	0.033	0.005
Antimony	0.000	0.000	0.000	0.000	0.000
Arsenic	0.000	0.000	0.000	0.000	0.000
Boron	0.312	0.700	0.900	0.637	0.299
Barium	0.018	0.013	0.011	0.014	0.004
Cadmium	0.000	0.000	0.000	0.000	0.000
Cobalt	0.000	0.000	0.000	0.000	0.000
Chrome	0.000	0.000	0.000	0.000	0.000
Copper	0.031	0.071	0.041	0.048	0.021
Iron	0.048	0.039	0.079	0.055	0.021
Manganese	0.000	0.000	0.000	0.000	0.000
Molybdenum	0.000	0.000	0.000	0.000	0.000
Nickel	0.005	0.003	0.007	0.005	0.002
Lead	0.023	0.021	0.017	0.020	0.003
Silver	0.013	0.024	0.017	0.018	0.006
Selenium	0.023	0.103	0.078	0.068	0.041
Titanium	0.000	0.000	0.000	0.000	0.000
Zinc	0.000	0.003	0.008	0.004	0.004
Strontium	0.130	0.178	0.163	0.157	0.025
Vanadium	0.000	0.000	0.000	0.000	0.000
Thallium	0.000	0.000	0.000	0.000	0.000
Silicon	2.123	1.232	1.642	1.666	0.446

The obtained concentrations from the blank are considered as an indication of measurement uncertainty, noise, interference with other substances and contaminants from the nitric acid, glassware, autoclave etc. Therefore, all results are corrected for these values.

The results from the directly analysis of the urine from person A are seen in *Table A-3*, while the results for the digested samples are seen in *Table A-4*. All results for digested samples have been multiplied with a dilution factor 1.25 due to the sample preparation.

Table A-3: Element concentration in human urine from Person A. The sample were shaken, filtered and split into three identical samples and measured by ICP-OES.

Element	Sample 1 [mg/L]	Sample 2 [mg/L]	Sample 3 [mg/L]	Mean [mg/L]	SD [mg/L]	Mean corrected for blank [mg/L]
Aluminium	0.000	0.000	0.000	0.000	0.000	0.000
Antimony	0.000	0.000	0.000	0.000	0.000	0.000
Arsenic	0.000	0.000	0.000	0.000	0.000	0.000
Boron	1.030	1.010	1.003	1.014	0.014	0.377
Barium	0.022	0.011	0.014	0.016	0.006	0.002
Cadmium	0.000	0.000	0.000	0.000	0.000	0.000
Cobalt	0.000	0.000	0.000	0.000	0.000	0.000
Chrome	0.000	0.000	0.000	0.000	0.000	0.000
Copper	0.033	0.028	0.031	0.031	0.003	0.000
Iron	0.000	0.000	0.000	0.000	0.000	0.000

Element	Sample 1 [mg/L]	Sample 2 [mg/L]	Sample 3 [mg/L]	Mean [mg/L]	SD [mg/L]	Mean corrected for blank [mg/L]
Manganese	0.002	0.001	0.001	0.001	0.001	0.001
Molybdenum	0.013	0.013	0.012	0.013	0.001	0.013
Nickel	0.000	0.000	0.000	0.000	0.000	0.000
Lead	0.000	0.000	0.000	0.000	0.000	0.000
Silver	0.020	0.020	0.020	0.020	0.000	0.002
Selenium	0.072	0.068	0.067	0.069	0.003	0.001
Titanium	0.000	0.000	0.000	0.000	0.000	0.000
Zinc	0.185	0.175	0.181	0.180	0.005	0.176
Strontium	0.153	0.144	0.145	0.147	0.005	0.000
Vanadium	0.000	0.000	0.000	0.000	0.000	0.000
Thallium	0.000	0.000	0.000	0.000	0.000	0.000
Silicon	9.655	9.425	9.400	9.493	0.141	7.827

Table A-4: Element concentration in human urine from Person A. The sample was split into three identical samples, digested, filtered and measured by ICP-OES.

Element	Sample 1 [mg/L]	Sample 2 [mg/L]	Sample 3 [mg/L]	Mean [mg/L]	SD [mg/L]	Mean corrected for blank [mg/L]
Aluminium	0.014	0.029	0.016	0.020	0.008	0.000
Antimony	0.000	0.000	0.000	0.000	0.000	0.000
Arsenic	0.000	0.000	0.000	0.000	0.000	0.000
Boron	1.098	1.135	1.133	1.122	0.021	0.485
Barium	0.000	0.004	0.016	0.007	0.009	0.000
Cadmium	0.000	0.000	0.000	0.000	0.000	0.000
Cobalt	0.000	0.000	0.000	0.000	0.000	0.000
Chrome	0.004	0.005	0.006	0.005	0.001	0.005
Copper	0.048	0.043	0.048	0.046	0.003	0.000
Iron	0.066	0.079	0.184	0.110	0.065	0.055
Manganese	0.000	0.013	0.004	0.005	0.006	0.005
Molybdenum	0.016	0.100	0.011	0.043	0.050	0.043
Nickel	0.000	0.000	0.000	0.000	0.000	0.000
Lead	0.025	0.025	0.034	0.028	0.005	0.008
Silver	0.023	0.024	0.025	0.024	0.001	0.006
Selenium	0.016	0.091	0.096	0.068	0.045	0.000
Titanium	0.000	0.000	0.000	0.000	0.000	0.000
Zinc	0.209	0.206	0.213	0.209	0.003	0.205
Strontium	0.146	0.151	0.154	0.150	0.004	0.000
Vanadium	0.000	0.000	0.000	0.000	0.000	0.000
Thallium	0.000	0.000	0.000	0.000	0.000	0.000
Silicon	9.718	10.58	10.18	10.16	0.429	8.492

It is seen that the obtained concentration were a bit higher for the digested samples than the directly measured samples. Therefore, all urine samples were digested prior to analysis in the following.

Table A-5: Element concentration in human urine from Person B. The sample was split into three identical samples, digested, filtered and measured by ICP-OES.

Element	Sample 1 [mg/L]	Sample 2 [mg/L]	Sample 3 [mg/L]	Mean [mg/L]	SD [mg/L]	Mean corrected for blank [mg/L]
Aluminium	0.247	0.101	0.072	0.140	0.094	0.107
Antimony	0.000	0.000	0.000	0.000	0.000	0.000
Arsenic	0.000	0.000	0.000	0.000	0.000	0.000
Boron	0.875	0.583	0.562	0.673	0.175	0.036
Barium	0.019	0.022	0.023	0.021	0.002	0.007
Cadmium	0.000	0.000	0.000	0.000	0.000	0.000
Cobalt	0.000	0.000	0.000	0.000	0.000	0.000
Chrome	0.003	0.002	0.007	0.004	0.003	0.004
Copper	0.031	0.037	0.040	0.036	0.005	0.000
Iron	0.099	0.091	0.120	0.103	0.015	0.048
Manganese	0.004	0.006	0.009	0.006	0.003	0.006
Molybdenum	0.000	0.000	0.000	0.000	0.000	0.000
Nickel	0.009	0.012	0.008	0.010	0.002	0.005
Lead	0.020	0.022	0.023	0.022	0.002	0.002
Silver	0.014	0.014	0.021	0.016	0.004	0.000
Selenium	0.013	0.026	0.020	0.020	0.007	0.000
Titanium	0.000	0.000	0.000	0.000	0.000	0.000
Zinc	0.220	0.214	0.287	0.240	0.041	0.236
Strontium	0.086	0.089	0.087	0.087	0.002	0.000
Vanadium	0.000	0.000	0.000	0.000	0.000	0.000
Thallium	0.000	0.000	0.000	0.000	0.000	0.000
Silicon	6.575	5.858	11.19	7.874	2.894	6.208

Table A-6: Element concentration in human urine from Person C. The sample was split into three identical samples, digested, filtered and measured by ICP-OES.

Element	Sample 1 [mg/L]	Sample 2 [mg/L]	Sample 3 [mg/L]	Mean [mg/L]	SD [mg/L]	Mean corrected for blank [mg/L]
Aluminium	0.248	0.250	0.213	0.237	0.021	0.204
Antimony	0.000	0.000	0.000	0.000	0.000	0.000
Arsenic	0.000	0.000	0.000	0.000	0.000	0.000
Boron	3.720	3.825	3.153	3.566	0.362	2.929
Barium	0.019	0.020	0.160	0.066	0.081	0.052
Cadmium	0.000	0.000	0.000	0.000	0.000	0.000
Cobalt	0.000	0.000	0.000	0.000	0.000	0.000
Chrome	0.004	0.004	0.003	0.004	0.001	0.004
Copper	0.038	0.037	0.041	0.039	0.002	0.000
Iron	0.084	0.084	0.096	0.088	0.007	0.033
Manganese	0.002	0.001	0.002	0.002	0.001	0.002
Molybdenum	0.002	0.001	0.001	0.001	0.001	0.001
Nickel	0.002	0.001	0.002	0.002	0.001	0.000
Lead	0.014	0.015	0.110	0.046	0.055	0.026
Silver	0.014	0.014	0.013	0.014	0.001	0.000
Selenium	0.032	0.030	0.029	0.030	0.002	0.000
Titanium	0.000	0.000	0.000	0.000	0.000	0.000
Zinc	0.174	0.170	0.223	0.189	0.030	0.185
Strontium	0.076	0.077	0.069	0.074	0.004	0.000

Element	Sample 1 [mg/L]	Sample 2 [mg/L]	Sample 3 [mg/L]	Mean [mg/L]	SD [mg/L]	Mean corrected for blank [mg/L]
Vanadium	0.000	0.000	0.000	0.000	0.000	0.000
Thallium	0.000	0.000	0.000	0.000	0.000	0.000
Silicon	10.21	10.32	9.351	9.960	0.531	8.294

Table A-7: Element concentration in human urine from Person D. The sample was split into three identical samples, digested, filtered and measured by ICP-OES.

Element	Sample 1 [mg/L]	Sample 2 [mg/L]	Sample 3 [mg/L]	Mean [mg/L]	SD [mg/L]	Mean corrected for blank [mg/L]
Aluminium	0.162	0.159	0.165	0.162	0.003	0.129
Antimony	0.000	0.000	0.000	0.000	0.000	0.000
Arsenic	0.000	0.000	0.000	0.000	0.000	0.000
Boron	5.649	4.955	5.542	5.382	0.374	4.745
Barium	0.003	0.002	0.002	0.002	0.001	0.000
Cadmium	0.000	0.000	0.000	0.000	0.000	0.000
Cobalt	0.002	0.000	0.000	0.001	0.001	0.001
Chrome	0.000	0.001	0.000	0.000	0.001	0.000
Copper	0.035	0.039	0.034	0.036	0.003	0.000
Iron	0.079	0.069	0.079	0.076	0.006	0.021
Manganese	0.000	0.000	0.000	0.000	0.000	0.000
Molybdenum	0.003	0.002	0.000	0.002	0.002	0.002
Nickel	0.000	0.001	0.000	0.000	0.001	0.000
Lead	0.000	0.003	0.006	0.003	0.003	0.000
Silver	0.014	0.011	0.015	0.013	0.002	0.000
Selenium	0.071	0.065	0.072	0.069	0.004	0.001
Titanium	0.000	0.000	0.000	0.000	0.000	0.000
Zinc	0.155	0.178	0.163	0.165	0.012	0.161
Strontium	0.126	0.119	0.127	0.124	0.004	0.000
Vanadium	0.000	0.000	0.000	0.000	0.000	0.000
Thallium	0.000	0.000	0.000	0.000	0.000	0.000
Silicon	11.47	10.36	11.60	11.14	0.681	9.477

No further urine analysis was carried out.

B Heavy metals in foodstuffs by ICP-OES

Some different types of foodstuffs were analysed by ICP-OES with the same method as described in the analysis of the urine in the previous appendix.

B.1 Sample preparation and analysis

All samples of foodstuff were dried at 105°C to a constant weight was obtained, as seen in *Figure B-1*.

Figure B-1: Drying of some different foodstuffs, more specifically tuna, salmon, pig cutlet and minced beef.

After drying the foodstuffs were milled in a mortar (see *Figure B-2*) and accurately weighed in a 100 mL autoclave bottle.

Figure B-2: Foodstuffs after drying (left) and milled tuna (right) prepared for digestion with nitric acid.

A triple determination was carried out by weighing three different amounts of the concerned foodstuff accurately. To each bottle 20 mL of 7 M nitric acid were added and the solutions were autoclaved at 120°C for 30 minutes. The solutions with tuna are seen in *Figure B-3*.

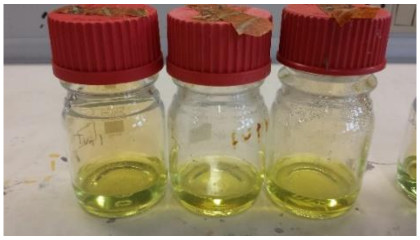


Figure B-3: Digestion solution with dried, milled tuna in nitric acid after autoclaving.

Three blanks were made by adding 20 mL of 7 M nitric acid to autoclave bottles and treating them as the samples.

B.2 Results

All blanks and samples were analysed with the same method as used for the urine analysis. The results for the element analysis of the blanks are seen in *Table B-1*.

Table B-1: Element analysis of blanks with triple determination.

Element	Blank 1 [mg/L]	Blank 2 [mg/L]	Blank 3 [mg/L]	Mean [mg/L]	SD [mg/L]
Aluminium	0.056	0.057	0.049	**0.054**	0.004
Antimony	0.000	0.000	0.000	**0.000**	0.000
Arsenic	0.000	0.000	0.000	**0.000**	0.000
Boron	0.132	0.000	0.078	**0.070**	0.066
Barium	0.000	0.000	0.000	**0.000**	0.000
Cadmium	0.000	0.000	0.000	**0.000**	0.000
Cobalt	0.000	0.000	0.000	**0.000**	0.000
Chrome	0.000	0.000	0.000	**0.000**	0.000
Copper	0.022	0.023	0.019	**0.021**	0.002
Iron	0.013	0.014	0.009	**0.012**	0.003
Manganese	0.000	0.000	0.000	**0.000**	0.000
Molybdenum	0.000	0.000	0.000	**0.000**	0.000
Nickel	0.003	0.002	0.003	**0.003**	0.001
Lead	0.022	0.022	0.023	**0.022**	0.001
Silver	0.014	0.014	0.015	**0.014**	0.001
Selenium	0.000	0.000	0.000	**0.000**	0.000
Titanium	0.000	0.000	0.000	**0.000**	0.000
Zinc	0.000	0.002	0.001	**0.001**	0.001
Strontium	0.009	0.002	0.005	**0.005**	0.004
Vanadium	0.000	0.016	0.009	**0.008**	0.008
Thallium	0.019	0.061	0.046	**0.042**	0.021
Silicon	0.744	0.000	0.000	**0.248**	0.430

The obtained concentrations from the blank are considered as an indication of contaminants from the nitric acid, glassware, autoclave etc. Therefore, all results are corrected for the mean value of each element. The results from the ICP [mg/L] are converted based on the weighed amount to mg per kg dry foodstuff.

Table B-2: Weighed amounts of dried foodstuff for triple determination of element analysis.

Salmon	Mass [g]	Canned tuna	Mass [g]	Pig cutlet	Mass [g]	Minced beef	Mass [g]
Sample 1	0.9908	**Sample 1**	0.9907	**Sample 2**	0.8122	**Sample 1**	0.8122
Sample 2	1.2229	**Sample 2**	1.2180	**Sample 3**	0.9874	**Sample 2**	0.9874
Sample 3	1.5366	**Sample 3**	1.5240	**Sample 1**	1.2664	**Sample 3**	1.2664

The results for the element analysis of dried salmon, canned tuna, pig cutlet and minced beef are seen in *Table B-3*, *Table B-4*, *Table B-5*, and *Table B-7*, respectively.

Table B-3: Element analysis by ICP-OES of dried salmon.

Element	Sample 1 [mg/L]	Sample 2 [mg/L]	Sample 3 [mg/L]	Sample 1 [mg/kg]	Sample 2 [mg/kg]	Sample 3 [mg/kg]	Mean [mg/kg]	SD [mg/kg]
Al	0.122	0.228	0.180	3.43	7.11	4.10	**4.88**	1.962
Sb	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
As	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
B	0.089	0.000	0.003	0.959	0.000	0.000	**0.320**	0.554
Ba	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Cd	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Co	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Cr	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Cu	0.053	0.060	0.071	1.61	1.59	1.63	**1.61**	0.016
Fe	0.376	0.491	0.566	18.4	19.6	18.0	**18.7**	0.819
Mn	0.011	0.016	0.018	0.555	0.654	0.586	**0.598**	0.051
Mo	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Ni	0.022	0.008	0.017	0.959	0.204	0.456	**0.540**	0.384
Pb	0.053	0.051	0.042	1.56	1.19	0.651	**1.13**	0.459
Ag	0.013	0.011	0.013	0.000	0.000	0.000	**0**	0
Se	0.015	0.015	0.051	0.757	0.613	1.66	**1.01**	0.567
Ti	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Zn	0.238	0.273	0.357	12.0	11.1	11.6	**11.6**	0.420
Sr	0.025	0.031	0.029	1.01	1.06	0.781	**1.0**	0.150
V	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Tl	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Si	1.375	102.000	4.272	56.9	4160	131	**1449**	2348

Table B-4: Element analysis by ICP-OES of dried canned tuna.

Element	Sample 1 [mg/L]	Sample 2 [mg/L]	Sample 3 [mg/L]	Sample 1 [mg/kg]	Sample 2 [mg/kg]	Sample 3 [mg/kg]	Mean [mg/kg]	SD [mg/kg]
Al	0.383	0.218	0.247	16.6	6.73	6.33	**9.89**	5.82
Sb	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
As	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
B	0.153	0.000	0.175	4.189	0.000	3.445	**2.54**	2.23
Ba	0.022	0.000	0.000	1.110	0.000	0.000	**0.370**	0.641
Cd	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Co	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Cr	0.000	0.000	0.016	0.000	0.000	0.525	**0.175**	0.303
Cu	0.074	0.078	0.093	2.67	2.34	2.36	**2.46**	0.187
Fe	1.393	1.825	1.998	69.7	74.4	65.2	**69.8**	4.63
Mn	0.025	0.040	0.029	1.26	1.64	0.951	**1.29**	0.346
Mo	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Ni	0.062	0.025	0.019	2.98	0.903	0.525	**1.47**	1.32
Pb	0.047	0.000	0.024	1.26	0.000	0.066	**0.442**	0.710
Ag	0.012	0.013	0.013	0.000	0.000	0.000	**0**	0
Se	0.021	0.050	0.055	1.06	2.05	1.80	**1.64**	0.517
Ti	0.004	0.000	0.000	0.202	0.000	0.000	**0.067**	0.117
Zn	0.584	0.680	0.864	29.4	27.9	28.3	**28.5**	0.799
Sr	0.030	0.037	0.039	1.26	1.31	1.12	**1.23**	0.103
V	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Tl	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Si	1.702	7.162	54.020	73	284	1764	**707**	921

Table B-5: Element analysis by ICP-OES of dried pig cutlet.

Element	Sample 1 [mg/L]	Sample 2 [mg/L]	Sample 3 [mg/L]	Sample 1 [mg/kg]	Sample 2 [mg/kg]	Sample 3 [mg/kg]	Mean [mg/kg]	SD [mg/kg]
Al	0.132	0.128	0.207	4.78	3.95	13.4	**7.36**	5.21
Sb	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
As	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
B	0.053	0.055	0.000	0.000	0.000	0.000	**0**	0
Ba	0.012	0.001	0.006	0.735	0.053	0.524	**0.437**	0.349
Cd	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Co	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Cr	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Cu	0.057	0.045	0.004	2.21	1.28	0.000	**1.16**	1.11
Fe	0.394	0.345	0.265	23.4	17.8	22.1	**21.1**	2.94
Mn	0.011	0.006	0.005	0.674	0.320	0.436	**0.477**	0.180
Mo	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Ni	0.023	0.000	0.006	1.225	0.000	0.262	**0.496**	0.645
Pb	0.079	0.008	0.041	3.49	0.000	1.66	**1.72**	1.75
Ag	0.012	0.016	0.014	0.000	0.107	0.000	**0.036**	0.062
Se	0.002	0.006	0.000	0.123	0.320	0.000	**0.148**	0.162
Ti	0.012	0.007	0.009	0.735	0.374	0.786	**0.631**	0.225
Zn	0.698	0.677	0.397	42.7	36.1	34.6	**37.8**	4.32
Sr	0.016	0.011	0.013	0.674	0.320	0.698	**0.564**	0.212
V	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Tl	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Si	70.130	50.390	92.500	4280	2676	8053	**5003**	2760

Table B-6: Element analysis of blanks with triple determination.

Element	Blank 1 [mg/L]	Blank 2 [mg/L]	Blank 3 [mg/L]	Mean [mg/L]	SD [mg/L]
Aluminium	0.134	0.137	0.135	0.135	0.002
Antimony	0.082	0.078	0.081	0.080	0.002
Arsenic	0.038	0.029	0.033	0.033	0.005
Boron	0.331	0.287	0.335	0.318	0.027
Barium	0.071	0.072	0.07	0.071	0.001
Cadmium	0.048	0.049	0.05	0.049	0.001
Cobalt	0.067	0.066	0.068	0.067	0.001
Chrome	0.074	0.074	0.073	0.074	0.001
Copper	0.103	0.103	1.102	0.436	0.577
Iron	0.090	0.087	0.088	0.088	0.002
Manganese	0.083	0.083	0.083	0.083	0.000
Molybdenum	0.075	0.071	0.072	0.073	0.002
Nickel	0.084	0.083	0.084	0.084	0.001
Lead	0.113	0.110	0.112	0.112	0.002
Silver	0.047	0.047	0.048	0.047	0.001
Selenium	0.040	0.045	0.039	0.041	0.003
Titanium	0.104	0.103	0.103	0.103	0.001
Zinc	0.069	0.070	0.069	0.069	0.001
Strontium	0.003	0.004	0.003	0.003	0.001
Vanadium	0.000	0.000	0.000	0.000	0.000
Thallium	0.089	0.086	0.087	0.087	0.002
Silicon	20.07	19.2	20.03	19.767	0.491

Table B-7: Element analysis by ICP-OES of dried minced beef.

Element	Sample 1 [mg/L]	Sample 2 [mg/L]	Sample 3 [mg/L]	Sample 1 [mg/kg]	Sample 2 [mg/kg]	Sample 3 [mg/kg]	Mean [mg/kg]	SD [mg/kg]
Al	0.205	0.237	0.240	4.29	5.15	4.13	**4.52**	0.547
Sb	0.071	0.073	0.073	0.000	0.00	0.00	**0**	0
As	0.000	0.000	0.000	0.000	0.00	0.00	**0**	0
B	0.224	0.442	0.188	0.000	6.30	0.00	**2.10**	3.63
Ba	0.095	0.100	0.108	1.48	1.47	1.46	**1.47**	0.008
Cd	0.044	0.049	0.047	0.000	0.00	0.00	**0**	0
Co	0.065	0.066	0.067	0.000	0.00	0.00	**0**	0
Cr	0.082	0.080	0.082	0.513	0.321	0.329	**0.388**	0.109
Cu	0.153	0.145	0.160	3.1	2.14	2.26	**2.50**	0.520
Fe	1.344	1.295	1.694	77.3	61.1	63.4	**67.3**	8.77
Mn	0.091	0.090	0.094	0.492	0.354	0.434	**0.427**	0
Mo	0.069	0.066	0.065	0.00	0.00	0.00	**0**	0
Ni	0.084	0.088	0.081	0.021	0.219	0.000	**0.080**	0.12
Pb	0.159	0.128	0.129	2.914	0.827	0.684	**1.48**	1.25
Ag	0.044	0.046	0.046	0.00	0.000	0.000	**0**	0
Se	0.093	0.111	0.099	3.18	3.53	2.28	**3.00**	0.646
Ti	0.107	0.105	0.108	0.226	0.084	0.184	**0.165**	0.073
Zn	2.324	2.297	2.846	139	113	110	**120**	16.0
Sr	0.029	0.024	0.024	1.58	1.05	0.82	**1.15**	0.392
V	0.000	0.000	0.000	0.00	0.00	0.00	**0**	0
Tl	0.000	0.000	0.000	0.00	0.00	0.00	**0**	0
Si	113.2	8.023	71.39	5752	0.000	2038	**2597**	2916

C Manure analysis

In this project, one sample of cattle manure and two samples of pig manure have been analysed. However, one of the samples of pig manure has been analysed by ICP-OES only. This is because a small amount was received only, and no other analyses were carried out before drying of the sample for ICP-OES analysis. In the following sections, analyses of one sample of pig manure and one sample of cattle manure therefore are presented only. Except for the section with ICP-OES where the two different samples of pig manure are included as well as the sample of cattle manure.

C.1 Sample preparation and analysis

Characterisation of manure

A weighed amount of the manure samples were dried at 105°C until a constant weight was obtained. The samples after drying are seen in *Figure C-1*.

Figure C-1: Drying of pig manure sample 1 to the right and pig manure sample 2 and cattle manure to the left.

The dry matter content was calculated by the following formula and the results are seen in *Table C-1*.

$$Dry\ Matter\ \left[\frac{g}{kg}\right] = \frac{a-b}{c} \cdot 10^3 = \frac{42.974 - 42.5735}{28.6575} \cdot 10^3 = 13.98\,\frac{g}{kg} = 1.40\ \%$$

Table C-1: Dry matter content in three samples of Pig Manure 2 and Cattle Manure, respectively.

Dry matter	Crucible [g] *b*	Crucible + wet sample [g]	Sample [g] *c*	Crucible + dry sample [g] *a*	Dry matter [g/kg]
Pig manure A	42.5735	71.231	28.6575	42.974	13.98
Pig manure B	43.3421	73.222	29.8799	43.687	11.54
Pig manure C	173.9672	418.32	244.3528	176.4734	10.26
Average					**11.92**
Cattle manure A	43.5003	90.887	44.8456	47.3867	28.39
Cattle manure B	44.2321	91.213	45.5563	46.9809	28.19
Cattle manure C	195.2582	549.2	205.26	353.9418	28.26
Average					**28.28**

After drying, the dried matter in the beakers were transferred to a mortar and milled to a homogeneous sample, as seen in *Figure C-2*.

Figure C-2: The dried manure was transferred to a mortar and milled to a homogenous sample.

The dried manure was kept for later metal analysis by ICP-OES.

C.2 Total N

The total N content in the manures were determined by FIA-STAR 5000 according Application Note (AN5202) from FOSS (51), see *Figure C-3*.

Figure C-3: FIAstar 5000 analyser and auto-sampler used for determination of total N.

A small amount of the fresh manures was accurately weighed into a 100 mL autoclave bottle and 8.0 mL potassium peroxodisulphate solution made according (51) were added. The bottles were autoclaved for 30 minutes at 120°C, see *Figure C-4*.

Figure C-4: Samples prepared for digestion with potassium peroxodisulphate solution.

After autoclaving, the samples were cooled to room temperature and filtered through ordinary filter paper. The samples were transferred quantitatively into a 50.00 mL volumetric flask and diluted with demineralised water to the mark, see *Figure C-5*.

Figure C-5: All samples were filtered after autoclaving and diluted to 50 mL with demineralised water.

Before analysis of the samples, the FIA were calibrated according the AN5202 (51). The standards used for the calibration were as follows:

0; 0.1; 0.5; 1.0; 3.0; 5.0 mg NO_3-N per L

In the programme on the PC the method *Total nitrogen 0.1-5 mg/L* NO_3*-N* were selected. The calibration was made and the samples were analysed.

C.2.1 Results

The total N analysis were made with a triple determination. The weighed amounts are seen in *Table C-2*.

Table C-2: Weighed amounts of pig and cattle manure for total N determination.

Pig manure	Amount [g]	Cattle manure	Amount [g]
Sample 1	0.1101	**Sample 1**	0.1139
Sample 2	0.1070	**Sample 2**	0.1424
Sample 3	0.1342	**Sample 3**	0.1480

Three blank samples were made with demineralised water and treated as the samples. The results for analysis of the blanks are seen in *Table C-3*.

Table C-3: Blank with demineralised water treated as the samples.

Blank	Concentration from FIA [mg N/L]
1	0.00
2	0.10
3	0.00
Mean	**0.03**

The results from the FIA were corrected for the blank and converted using the formula listed below.

$$\frac{mg\ N}{kg\ sample} = \frac{C \cdot 50\ mL \cdot 1000\,\frac{g}{kg}}{a \cdot 1000\ mL/L}$$

C = Concentration obtained from FIA [mg N/L]
a = mass of dried sample [g]

The results of the total N determination in pig and cattle manure are seen in *Table C-4*.

Table C-4: Total N in wet pig and cattle manure.

	Sample	Mass [g]	Concentration from FIA [mg N/L]	Corrected for blank [mg N/L]	Total N [mg N/kg]	Mean [mg N/kg]	SD [mg N/kg]
Pig manure	**1**	0.1139	4.1	4.0	1776	**1739**	217
	2	0.1424	4.3	4.3	1505		
	3	0.1480	5.8	5.7	1935		
Cattle manure	**1**	0.1101	4.9	4.9	2219	**2406**	283
	2	0.1070	5.9	5.8	2732		
	3	0.1342	6.1	6.1	2268		

C.3 ICP-OES

The dried manure samples are digested prior to ICP-OES analysis in the following way: An accurately weighed amount of dried manure was transferred to a 100 mL autoclave bottle and 20 mL of 7 M nitric acid are added. The samples were digested at 120°C for 30 minutes, as seen in *Figure C-6*.

Figure C-6: Some manure samples before and after digestion.

After the digestion and cooling to room temperature, the samples were filtered into 50 mL volumetric flasks and diluted to the mark with demineralised water, as seen in *Figure C-7*.

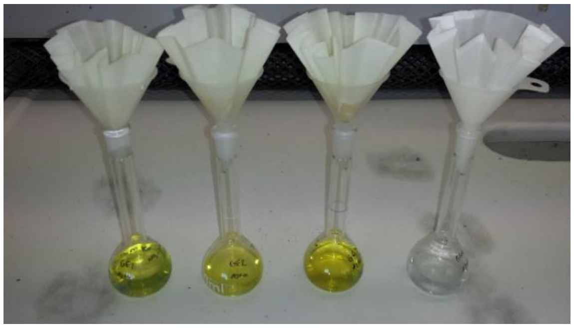


Figure C-7: All samples are filtered and diluted to 50.00 mL with demineralised water after digestion.

All samples were analysed with a triple determination. A new method that measures 15 metals and P and K was developed. The 15 elements and P were calibrated as the previous method; 0.1; 0.5; 1.0; 5.0; 10.0 mg/L, while K was calibrated with a factor 10 higher: 1.0; 5.0; 10.0; 50.0; 100.0 mg/L.

C.3.1 Results

Blanks

Three blanks were made with demineralised water, nitric acid and treated as the samples. The results for the analysis of the blanks are seen in *Table C-5*.

Table C-5: Element concentration in three blanks made of 20 mL 7 M HNO_3 and treated as the samples.

Element	Blank 1 [mg/L]	Blank 2 [mg/L]	Blank 3 [mg/L]	Mean [mg/L]	SD [mg/L]
Al	0.171	0.197	0.187	**0.185**	0.013
Sb	0.048	0.045	0.043	**0.045**	0.003
As	0.000	0.000	0.000	**0.000**	0.000
Ba	0.055	0.055	0.053	**0.054**	0.001
Cd	0.063	0.065	0.067	**0.065**	0.002
Co	0.052	0.051	0.053	**0.052**	0.001
Cr	0.063	0.060	0.068	**0.064**	0.004
Cu	0.180	0.094	0.121	**0.132**	0.044
Fe	0.000	0.000	0.000	**0.000**	0.000
Mn	0.067	0.067	0.068	**0.067**	0.001
Mo	0.052	0.049	0.053	**0.051**	0.002
Ni	0.072	0.068	0.067	**0.069**	0.003
Pb	0.104	0.094	0.098	**0.099**	0.005
Ag	0.043	0.039	0.041	**0.041**	0.002
Zn	0.046	0.043	0.042	**0.044**	0.002
P	0.055	0.061	0.060	**0.059**	0.003
K	1.792	1.643	1.712	**1.716**	0.075

Manures

The weighed amounts of the dried manure are seen in *Table C-6*.

Table C-6: Weighed amounts of dried manure for ICP-OES analysis.

Pig manure 1	Amount [g]	Pig manure 2	Amount [g]	Cattle manure	Amount [g]
Sample 1	0.4964	**Sample 1**	0.8293	**Sample 1**	0.7707
Sample 2	0.6768	**Sample 2**	1.0505	**Sample 2**	1.0016
Sample 3	1.0530	**Sample 3**	1.2503	**Sample 3**	1.2077

The results were corrected for the blank mean value and converted to mg/kg dry matter. Some of the results are multiplied with a dilution factor (not shown). The dilution was made to ensure analysis within the calibration.

Table C-7: Metal analysis of pig manure sample 1 with triple determination.

Element	1 [mg/L]	2 [mg/L]	3 [mg/L]	1 [mg/kg]	2 [mg/kg]	3 [mg/kg]	Mean [mg/kg]	SD [mg/kg]
Al	1.699	2.712	4.244	76.2	93.3	96.4	**88.7**	10.8
Sb	0.048	0.047	0.038	0.151	0.074	0.000	**0.075**	0.076
As	0.000	0.000	0.000	0	0	0	**0**	0
Ba	0.213	0.282	0.396	8.01	8.42	8.12	**8.18**	0.214
Cd	0.065	0.069	0.069	0.000	0.148	0.095	**0.081**	0.075
Co	0.012	0.070	0.077	0.000	0.665	0.594	**0.419**	0.365
Cr	0.085	0.099	0.113	1.06	1.29	1.16	**1.17**	0.118
Cu	1.347	1.783	2.736	61.2	61.0	61.8	**61.3**	0.437
Fe	8.561	12.01	17.63	431	444	419	**431**	12.5
Mn	1.184	2.485	3.638	56.2	89.3	84.8	**76.8**	17.9

Element	1 [mg/L]	2 [mg/L]	3 [mg/L]	1 [mg/kg]	2 [mg/kg]	3 [mg/kg]	Mean [mg/kg]	SD [mg/kg]
Mo	0.107	0.121	0.157	2.82	2.59	2.52	**2.64**	0.159
Ni	0.193	0.226	0.273	6.24	5.80	4.84	**5.63**	0.716
Pb	0.116	0.156	0.149	0.856	2.11	1.19	**1.38**	0.647
Ag	0.035	0.034	0.031	0	0	0	**0**	0
Zn	3.894	5.529	7.870	194	203	186	**194**	8.40
P	12.450	11.560	9.776	24970	42498	23079	**30182**	10707
K	79.610	89.710	68.240	156765	324756	157759	**213093**	96704

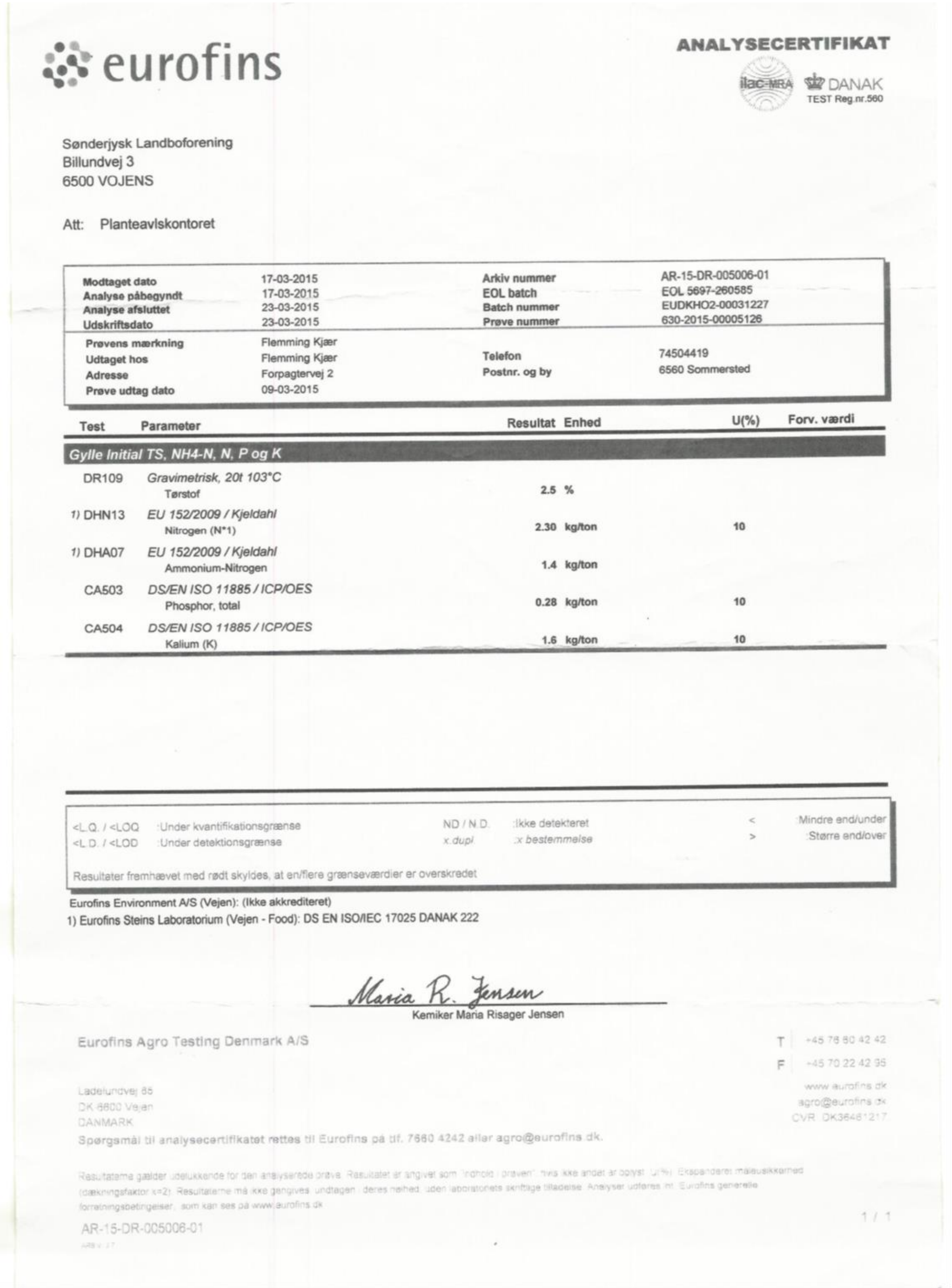
Table C-8: Metal analysis of pig manure sample 2 with triple determination.

Element	1 [mg/L]	2 [mg/L]	3 [mg/L]	1 [mg/kg]	2 [mg/kg]	3 [mg/kg]	Mean [mg/kg]	SD [mg/kg]
Al	5.317	6.823	7.819	309	316	305	**310**	5.37
Sb	0.032	0.034	0.033	0	0	0	**0**	0
As	0.000	0.000	0.000	0	0	0	**0**	0
Ba	0.182	0.211	0.250	7.72	7.47	7.84	**7.68**	0.186
Cd	0.069	0.068	0.068	0.241	0.143	0.120	**0.168**	0.064
Co	0.066	0.073	0.077	0.844	1.00	1.00	**0.948**	0.090
Cr	0.085	0.093	0.097	1.27	1.38	1.32	**1.32**	0.057
Cu	2.595	3.321	3.810	148	152	147	**149**	2.41
Fe	11.120	14.270	16.470	670	679	659	**669**	10.3
Mn	2.825	3.588	4.238	166	168	167	**167**	0.656
Mo	0.075	0.084	0.089	1.45	1.57	1.52	**1.51**	0.062
Ni	0.185	0.215	0.236	6.99	6.95	6.68	**6.87**	0.171
Pb	0.122	0.115	0.107	1.387	0.762	0.320	**0.823**	0.536
Ag	0.031	0.031	0.029	0	0	0	**0**	0
Zn	10.690	13.910	15.520	642	660	619	**640**	20.6
P	8.777	9.899	5.080	21035	18742	20095	**19957**	1153
K	87.420	100.200	44.040	206507	187355	168951	**187604**	18779

Table C-9: Metal analysis of cattle manure with triple determination.

Element	1 [mg/L]	2 [mg/L]	3 [mg/L]	1 [mg/kg]	2 [mg/kg]	3 [mg/kg]	Mean [mg/kg]	SD [mg/kg]
Al	9.810	13.740	15.260	624	677	624	**642**	30.2
Sb	0.043	0.043	0.042	0	0	0	**0**	0
As	0.000	0.000	0.000	0	0	0	**0**	0
Ba	0.413	0.528	0.653	23.3	23.7	24.8	**23.9**	0.786
Cd	0.067	0.068	0.065	0.130	0.150	0.000	**0.093**	0.081
Co	0.063	0.067	0.069	0.714	0.749	0.704	**0.722**	0.024
Cr	0.118	0.139	0.162	3.50	3.74	4.06	**3.77**	0.278
Cu	2.898	3.766	4.816	179	181	194	**185**	7.85
Fe	14.25	18.92	23.63	924	944	978	**949**	27.2
Mn	3.160	4.212	5.322	201	207	218	**208**	8.54
Mo	0.063	0.061	0.068	0.779	0.499	0.704	**0.661**	0.145
Ni	0.119	0.321	0.143	3.24	12.6	3.06	**6.30**	5.44
Pb	0.106	0.109	0.123	0.454	0.499	0.994	**0.649**	0.299
Ag	0.029	0.032	0.024	0	0	0	**0**	0
Zn	2.909	3.797	4.798	186	187	197	**190**	5.94
P	8.148	10.610	6.913	10501	10538	11357	**10799**	484
K	43.990	52.650	30.460	54753	50777	47475	**51002**	3644

C.4 Test results Eurofins



eurofins

ANALYSECERTIFIKAT

ilac-MRA DANAK
TEST Reg.nr.560

Sønderjysk Landboforening
Billundvej 3
6500 VOJENS

Att: Planteavlskontoret

Modtaget dato	17-03-2015	**Arkiv nummer**	AR-15-DR-005006-01
Analyse påbegyndt	17-03-2015	**EOL batch**	EOL 5697-260585
Analyse afsluttet	23-03-2015	**Batch nummer**	EUDKHO2-00031227
Udskriftsdato	23-03-2015	**Prøve nummer**	630-2015-00005126
Prøvens mærkning	Flemming Kjær		
Udtaget hos	Flemming Kjær	**Telefon**	74504419
Adresse	Forpagtervej 2	**Postnr. og by**	6560 Sommersted
Prøve udtag dato	09-03-2015		

Test	Parameter	Resultat	Enhed	U(%)	Forv. værdi
Gylle Initial TS, NH4-N, N, P og K					
DR109	*Gravimetrisk, 20t 103°C* Tørstof	2.5	%		
1) DHN13	*EU 152/2009 / Kjeldahl* Nitrogen (N*1)	2.30	kg/ton	10	
1) DHA07	*EU 152/2009 / Kjeldahl* Ammonium-Nitrogen	1.4	kg/ton		
CA503	*DS/EN ISO 11885 / ICP/OES* Phosphor, total	0.28	kg/ton	10	
CA504	*DS/EN ISO 11885 / ICP/OES* Kalium (K)	1.6	kg/ton	10	

<L.Q. / <LOQ	:Under kvantifikationsgrænse	ND / N.D.	:Ikke detekteret	<	:Mindre end/under
<L.D. / <LOD	:Under detektionsgrænse	x.dupl.	:x bestemmelse	>	:Større end/over

Resultater fremhævet med rødt skyldes, at en/flere grænseværdier er overskredet

Eurofins Environment A/S (Vejen): (Ikke akkrediteret)
1) Eurofins Steins Laboratorium (Vejen - Food): DS EN ISO/IEC 17025 DANAK 222

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Resultaterne gælder udelukkende for den analyserede prøve. Resultatet er angivet som "Indhold i prøven", hvis ikke andet er oplyst. U(%): Ekspanderet måleusikkerhed (dækningsfaktor k=2). Resultaterne må ikke gengives, undtagen i deres helhed, uden laboratoriets skriftlige tilladelse. Analyser udføres iht. Eurofins generelle forretningsbetingelser, som kan ses på www.eurofins.dk

AR-15-DR-005006-01

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D Soil analysis

Some different soil samples were collected and analysed. In the following the sampling, sample preparation and analysis procedure as well as obtained results are presented.

D.1 Sampling

Sampling and characterisation of soil

Soil samples were collected at three different places, including two agricultural fields, which have been applied cattle and pig manure, respectively and a forest floor, as seen in *Figure D-1*.

Figure D-1: From left to right: Field that has received cattle manure and pig manure, respectively and a forest floor.

Three samples were collected from each place in order to ensure a representative sampling from the fields and the forest, giving nine samples totally, see *Figure D-2*.

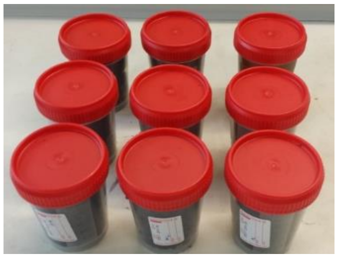

Figure D-2: Soil samples collected for different analyses.

All soil samples were dried at 105°C in a stove for at least 24 hours to a constant weight, and dry matter yields were obtained, see *Figure D-3*.

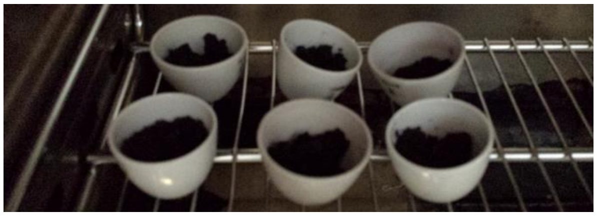

Figure D-3: Soil samples weighed and prepared for drying at 105°C for at least 24 hours.

The dry matter (DM) content in the different soil samples are seen in *Table D-1*.

Table D-1: Dry matter (DM) content in the soil samples.

Sample	Crucible [g]	Crucible incl. Sample [g]	Sample [g]	After drying [g]	Dry matter (DM) [g/kg]	Average DM [g/kg]
Cattle field 1	64.5568	102.3352	37.7784	96.3098	**840.5**	**847**
Cattle field 2	73.2216	108.8285	35.6069	103.7534	**857.5**	
Cattle field 3	68.4854	102.2727	33.7873	96.9208	**841.6**	
Pig field site 1	70.7403	100.3077	29.5674	95.7448	**845.7**	**853**
Pig field site 2	74.5843	103.0539	28.4696	99.1784	**863.9**	
Pig field site 3	68.9414	96.8815	27.9401	92.6753	**849.5**	
Forest site 1	62.3511	104.93	42.5789	91.4742	**684.0**	**657**
Forest site 2	68.7012	114.95	46.2488	93.5342	**536.9**	
Forest site 3	61.3701	95.060	33.6899	86.5963	**748.8**	

A small amount (accurately weighed) of each dried sample were transferred to crucibles for determination of organic matter by ignition at 550°C for two hours, *Figure D-4*.

Figure D-4: Some soil samples before and after ignition at 550°C for two hours.

The organic matter (OM) content in the different soil samples are seen in *Table D-2*.

Table D-2: Organic matter (OM) content in the soil samples.

Sample	Crucible [g]	Sample [g]	After ignition [g]	Residue on ignition [g]	Loss on ignition (OM) [g]	Average OM [g/kg]
Cattle field 1	15.1835	0.7711	15.5095	422.8	**417.7**	**426**
Cattle field 2	17.4103	0.6817	17.6975	421.3	**436.2**	
Cattle field 3	16.6886	0.7456	16.9997	417.2	**424.4**	
Pig field site 1	17.0998	0.6588	17.3659	403.9	**441.8**	**449**
Pig field site 2	16.0259	0.7252	16.3401	433.3	**430.6**	
Pig field site 3	16.0669	0.6238	16.2999	373.5	**475.9**	
Forest site 1	15.1302	0.5053	15.2370	211.4	**472.6**	**510**
Forest site 2	14.8898	0.531	14.9078	33.90	**503.0**	
Forest site 3	17.1175	0.5126	17.2169	193.9	**554.9**	

D.2 ICP-OES

The content of some selected metals in the soil samples were determined by ICP-OES as previously described with the method including 15 elements as well as P and K. Prior to analysis the samples were digested as described in the following.

Digestion

An accurately weighed amount of the dried soil samples were transferred into a 100 mL autoclave bottle and added 20 mL 7 M HNO_3 for digestion, as seen in *Figure D-5*.

Figure D-5: Soil samples after drying, weighed and added 20 mL 7M HNO_3 for digestion.

The digestion of the soil samples were carried out by heating at 120°C for 30 min in an autoclave, see *Figure D-6*.

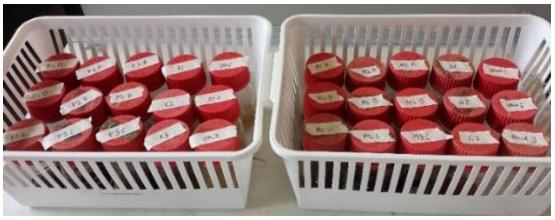

Figure D-6: Soil samples prepared for autoclaving at 120°C in 30 minutes and subsequent ICP-analysis.

All samples were filtered after autoclaving into a volumetric flask (50 mL) and demineralised water was added to reach a fixed volume. The samples were then analysed using ICP-OES.

D.2.1 Results

Blanks

Three blanks were made with demineralised water, nitric acid and treated as the samples. The results for the analysis of the blanks are seen in *Table D-3*.

Table D-3: Element concentration in three blanks made of 20 mL HNO_3 and treated as the samples.

Element	Blank 1 [mg/L]	Blank 2 [mg/L]	Blank 3 [mg/L]	Mean [mg/L]	SD [mg/L]
Al	0.171	0.197	0.187	0.185	0.013
Sb	0.048	0.045	0.043	0.045	0.003
As	0.000	0.000	0.000	0.000	0.000
Ba	0.055	0.055	0.053	0.054	0.001
Cd	0.063	0.065	0.067	0.065	0.002
Co	0.052	0.051	0.053	0.052	0.001
Cr	0.063	0.060	0.068	0.064	0.004
Cu	0.180	0.094	0.121	0.132	0.044
Fe	0.000	0.000	0.000	0.000	0.000
Mn	0.067	0.067	0.068	0.067	0.001
Mo	0.052	0.049	0.053	0.051	0.002
Ni	0.072	0.068	0.067	0.069	0.003
Pb	0.104	0.094	0.098	0.099	0.005
Ag	0.043	0.039	0.041	0.041	0.002
Zn	0.046	0.043	0.042	0.044	0.002
P	0.017	0.025	0.023	0.022	0.004
K	1.523	1.539	1.525	1.529	0.009

Field that has received pig manure

The analysis was carried out with a triple determination of all samples. The dried soil samples were weighed and the amounts for the field that has received pig manure are seen in *Table D-4*.

Table D-4: Weighed amounts of soil samples from the field that has received pig manure.

Site 1	Mass [g]	Site 2	Mass [g]	Site 3	Mass [g]
Sample 1	0.7428	**Sample 1**	0.7829	**Sample 1**	0.7944
Sample 2	1.005	**Sample 2**	1.019	**Sample 2**	1.012
Sample 3	1.235	**Sample 3**	1.207	**Sample 3**	1.227

All results from ICP [mg/L] are corrected for the blank mean value and converted with the weighed amount and dilution to mg per kg dry soil. The results for the field that has received pig manure are seen in *Table D-5*, *Table D-6* and *Table D-7*.

Table D-5: Metals in soil sample from site 1 at the field that has received pig manure.

Element	ICP 1 [mg/L]	ICP 2 [mg/L]	ICP 3 [mg/L]	1 [mg/kg dry soil]	2 [mg/kg dry soil]	3 [mg/kg dry soil]	Mean	SD
Al	52.59	83.55	91.93	3528	4146	3715	**3796**	317
Sb	0.046	0.046	0.045	0.067	0.050	0.000	**0.039**	0.035
As	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Ba	0.254	0.359	0.377	13.5	15.2	13.1	**13.9**	1.11
Cd	0.063	0.065	0.063	0.000	0.000	0.000	**0**	0
Co	0.064	0.072	0.073	0.808	0.995	0.850	**0.884**	0.098
Cr	0.134	0.173	0.183	4.71	5.42	4.82	**4.98**	0.382
Cu	0.311	0.393	0.397	12.05	13.0	10.7	**11.9**	1.13
Fe	58.44	87.00	99.85	3933	4327	4043	**4101**	203
Mn	2.668	4.034	4.615	175	197	184	**186**	11.2
Mo	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Ni	0.109	0.126	0.128	2.69	2.83	2.39	**2.64**	0.228
Pb	0.223	0.344	0.299	8.35	12.2	8.10	**9.54**	2.29
Ag	0.001	0.000	0.000	0.000	0.000	0.000	**0**	0
Zn	0.507	0.747	0.792	31.2	35.0	30.3	**32.1**	2.48
P	7.892	8.298	10.332	530	412	418	**453**	66.6
K	7.921	9.532	10.924	430	398	380	**403**	25.2

Table D-6: Metals in soil sample from site 2 at the field that has received pig manure.

Element	ICP 1 [mg/L]	ICP 2 [mg/L]	ICP 3 [mg/L]	1 [mg/kg dry soil]	2 [mg/kg dry soil]	3 [mg/kg dry soil]	Mean	SD
Al	55.760	61.740	67.340	3549	3021	2783	**3118**	392
Sb	0.049	0.049	0.064	0.255	0.196	0.787	**0.413**	0.325
As	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Ba	0.270	0.298	0.325	13.8	12.0	11.2	**12.3**	1.32
Cd	0.062	0.062	0.063	0.000	0.000	0.000	**0**	0
Co	0.065	0.067	0.067	0.830	0.736	0.622	**0.729**	0.105
Cr	0.137	0.144	0.156	4.66	3.93	3.81	**4.13**	0.461
Cu	0.199	0.204	0.222	4.28	3.53	3.73	**3.85**	0.387
Fe	68.880	72.960	83.590	4399	3580	3464	**3814**	510
Mn	2.597	2.957	3.313	162	142	134	**146**	14.0
Mo	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Ni	0.107	0.150	0.113	2.43	3.97	1.82	**2.74**	1.11
Pb	0.202	0.216	0.231	6.58	5.74	5.47	**5.93**	0.578
Ag	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Zn	0.321	0.352	0.390	17.7	15.1	14.3	**15.7**	1.76
P	8.902	9.923	13.320	567	486	551	**535**	43.0
K	8.965	10.021	13.582	475	417	499	**464**	42.5

Table D-7: Metals in soil sample from site 3 at the field that has received pig manure.

Element	ICP 1 [mg/L]	ICP 2 [mg/L]	ICP 3 [mg/L]	1 [mg/kg dry soil]	2 [mg/kg dry soil]	3 [mg/kg dry soil]	Mean	SD
Al	51.270	69.670	88.520	3215	3434	3600	**3416**	193
Sb	0.048	0.047	0.048	0.189	0.099	0.122	**0.137**	0.047
As	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Ba	0.249	0.294	0.356	12.3	11.9	12.3	**12.1**	0.249
Cd	0.061	0.063	0.062	0.000	0.000	0.000	**0**	0
Co	0.066	0.068	0.074	0.881	0.791	0.896	**0.856**	0.057
Cr	0.138	0.160	0.170	4.66	4.74	4.32	**4.57**	0.224
Cu	0.286	0.310	0.356	9.69	8.80	9.13	**9.21**	0.453
Fe	60.870	72.770	89.480	3831	3596	3646	**3691**	124
Mn	2.814	3.391	4.518	173	164	181	**173**	8.56
Mo	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Ni	0.091	0.113	0.119	1.38	2.17	2.04	**1.87**	0.422
Pb	0.203	0.235	0.255	6.55	6.72	6.36	**6.54**	0.182
Ag	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Zn	0.476	0.565	0.680	27.2	25.7	25.9	**26.3**	0.789
P	7.252	8.942	10.337	455	441	420	**439**	17.5
K	6.982	8.983	9.761	343	368	335	**349**	17.2

Field that has received cattle manure

The weighed amounts and results for the field that has received cattle manure are seen in *Table D-8*, *Table D-9*, *Table D-10* and *Table D-11*.

Table D-8: Weighed amounts of soil samples from the field that has received cattle manure.

Site 1	Mass [g]	Site 2	Mass [g]	Site 3	Mass [g]
Sample 1	0.8825	**Sample 1**	0.8491	**Sample 1**	0.8272
Sample 2	1.083	**Sample 2**	1.070	**Sample 2**	1.015
Sample 3	1.306	**Sample 3**	1.232	**Sample 3**	1.280

Table D-9: Metals in soil sample from site 1 at the field that has received cattle manure.

Element	ICP 1 [mg/L]	ICP 2 [mg/L]	ICP 3 [mg/L]	1 [mg/kg dry soil]	2 [mg/kg dry soil]	3 [mg/kg dry soil]	Mean	SD
Al	50.550	59.120	75.450	2854	2722	2882	**2819**	85.2
Sb	0.054	0.044	0.047	0.510	0.000	0.077	**0.195**	0.275
As	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Ba	0.293	0.311	0.374	13.5	11.9	12.3	**12.6**	0.875
Cd	0.063	0.062	0.061	0.000	0.000	0.000	**0**	0
Co	0.064	0.066	0.070	0.680	0.647	0.689	**0.672**	0.022
Cr	0.126	0.137	0.155	3.51	3.37	3.48	**3.46**	0.074
Cu	0.185	0.195	0.250	3.00	2.91	4.52	**3.48**	0.903
Fe	56.40	65.85	81.29	3195	3042	3112	**3116**	77.0
Mn	4.222	5.012	6.218	235	228	236	**233**	4.07
Mo	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Ni	0.095	0.099	0.106	1.47	1.39	1.42	**1.43**	0.044
Pb	0.220	0.242	0.289	6.86	6.61	7.27	**6.9**	0.34
Ag	0.007	0.000	0.000	0.000	0.000	0.000	**0**	0
Zn	0.319	0.366	0.435	15.6	14.9	15.0	**15.1**	0.384
P	6.213	7.830	8.421	351	361	322	**344**	20.3
K	5.983	7.983	9.121	252	298	291	**280**	24.6

Table D-10: Metals in soil sample from site 2 at the field that has received cattle manure

Element	ICP 1 [mg/L]	ICP 2 [mg/L]	ICP 3 [mg/L]	1 [mg/kg dry soil]	2 [mg/kg dry soil]	3 [mg/kg dry soil]	Mean	SD
Al	50.810	63.210	70.760	2981	2945	2864	**2930**	59.8
Sb	0.045	0.043	0.050	0.000	0.000	0.203	**0.068**	0.117
As	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Ba	0.258	0.315	0.332	12.0	12.2	11.3	**11.8**	0.483
Cd	0.065	0.064	0.064	0.000	0.000	0.000	**0.000**	0.000
Co	0.063	0.068	0.067	0.648	0.748	0.609	**0.668**	0.072
Cr	0.124	0.140	0.148	3.53	3.55	3.41	**3.50**	0.077
Cu	0.177	0.192	0.202	2.65	2.80	2.84	**2.76**	0.101
Fe	54.30	68.66	77.02	3198	3208	3126	**3177**	44.7
Mn	4.013	5.795	5.916	232	268	237	**246**	19.1
Mo	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Ni	0.092	0.100	0.118	1.35	1.45	1.99	**1.60**	0.342
Pb	0.212	0.525	0.266	6.65	19.9	6.78	**11.1**	7.61
Ag	0.002	0.000	0.000	0.000	0.000	0.000	**0**	0.0
Zn	0.293	0.355	0.389	14.7	14.5	14.0	**14.4**	0.350
P	6.126	7.492	8.821	359	349	357	**355**	5.48
K	6.982	7.982	8.931	321	301	300	**308**	11.7

Table D-11: Metals in soil sample from site 3 at the field that has received cattle manure

Element	ICP 1 [mg/L]	ICP 2 [mg/L]	ICP 3 [mg/L]	1 [mg/kg dry soil]	2 [mg/kg dry soil]	3 [mg/kg dry soil]	Mean	SD
Al	43.160	56.510	68.330	2598	2774	2661	**2678**	89.2
Sb	0.055	0.050	0.046	0.604	0.246	0.039	**0.297**	0.286
As	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Ba	0.254	0.294	0.342	12.1	11.8	11.2	**11.7**	0.430
Cd	0.065	0.064	0.063	0.000	0.000	0.000	**0**	0
Co	0.062	0.065	0.069	0.604	0.640	0.664	**0.636**	0.030
Cr	0.121	0.134	0.144	3.45	3.45	3.12	**3.34**	0.186
Cu	0.208	0.208	0.217	4.59	3.74	3.32	**3.89**	0.649
Fe	61.08	65.55	71.60	3692	3228	2796	**3239**	448
Mn	3.972	4.806	5.984	236	233	231	**233**	2.49
Mo	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0
Ni	0.092	0.093	0.101	1.39	1.18	1.25	**1.27**	0.106
Pb	0.205	0.224	0.256	6.41	6.16	6.13	**6.23**	0.153
Ag	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0.0
Zn	0.304	0.380	0.429	15.7	16.5	15.0	**15.8**	0.757
P	6.248	7.821	8.021	376	384	312	**358**	39
K	6.132	6.591	7.322	278	249	226	**251**	26

Forest

These samples were analysed on a day different from the previous samples. Therefore, blanks were measured again, and the results for the analysis of the blanks are seen in *Table D-12*.

Table D-12: Element concentration in three blanks made of 20 mL HNO3 and treated as the samples.

Element	Blank 1 [mg/L]	Blank 2 [mg/L]	Blank 3 [mg/L]	Mean [mg/L]	SD [mg/L]
Al	0	0	0	0	0
Sb	0	0	0	0	0
As	0	0	0	0	0
Ba	0	0	0	0	0
Cd	0	0	0	0	0
Co	0	0	0	0	0
Cr	0	0	0	0	0
Cu	0.017	0.016	0.017	0.017	0.001
Fe	0	0	0	0	0
Mn	0	0	0	0	0
Mo	0	0	0	0	0
Ni	0	0	0	0	0
Pb	0.033	0.026	0.037	0.032	0.006
Ag	0	0	0	0	0
Zn	0	0	0	0	0
P	0.48	0.495	0.475	0.483	0.010
K	1.228	1.216	1.576	1.34	0.204

The weighed amounts and results for the forest soils are seen in *Table D-13, Table D-14*, *Table D-15*, and *Table D-16*.

Table D-13: Weighed amounts of soil samples from the forest.

Site 1	Mass [g]	Site 2	Mass [g]	Site 3	Mass [g]
Sample 1	11787	**Sample 1**	0.9106	**Sample 1**	0.9347
Sample 2	1.0121	**Sample 2**	1.0098	**Sample 2**	1.0262
Sample 3	0.8183	**Sample 3**	1.2212	**Sample 3**	1.2246

Table D-14: Metals in soil sample from site 1 in the forest.

Element	ICP 1 [mg/L]	ICP 2 [mg/L]	ICP 3 [mg/L]	1 [mg/kg dry soil]	2 [mg/kg dry soil]	3 [mg/kg dry soil]	Mean	SD
Al	29.26	36.58	31.99	1241	1807	1955	**1668**	377
Sb	0	0	0	0	0	0	**0**	0
As	0	0	0	0	0	0	**0**	0
Ba	0.079	0.134	0.109	3.35	6.62	6.66	**5.54**	1.90
Cd	0	0	0	0	0	0	**0**	0
Co	0	0	0	0	0	0	**0**	0
Cr	0.044	0.063	0.05	1.87	3.11	3.06	**2.68**	0.70
Cu	0.053	0.083	0.07	1.54	3.28	3.26	**2.69**	1.00
Fe	37.47	53.6	48.03	1589	2648	2935	**2391**	709
Mn	0.76	1.38	1.627	32.2	68.2	99.4	**66.6**	33.6
Mo	0	0	0	0	0	0	**0**	0
Ni	0.016	0.035	0.031	0.679	1.73	1.89	**1.434**	0.659
Pb	0.178	0.289	0.267	6.19	12.7	14.4	**11.08**	4.32
Ag	0	0	0	0	0	0	**0**	0
Zn	0.058	0.11	0.088	2.46	5.43	5.38	**4.42**	1.70
P	3.721	5.469	5.055	137	246	279	**221**	74
K	5.768	7.564	6.461	188	307	313	**269**	71

Table D-15: Metals in soil sample from site 2 in the forest.

Element	ICP 1 [mg/L]	ICP 2 [mg/L]	ICP 3 [mg/L]	1 [mg/kg dry soil]	2 [mg/kg dry soil]	3 [mg/kg dry soil]	Mean	SD
Al	20.81	26.12	23.74	1143	1293	972	**1136**	161
Sb	0	0	0	0	0	0	**0**	0
As	0	0	0	0	0	0	**0**	0
Ba	0.11	0.148	0.133	6.04	7.33	5.45	**6.27**	0.962
Cd	0	0	0	0	0	0	**0**	0
Co	0	0	0	0	0	0	**0**	0
Cr	0.037	0.049	0.04	2.03	2.43	1.64	**2.03**	0.394
Cu	0.074	0.089	0.076	3.15	3.58	2.43	**3.05**	0.582
Fe	25.06	35.71	27.93	1376	1768	1144	**1429**	316
Mn	1.024	1.582	1.208	56.2	78.3	49.5	**61.3**	15.1
Mo	0	0	0	0	0	0	**0**	0
Ni	0.024	0.037	0.025	1.32	1.83	1.02	**1.39**	0.409
Pb	0.255	0.346	0.265	12.2	15.5	9.5	**12.4**	3.01
Ag	0	0	0	0	0	0	**0**	0
Zn	0.099	0.135	0.095	5.44	6.68	3.89	**5.34**	1.40
P	3.903	4.931	4.287	188	220	156	**188**	32.2
K	4.838	5.69	5.409	192	215	167	**191**	24.4

Table D-16: Metals in soil sample from site 3 in the forest.

Element	ICP 1 [mg/L]	ICP 2 [mg/L]	ICP 3 [mg/L]	1 [mg/kg dry soil]	2 [mg/kg dry soil]	3 [mg/kg dry soil]	Mean	SD
Al	31.69	41.7	33.84	1695	2032	1382	**1703**	325
Sb	0	0	0	0	0	0	**0**	0
As	0	0	0	0	0	0	**0**	0
Ba	0.077	0.093	0.416	4.12	4.53	16.99	**8.55**	7.31
Cd	0	0	0	0	0	0	**0**	0
Co	0	0	0	0	0	0	**0**	0
Cr	0.051	0.067	0.05	2.73	3.26	2.04	**2.68**	0.61
Cu	0.049	0.06	0.081	1.73	2.11	2.63	**2.15**	0.45
Fe	43.51	51.82	46.45	2327	2525	1897	**2250**	321
Mn	0.596	0.702	1.048	31.9	34.2	42.8	**36.3**	5.7
Mo	0	0	0	0	0	0	**0**	0
Ni	0.022	0.025	0.043	1.18	1.22	1.76	**1.38**	0.323
Pb	0.172	0.209	0.165	7.49	8.62	5.43	**7.18**	1.62
Ag	0	0	0	0	0	0	**0**	0
Zn	0.071	0.110	0.175	3.80	5.36	7.15	**5.43**	1.67
P	3.3865	4.681	7.273	155	205	277	**212**	61
K	5.759	7.100	11.72	236	281	424	**314**	98

D.3 Total N

The total N content in the soils were determined in a similar way as in the manures. The total N determination was made on the dried soil samples, though. The soil was digested by weighing approximately 500.00 mg into a 100 mL autoclave bottle. Then 40 mL demineralised water and 8.0 mL potassium peroxodisulphate solution was added, see *Figure D-7*.

Figure D-7: Soil samples prepared for digestion with demineralised water and digestion solution (potassium peroxodisulphate solution).

The solution was autoclaved for 30 minutes at 120°C, see *Figure D-8*. After cooling to room temperature the samples were filtered into 50.00 mL volumetric flasks and diluted to the mark.

Figure D-8: Samples ready for autoclaving for 30 minutes at 120°C.

After filtration the samples were analysed by FIA as previously described, see *Figure D-9*. The method *Total nitrogen 0.1-5 mg/L* NO_3*-N* were used this time too.

Figure D-9: Soil samples being analysed by FIAstar 5000.

All samples were measured directly and since their concentrations were higher than the measuring range, all samples were diluted with a factor 5 and analysed again.

D.3.1 Results

Three blanks were made of demineralised water and digestion solution and treated as the samples. The results for the blanks are seen in *Table D-17*.

Table D-17: Blanks of demineralised water and digestion solution treated as the samples.

Blank	Concentration from FIA [mg N/L]
1	0.2
2	0.2
3	0.1
Mean	0.17

All samples were analysed with a triple determination and the results are seen in *Table D-18*, *Table D-19* and *Table D-20*. The results are converted with the dilution factor, corrected for blank and converted to total mg N per kg dry soil.

Table D-18: Total N in three soil samples from the field that has received cattle manure.

	Sample	Mass [g]	Concentration from FIA [mg N/L]	Converted with dilution factor [mg N/L]	Corrected for blank [mg N/L]	Total N [mg N/kg dry soil]
Site 1	1	0.4563	1.6	8.0	7.8	**858**
	2	0.5011	1.5	7.5	7.3	**732**
	3	0.5539	1.6	8.0	7.8	**707**
Site 2	1	0.4500	1.4	7.0	6.8	**759**
	2	0.5032	1.5	7.5	7.3	**729**
	3	0.5545	1.7	8.5	8.3	**751**
Site 3	1	0.4572	1.4	7.0	6.8	**747**
	2	0.5033	1.5	7.5	7.3	**729**
	3	0.5711	1.5	7.5	7.3	**642**

Table D-19: Total N in three soil samples from the field that has received pig manure.

	Sample	Mass [g]	Concentration from FIA [mg N/L]	Converted with dilution factor [mg N/L]	Corrected for blank [mg N/L]	Total N [mg N/kg dry soil]
Site 1	1	0.4493	1.0	5.0	4.8	**538**
	2	0.5017	1.2	6.0	5.8	**581**
	3	0.5709	1.4	7.0	6.8	**598**
Site 2	1	0.4512	1.0	5.0	4.8	**536**
	2	0.5017	1.1	5.5	5.3	**532**
	3	0.5539	0.8	4.0	3.8	**346**
Site 3	1	0.4498	1.4	7.0	6.8	**760**
	2	0.5089	1.7	8.5	8.3	**819**
	3	0.5584	1.9	9.5	9.3	**836**

Table D-20: Total N in three soil samples from the forest floor.

	Sample	Mass [g]	Concentration from FIA [mg N/L]	Converted with dilution factor [mg N/L]	Corrected for blank [mg N/L]	Total N [mg N/kg dry soil]
Site 1	1	0.4558	1.6	8.0	7.8	**859**
	2	0.5082	1.3	6.5	6.3	**623**
	3	0.5579	1.8	9.0	8.8	**792**
Site 2	1	0.4507	1.6	8.0	7.8	**869**
	2	0.5017	1.4	7.0	6.8	**681**
	3	0.5611	1.8	9.0	8.8	**787**
Site 3	1	0.4513	1.4	7.0	6.8	**757**
	2	0.5028	1.2	6.0	5.8	**580**
	3	0.5505	1.9	9.5	9.3	**848**

E Preparation of plant experiments

This appendix covers the analysis procedure and raw data results of the planting soil and watering water used for the plant experiment.

E.1 Planting soil

Dry and Organic Matter

The content of dry matter and organic matter in the planting soil were determined as previously described. The results are presented in *Table E-1* and *Table E-2*, respectively.

Table E-1: Dry matter content in the planting soil.

Sample	Crucible [g]	Crucible incl. Sample [g]	Sample [g]	After drying [g]	Dry matter (DM) [g/kg]	Average DM [g/kg]
Planting soil 1	75.1103	87.110	12.00	83.1241	**667.8**	**585**
Planting soil 2	62.3196	72.5678	10.2482	67.9959	**553.9**	
Planting soil 3	67.1654	75.5641	8.3987	71.6459	**533.5**	

Table E-2: Organic matter content in the planting soil.

Sample	Crucible [g]	Sample [g]	After ignition [g]	Residue on ignition [g]	Loss on ignition (OM) [g]	Average OM [g/kg]
Planting soil site 1	17.4681	0.4711	17.5015	70.9	**596.9**	**556**
Planting soil site 2	16.7585	0.5438	16.7641	10.3	**543.6**	
Planting soil site 3	16.1499	0.5266	16.1536	7.0	**526.4**	

Total N

Total N in the planting soil was determined on the dried soil samples as described in the previous appendix. A triple determination was carried out and the results are seen in *Table E-3*.

Table E-3: Total N in planting soil by FIA.

Sample	Mass [g]	Concentration from FIA [mg N/L]	Converted with dilution factor [mg N/L]	Corrected for blank [mg N/L]	Total N [mg N/kg dry soil]	Mean [mg N/kg dry soil]
1	0.4635	0.5	2.5	2.3	252	**264**
2	0.5008	0.6	3.0	2.8	283	
3	0.5500	0.6	3.0	2.8	258	

ICP-OES

The content of some selected metals in the planting soil were determined by ICP-OES as previously described with the method including 15 elements as well as P and K. Three soil samples were digested and the weighed amounts are seen in *Table E-4*.

Table E-4: Weighed amounts of planting soil samples for ICP-OES analysis.

Sample	Mass [g]
1	1.0357
2	0.9057
3	1.0355

The results are seen in *Table E-5*. The samples were analysed at the same time as the agricultural and forest soils, therefore the same blanks (*Table D-12*) are used in these calculations.

Table E-5: Metals in planting soil by ICP-OES.

Element	ICP 1 [mg/L]	ICP 2 [mg/L]	ICP 3 [mg/L]	1 [mg/kg dry soil]	2 [mg/kg dry soil]	3 [mg/kg dry soil]	Mean	SD
Al	30.78	28.58	25.91	1486	1578	1251	**1438**	168
Sb	0	0	0	0	0	0	**0**	0
As	0	0	0	0	0	0	**0**	0
Ba	0.0404	0.383	0.343	1.95	21.14	16.56	**13.22**	10.0
Cd	0	0	0	0	0	0	**0**	0
Co	0	0	0	0	0	0	**0**	0
Cr	0.045	0.042	0.038	2.17	2.32	1.83	**2.11**	0.248
Cu	0.072	0.062	0.062	2.67	2.50	2.19	**2.45**	0.245
Fe	37.94	36.1	33.99	1832	1993	1641	**1822**	176
Mn	0.976	0.912	0.85	47.1	50.3	41.0	**46.2**	4.72
Mo	0	0	0	0	0	0	**0**	0
Ni	0.036	0.032	0.029	1.74	1.77	1.40	**1.63**	0.204
Pb	0.119	0.113	0.109	4.20	4.47	3.72	**4.13**	0.382
Ag	0	0	0	0	0	0	**0**	0
Zn	0.139	0.125	0.114	6.71	6.90	5.50	**6.37**	0.757
P	6.956	6.239	5.906	312	318	262	**297**	30.9
K	13.28	10.38	9.925	576	499	415	**497**	81.0

E.2 Watering water (tap water)

The water used for the plant experiments were analysed by ICP-OES. Three blanks were made of 40 mL demineralised water and 10 mL concentrated HNO_3 and treated as the samples. The concentrations in the blanks are seen in *Table E-6*.

Table E-6: Element concentration in three blanks made of 40 mL demineralised water and 10 mL concentrated HNO_3 and treated as the samples.

Element	Blank 1 [mg/L]	Blank 2 [mg/L]	Blank 3 [mg/L]	Mean [mg/L]	SD [mg/L]
Al	0.134	0.137	0.135	0.135	0.002
Sb	0.082	0.078	0.081	0.080	0.002
As	0.038	0.029	0.033	0.033	0.005
B	0.331	0.287	0.335	0.318	0.027
Ba	0.071	0.072	0.07	0.071	0.001
Cd	0.048	0.049	0.05	0.049	0.001
Co	0.067	0.066	0.068	0.067	0.001
Cr	0.074	0.074	0.073	0.074	0.001
Cu	0.103	0.103	1.102	0.436	0.577
Fe	0.090	0.087	0.088	0.088	0.002
Mn	0.083	0.083	0.083	0.083	0.000
Mo	0.075	0.071	0.072	0.073	0.002
Ni	0.084	0.083	0.084	0.084	0.001
Pb	0.113	0.110	0.112	0.112	0.002
Ag	0.047	0.047	0.048	0.047	0.001
Se	0.040	0.045	0.039	0.041	0.003
Ti	0.104	0.103	0.103	0.103	0.001
Zn	0.069	0.070	0.069	0.069	0.001
Sr	0.003	0.004	0.003	0.003	0.001
V	0.000	0.000	0.000	0.000	0.000

Element	Blank 1 [mg/L]	Blank 2 [mg/L]	Blank 3 [mg/L]	Mean [mg/L]	SD [mg/L]
Tl	0.089	0.086	0.087	0.087	0.002
Si	20.07	19.2	20.03	19.767	0.491
P	0.122	0.207	0.187	0.172	0.044
K	1.016	1.324	1.293	1.211	0.170

The watering water were analysed by taking 40 mL water and 10 mL concentrated nitric acid. The solution was autoclaved at 120°C for 30 minutes. After cooling to room temperature, the samples were transferred to 100 mL volumetric flasks and diluted to the mark. The results are seen in *Table E-7*.

Table E-7: Metals in tap water used for watering in plant experiments. The concentration from ICP is corrected for the blank mean value and converted with dilution factor 2.5.

Element	ICP 1 [mg/L]	ICP 2 [mg/L]	ICP 3 [mg/L]	Sample 1 [mg/L]	Sample 2 [mg/L]	Sample 3 [mg/L]	Mean [mg/L]	SD [mg/L]
Al	0.117	0.129	0.174	0.000	0.000	0.097	**0.032**	0.056
Sb	0.082	0.084	0.082	0.004	0.009	0.004	**0.006**	0.003
As	0.043	0.032	0.053	0.024	0.000	0.049	**0.024**	0.025
B	0.195	0.296	0.257	0.000	0.000	0	**0**	0.000
Ba	0.118	0.119	0.134	0.118	0.120	0.158	**0.132**	0.022
Cd	0.049	0.049	0.05	0.000	0.000	0.002	**0.001**	0.001
Co	0.067	0.066	0.067	0.000	0.000	0	**0**	0.000
Cr	0.074	0.074	0.073	0.001	0.001	0	**0.001**	0.000
Cu	0.107	0.11	0.102	0.011	0.018	0	**0.010**	0.009
Fe	0.103	0.106	0.088	0.037	0.044	0.000	**0.027**	0.024
Mn	0.083	0.084	0.083	0.000	0.003	0.000	**0.001**	0.001
Mo	0.071	0.07	0.07	0.000	0.000	0.000	**0**	0.000
Ni	0.082	0.081	0.083	0.000	0.000	0.000	**0**	0.000
Pb	0.103	0.111	0.102	0.000	0.000	0.000	**0**	0.000
Ag	0.046	0.46	0.046	0.000	1.032	0.000	**0.344**	0.596
Se	0.048	0.049	0.05	0.017	0.019	0.022	**0.019**	0.003
Ti	0.106	0.102	0.103	0.007	0.000	-0.001	**0.002**	0.004
Zn	0.116	0.117	0.113	0.117	0.119	0.109	**0.115**	0.005
Sr	0.087	0.087	0.088	0.209	0.209	0.212	**0.210**	0.001
V	0.000	0.000	0.000	0.000	0.000	0.000	**0**	0.000
Tl	0.073	0.068	0.061	0.000	0.000	0.000	**0**	0.000
Si	5.031	17.86	4.181	0.000	0.000	0.000	**0**	0.000
P	0.137	0.175	0.140	0.000	0.000	0.000	**0**	0.000
K	1.632	1.622	1.607	1.053	1.028	0.990	**1.02**	0.031

F Radish experiment

This appendix covers the procedure as well as the obtained raw data results of the radish experiment.

F.1 Seeding procedure

Soil preparation

Cu and Zn were supplied in the form of the sulphate compounds $CuSO_4$ and $ZnSO_4{\cdot}7H_2O$ respectively. The content of Cu in Zn in the respective sources were calculated in order to know how much of the compounds that needs to be weighed in order to add 60 mg Cu per kg soil and 150 mg Zn per kg soil.

Table F-1: Calculation of Cu content (w/w %) in $CuSO_4$.

Element	Atomic weight	# of atoms	Mass percent
Cu	63.546	1	39.81
S	32.065	1	20.09
O	15.999	4	40.10
M($CuSO_4$) 159.6086 g/mole			

Amount of $CuSO_4$ that must be weighed in order to get 60 mg of Cu per kg soil:

$$\frac{60\,\frac{mg\ Cu}{kg\ soil}}{0.3981} = 150.72\,\frac{mg}{kg\ soil} = 0.1507\,\frac{g}{kg\ soil}$$

Table F-2: Calculation of Zn content (w/w %) in $ZnSO_4{\cdot}7H_2O$.

Element	Atomic weight	# of atoms	Mass percent
Zn	65.380	1	22.74
S	32.065	1	11.15
O	15.999	11	61.21
H	1.008	14	4.91
M($ZnSO_4{\cdot}7H_2O$) 287.53 g/mole			

Amount of $ZnSO_4{\cdot}7H_2O$ that must be weighed in order to get 150 mg of Zn per kg soil:

$$\frac{150\,\frac{mg\ Zn}{kg\ soil}}{0.2274} = 659.67\,\frac{mg}{kg\ soil} = 0.6598\,\frac{g}{kg\ soil}$$

Each plastic pot was filled with 1 kg soil, which was added the above-calculated amounts of Cu and Zn as well as 1.75 g KH_2PO_4 and 1.00 g NH_4NO_3 per kg soil according to the plan from design of experiment. The pot experiments were carried out with a triple determination. The actual weighed amounts are seen in *Table F-3*.

Table F-3: Actual weighed amounts for preparation of soil for radish experiment.

		KH_2PO_4 [g]	NH_4NO_3 [g]	$ZnSO_4 \cdot 7H_2O$ [g]	$CuSO_4$ [g]
Treatment 1	Run 11	1.7462	1.0044	0	0
	Run 12	1.7498	0.9953	0	0
	Run 13	1.7504	1.0002	0	0
Treatment 2	Run 21	1.7510	1.0141	0.6591	0.1505
	Run 22	1.7498	0.9954	0.6595	0.1507
	Run 23	1.7555	1.0017	0.6598	0.1509
Treatment 3	Run 31	1.7568	1.0018	0	0.1504
	Run 32	1.7494	1.0040	0	0.1509
	Run 33	1.7506	0.9954	0	0.1507
Treatment 4	Run 41	1.7517	0.9996	0.6596	0
	Run 42	1.7491	1.0057	0.6597	0
	Run 43	1.7511	1.0026	0.6598	0

The chemicals were added the soil by mixing them thoroughly in a mortar and continuously increasing the amount of soil. The procedure was carried on until 1 kg of soil were mixed with the weighed portion of chemicals. The procedure is seen in *Figure F-1*.

Figure F-1: The chemicals were added by thorough mixing with a continually increasing amount of soil in a mortar.

Seeding

The radish seeds were purchased in the locale supermarket and are seen in *Figure F-2*. The radishes were sown at a rate of 15 seeds per pot in about 1 cm of depth. All pots were watered with tap water. The amount was not noted down, but all pots received exactly the same amount of water.

Figure F-2: Radish (Raphanus sativus) seeds used for the experiment.

The pots were placed randomly in a sunroom with no controlled climate. The seeds were allowed to germinate and establish for 10 days and then the seedlings were thinned to nine seedlings per pot. The pots after seeding and 10 days respectively are seen in *Figure F-3 and Figure F-4*.

Figure F-3: Pot experiments with radish after seeding and 10 days growth in a sunroom.

Figure F-4: Plastic pot with radish seedlings after 10 days growth in a sunroom.

After 21 days, the pots were moved to a greenhouse due to better and warmer weather. The radish plants are seen in *Figure F-5*.

Figure F-5: Radish plants (after 36 days of growth) placed randomly in a greenhouse.

The radishes were harvested after 36 days and the soils were sampled at the same time.

F.2 Cu and Zn in radish plants

The radish were divided into two parts, the aboveground part and root, as seen in *Figure F-6*.

Figure F-6: Radish plants were divided in two parts; aboveground part and roots.

The plants were washed with tap water to remove any attached soil particles, and rinsed twice with demineralised water. Fresh plant weights were weighed and the plant samples were dried at 105°C to a constant weight after cutting to smaller pieces. The plants were dried in aluminium trays as seen in *Figure F-7*.

Figure F-7: Radish plants prepared for drying.

Radish plant (aboveground) after drying are seen in *Figure F-8*.

Figure F-8: Radish plant (aboveground) after drying.

After drying the plant material was milled in a mortar to powder as seen in *Figure F-9*.

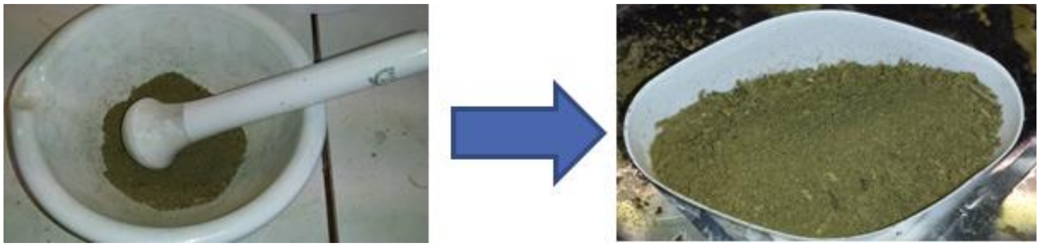

Figure F-9: The dried plant material was milled in a mortar to a powder.

A small amount was accurately weighed in an autoclave bottle and added 20 mL of 7 M HNO_3 for digestion at 120°C for 30 minutes. After cooling to room temperature, the samples were filtered through ordinary filter paper and diluted to 50.00 mL with demineralised water.

All samples were analysed by ICP-OES with a similar method as the previous only measuring the elements Cu and Zn, though.

F.2.1 Results

Biomass

The fresh weight of the radish plants are seen in *Table F-4*.

Table F-4: Biomass (fresh weight) of the aboveground part and root of the radish plants.

Fresh weight	Aboveground					Root				
	Tray [g]	Tray + sample [g]	Mass [g]	Mean [g]	SD [g]	Tray [g]	Tray + sample [g]	Mass [g]	Mean [g]	SD [g]
Run 11	11.36	98.79	87.43	**80.9**	7.62	5.91	13.24	7.33	**7.31**	0.475
Run 12	11.33	83.83	72.50			5.92	12.74	6.82		
Run 13	11.32	93.96	82.64			5.90	13.67	7.77		
Run 21	21.28	173.81	152.53	**135.8**	18.2	5.91	80.26	74.35	**65.44**	8.86
Run 22	21.36	159.74	138.38			5.91	71.24	65.33		
Run 23	21.35	137.70	116.35			5.90	62.54	56.64		
Run 31	21.4	167.12	145.72	**149.6**	4.34	6.08	64.52	58.44	**52.07**	5.56
Run 32	21.31	175.62	154.31			6.10	54.29	48.19		
Run 33	21.28	170.18	148.90			6.05	55.63	49.58		
Run 41	11.21	110.37	99.16	**98.3**	8.06	5.92	13.46	7.54	**11.16**	3.15
Run 42	11.32	117.28	105.96			5.89	18.56	12.67		
Run 43	11.21	101.11	89.90			5.91	19.19	13.28		

Table F-5: Total biomass (fresh weight) of the radish plants.

	Total Biomass [g]	Mean [g]	SD [g]
Run 11	94.76	**88.16**	7.96
Run 12	79.32		
Run 13	90.41		
Run 21	226.9	**201.2**	27.0
Run 22	203.7		
Run 23	173.0		
Run 31	204.2	**201.7**	2.92
Run 32	202.5		
Run 33	198.5		
Run 41	106.7	**109.5**	8.10
Run 42	118.6		
Run 43	103.2		

ICP

The measured concentration of Cu and Zn in the blanks are seen in *Table F-6*.

Table F-6: Measured concentration of Cu and Zn in blank samples.

Element	Blank 1 [mg/L]	Blank 2 [mg/L]	Blank 3 [mg/L]	Average [mg/L]
Cu	0.001	0.004	0.003	**0.003**
Zn	0.000	0.000	0.000	**0.000**

The concentration of Cu and Zn in the aboveground part of radish plants are seen in *Table F-7*.

Table F-7: Cu and Zn in aboveground part of radish plants.

	Mass [g]	Cu ICP [mg/L]	Zn ICP [mg/L]	Cu corrected for blank [mg/L]	Zn corrected for blank [mg/L]	Cu in radish [mg/kg]	Zn in radish [mg/kg]
Run 11A	0.9913	0.008	0.503	0.005	0.503	**0.252**	**25.4**
Run 11B	0.7451	0.018	0.538	0.015	0.538	**1.007**	**36.1**
Run 12A	0.8062	0.009	0.467	0.006	0.467	**0.372**	**29.0**
Run 12B	0.9954	0.022	0.59	0.019	0.59	**0.954**	**29.6**
Run 13A	0.7945	0.032	0.677	0.029	0.677	**1.825**	**42.6**
Run 13B	1.0015	0.017	0.541	0.014	0.541	**0.699**	**27.0**
Run 21A	0.7456	0.062	2.21	0.059	2.21	**3.96**	**148.2**
Run 21B	0.9555	0.073	2.493	0.07	2.49	**3.66**	**130.5**
Run 22A	0.7676	0.051	1.752	0.048	1.75	**3.13**	**114.1**
Run 22B	0.9496	0.046	1.359	0.043	1.36	**2.26**	**71.6**
Run 23A	0.8186	0.056	1.499	0.053	1.50	**3.24**	**91.6**
Run 23B	0.9972	0.058	1.779	0.055	1.78	**2.76**	**89.2**
Run 31A	0.7123	0.055	0.245	0.052	0.245	**3.65**	**17.2**
Run 31B	0.9935	0.085	0.357	0.082	0.357	**4.13**	**18.0**
Run 32A	0.7260	0.073	0.345	0.07	0.345	**4.82**	**23.8**
Run 32B	0.9519	0.102	0.421	0.099	0.421	**5.20**	**22.1**
Run 33A	0.7220	0.068	0.337	0.065	0.337	**4.50**	**23.3**
Run 33B	0.9653	0.084	0.391	0.081	0.391	**4.20**	**20.3**
Run 41A	0.7083	0.012	1.536	0.009	1.54	**0.635**	**108.4**
Run 41B	0.9567	0.014	1.915	0.011	1.915	**0.575**	**100.1**
Run 42A	0.7138	0.017	1.608	0.014	1.61	**0.981**	**112.6**
Run 42B	0.9654	0.019	2.160	0.016	2.16	**0.829**	**111.9**
Run 43A	0.7032	0.008	1.958	0.005	1.958	**0.356**	**139.2**
Run 43B	0.9573	0.012	2.661	0.009	2.66	**0.470**	**139.0**

The concentration of Cu and Zn in root of the radish plants are seen in *Table F-8*. In run 1 and 4 the amount of radish roots only was enough to make a duplicate determination.

Table F-8: Cu and Zn in root of radish plant.

	Mass [g]	Cu ICP [mg/L]	Zn ICP [mg/L]	Cu corrcted for blank [mg/L]	Zn corrected for blank [mg/L]	Cu in radish [mg/kg]	Zn in radish [mg/kg]
Run 1A	0.7976	0.023	0.748	0.020	0.748	**1.25**	**46.9**
Run 1B	0.7695	0.014	0.546	0.011	0.546	**0.71**	**35.5**
Run 21A	0.9325	0.037	1.585	0.034	1.585	**1.82**	**85.0**
Run 21B	1.0666	0.046	1.785	0.043	1.785	**2.02**	**83.7**
Run 22A	0.7867	0.033	0.884	0.03	0.884	**1.91**	**56.2**
Run 22B	1.0098	0.045	1.062	0.042	1.062	**2.08**	**52.6**
Run 23A	0.8228	0.034	0.986	0.031	0.986	**1.88**	**59.9**
Run 23B	0.9795	0.037	1.164	0.034	1.164	**1.74**	**59.4**
Run 31A	0.7452	0.042	0.333	0.039	0.333	**2.62**	**22.3**
Run 31B	1.0099	0.053	0.343	0.05	0.343	**2.48**	**17.0**
Run 32A	0.7257	0.039	0.413	0.036	0.413	**2.48**	**28.5**
Run 32B	0.9847	0.052	0.507	0.049	0.507	**2.49**	**25.7**
Run 33A	0.7602	0.039	0.3	0.036	0.300	**2.37**	**19.7**
Run 33B	1.0208	0.053	0.421	0.05	0.421	**2.45**	**20.6**
Run 4A	0.8322	0.014	1.711	0.011	1.711	**0.66**	**102.8**
Run 4B	0.9566	0.03	2.018	0.027	2.018	**1.41**	**105.5**

F.3 Cu and Zn in pot experiment soil

The soil samples were dried in aluminium trays at 105°C to a constant weight was obtained, as seen in *Figure F-10*.

Figure F-10: Drying of soil samples for determination of Cu and Zn concentration.

After drying, a small amount was accurately weighed in an autoclave bottle and added 20 mL of 7 M HNO_3 for digestion at 120°C for 30 minutes. After cooling to room temperature, the samples were filtered through ordinary filter paper and diluted to 50.00 mL with demineralised water. All samples were analysed using ICP-OES for the concentration of Cu and Zn.

F.3.1 Results

The concentration of Cu and Zn in the soil before seeding are seen in *Table F-9*.

Table F-9: M: Concentration of Cu and Zn in soil from pot experiment before seeding.

Sample	Mass [g]	Cu ICP [mg/L]	Zn ICP [mg/L]	Cu in soil [mg/kg]	Zn in soil [mg/kg]	Mean Cu [mg/kg]	Mean Zn [mg/kg]
Run 11	0.9033	0.078	0.153	4.32	8.5	**4.60**	**8.77**
Run 12	0.8744	0.081	0.162	4.63	9.3		
Run 13	0.8442	0.082	0.145	4.86	8.6		
Run 21	0.9462	1.206	3.231	63.7	171	**66.3**	**171**
Run 22	0.8694	1.198	2.921	68.9	168		
Run 23	0.9034	1.198	3.125	66.3	173		
Run 31	0.7877	1.225	0.168	77.8	10.7	**80.0**	**10.1**
Run 32	0.9537	1.621	0.189	85.0	9.91		
Run 33	0.9980	1.545	0.192	77.4	9.62		
Run 41	0.8642	0.085	2.802	4.92	162	**4.87**	**166**
Run 42	0.9075	0.092	2.995	5.07	165		
Run 43	0.8762	0.081	2.987	4.62	170		

The concentration of Cu and Zn in the soil after harvest are seen in *Table F-10*.

Table F-10: Concentration of Cu and Zn in soil from pot experiment after harvest of radish plant.

Sample	Mass [g]	Cu ICP [mg/L]	Zn ICP [mg/L]	Cu in soil [mg/kg]	Zn in soil [mg/kg]	Mean Cu [mg/kg]	Mean Zn [mg/kg]
Run 11	0.9021	0.069	0.143	3.82	7.93	**3.82**	**8.77**
Run 12	0.8689	0.062	0.143	3.57	8.23		
Run 13	0.8512	0.069	0.144	4.05	8.46		
Run 21	0.9482	1.159	3.127	61.1	165	**64.5**	**166**
Run 22	0.8709	1.195	2.886	68.6	166		
Run 23	0.9051	1.157	3.005	63.9	166		
Run 31	0.7902	1.339	0.145	84.7	9.17	**85.1**	**8.94**
Run 32	0.9487	1.556	0.185	82.0	9.75		
Run 33	0.9835	1.745	0.155	88.7	7.88		
Run 41	0.8731	0.064	2.504	3.67	143	**4.09**	**132**
Run 42	0.9032	0.077	2.760	4.26	153		
Run 43	0.8751	0.076	1.768	4.34	101		

F.4 Plant-available concentration of Cu and Zn in soil

About 5.00 g of dried soil were suspended in 50 mL of 0.01 M $CaCl_2$ and heated at 90°C for 30 minutes in a water bath, as seen in *Figure F-11*.

Figure F-11: The dried soil were mixed with 0.01 M $CaCl_2$ and heated at 90°C for 30 minutes.

After heating, the suspensions were vacuum filtered hot through Whatman 42 filter paper, as seen in *Figure F-12*.

Figure F-12: All samples were vacuum filtered hot trough Whatman 42 filter paper.

After filtration of the suspension, 4 drops of 1 M HNO_3 were added to prevent metal precipitation. The filtered solutions (seen in *Figure F-13*) were analysed by ICP-OES.

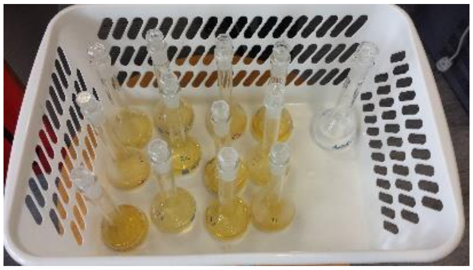

Figure F-13: All samples were added 4 drops of 1 M HNO_3 after filtration to prevent metal precipitation.

A blank was prepared with 50 mL of 0.01 M $CaCl_2$ and treated as the samples, by heating and addition of 4 drops HNO_3 after heating.

F.4.1 Results

The concentration of plant-available Cu and Zn in soil after harvest are seen in *Table F-11*. The analysis of the *blank samples* did not show any concentration of Cu or Zn.

Table F-11: Plant-available Cu and Zn in soil after harvest.

Sample	Mass [g]	Cu ICP [mg/L]	Zn ICP [mg/L]	Cu in soil [mg/kg]	Zn in soil [mg/kg]	Mean Cu [mg/kg]	Mean Zn [mg/kg]
Run 11	4.9020	0	0	0	0	**0.025**	**0.035**
Run 12	5.2333	0.008	0.011	0.076	0.105		
Run 13	5.8576	0	0	0	0		
Run 21	5.0753	0.057	0.273	0.562	2.69	**0.703**	**2.74**
Run 22	4.9993	0.075	0.272	0.750	2.72		
Run 23	5.0702	0.081	0.284	0.799	2.80		
Run 31	4.9392	0.073	0.013	0.739	0.13	**0.699**	**0.044**
Run 32	4.6168	0.074	0	0.801	0		
Run 33	5.2032	0.058	0	0.557	0		
Run 41	4.8322	0	0.183	0	1.89	**0.00**	**2.30**
Run 42	4.9437	0	0.296	0	2.99		
Run 43	4.8684	0	0.195	0	2.00		

F.5 Statistical analysis – MATLAB

The full factorial radish experiment is analysed using MATLAB. In this analyse a function named *genff2n* are used. The MATLAB script for this function is shown below:

```
function m = genff2n(factors)

% GENFF2N generates matrices for 2^n full factorial design
%
% Inputs:
% ------
% FACTORS ? nlevels x nfactors matrix with factors
%
% Outputs:
% -------
% M ? a structure with 5 elements
% M.DM - a design matrix with 0 and 1 values
% M.DMB - a design matrix with -1 and +1 values
% M.DMF - a design matrix with factor levels
% M.CMB - a contrast matrix with all factor interactions
% M.CMB_IDX - a matrix with factor indices for each interaction
%

   [nlevels, nfact] = size(factors);

   if nlevels ~= 2
      error('Factors shall have 2 levels');
   end

   dm = ff2n(nfact);
   dm = dm(:, end:-1:1);
   dmb = dm * 2 - 1;
```

```
    ntrials = size(dm , 1);

    dmf = zeros(ntrials, nfact);
    for i = 1:nfact
       dmf(:, i) = factors(dm(:, i) + 1, i);
    end

    cmb = [];
    cmb_idx = {};
    for k = 2:nfact
       idx = combnk(1:nfact, k);
       if idx(1, 1) > idx(end, 1)
          idx = idx(end:-1:1, :);
       end
       for i = 1:size(idx, 1)
          cmb = [cmb prod(dmb(:, idx(i, :)), 2)];
          cmb_idx = [cmb_idx; {idx(i, :)}];
       end
    end

    m.dm = dm;
    m.dmb = dmb;
    m.dmf = dmf;
    m.cmb = cmb;
    m.cmb_idx = cmb_idx;
end
```

F.5.1 Radish biomass

The full factorial radish experiment is analysed with different results as the response. In this section biomass is used as the response. The biomass is split into three responses, including: total, aboveground and root biomass. The MATLAB script are presented below.

```
clear
clc
close all

factors=[0 0;60 150];
factor_names={'Cu','Zn'};
y=[88.21; 201.67; 109.46; 201.24]; % Total Biomass [Run1 Run3 Run4 Run2]

% Generate DM
m=genff2n(factors);

dm=m.dmb;
cm=m.cmb;

% Calculate effects
fme=y'*dm/2;
fie=y'*cm/2;

disp(fme)
```

```
disp(fie)

figure
maineffectsplot(y, dm, 'varnames', factor_names
title('Main Effect Total Biomass','Fontsize',24,'FontWeight','bold')

figure
interactionplot(y, dm, 'varnames', factor_names)
title('Interaction Effect Total Biomass', 'Fontsize',24,'Font-
Weight','bold')

%% Aboveground part
y1=[80.9; 149.6; 98.3; 135.8]; %Biomass Aboveground [Run1 Run3 Run4 Run2]

m=genff2n(factors);

dm=m.dmb;
cm=m.cmb;

fme=y1'*dm/2;
fie=y1'*cm/2;

disp(fme)
disp(fie)

figure
maineffectsplot(y1, dm, 'varnames', factor_names
title('Main Effect Aboveground Part','Fontsize',24,'FontWeight','bold')

figure
interactionplot(y1, dm, 'varnames', factor_names)
title('Interaction Effect Aboveground Part', 'Fontsize',24,'Font-
Weight','bold')

%% Root
y1=[7.31; 52.07; 11.16; 65.44]; % Root Biomass [Run1 Run3 Run4 Run2]

m=genff2n(factors);

dm=m.dmb;
cm=m.cmb;

fme=y1'*dm/2;
fie=y1'*cm/2;

disp(fme)
disp(fie)

figure
maineffectsplot(y1, dm, 'varnames', factor_names
title('Main Effect Root','Fontsize',24,'FontWeight','bold')

figure
```

```
interactionplot(y1, dm, 'varnames', factor_names)
title('Interaction Effect Root', 'Fontsize',24,'FontWeight','bold')
```

F.5.2 Cu and Zn uptake in radish

In this section, the uptake of Cu and Zn, respectively is used as the response. The MATLAB script are presented below.

```
clear
clc
close all

%% Cu i Radish
factors=[0 0;60 150];
factor_names={'Cu','Zn'};
y=[0.852; 4.42; 0.641; 3.17]; %  Cu in Radish [Run1 Run3 Run4 Run2]

% 1. Generate DM
m=genff2n(factors);

dm=m.dmb;
cm=m.cmb;

% Calculate effects
fme=y'*dm/2;
fie=y'*cm/2;

disp(fme)
disp(fie)

figure
maineffectsplot(y, dm, 'varnames', factor_names
title('Main Effect - Cu in Aboveground Part of Ra-
sish','Fontsize',24,'FontWeight','bold')

figure
interactionplot(y, dm, 'varnames', factor_names)
title('Interaction Effect - Cu in Aboveground Part of Radish',
'Fontsize',24,'FontWeight','bold')

% Cu in Radish root
y1=[0.984; 2.48; 1.04; 1.91]; % Cu in radish root [Run1 Run3 Run4 Run2]

% 1. Generate DM
m=genff2n(factors);

dm=m.dmb;
cm=m.cmb;

% Calculate effects
fme=y1'*dm/2;
fie=y1'*cm/2;
```

```
disp(fme)
disp(fie)

figure
maineffectsplot(y1, dm, 'varnames', factor_names
title('Main Effect - Cu in Radish Root','Fontsize',24,'Font-
Weight','bold')

figure
interactionplot(y1, dm, 'varnames', factor_names)
title('Interaction Effect - Cu in Radish Root', 'Fontsize',24,'Font-
Weight','bold')

%% Zn in Radish
factors=[0 0;60 150];
factor_names={'Cu','Zn'};
y=[31.6; 20.8; 119; 108]; %  Zn in Radish [Run1 Run3 Run4 Run2]

% 1. Generate DM
m=genff2n(factors);

dm=m.dmb;
cm=m.cmb;

% Calculate effects
fme=y'*dm/2;
fie=y'*cm/2;

disp(fme)
disp(fie)

figure
maineffectsplot(y, dm, 'varnames', factor_names
title('Main Effect - Zn in Aboveground Part of Ra-
dish','Fontsize',24,'FontWeight','bold')

figure
interactionplot(y, dm, 'varnames', factor_names)
title('Interaction Effect - Zn in Aboveground Part of Radish ',
'Fontsize',24,'FontWeight','bold')

% Zn Radish root
y1=[41.2; 22.3; 104; 66.1]; % Zn in Radish Root [Run1 Run3 Run4 Run2]

% 1. Generate DM
m=genff2n(factors);

dm=m.dmb;
cm=m.cmb;

% Calculate effects
fme=y1'*dm/2;
```

```
fie=y1'*cm/2;

disp(fme)
disp(fie)

figure
maineffectsplot(y1, dm, 'varnames', factor_names
title('Main Effect - Zn in Radish Root','Fontsize',24,'Font-
Weight','bold')

figure
interactionplot(y1, dm, 'varnames', factor_names)
title('Interaction Effect - Zn in Radish Root', 'Fontsize',24,'Font-
Weight','bold')
```

G Cu and Zn in cress by application of pig manure

This appendix covers the procedure as well as the obtained raw data results of the cress experiment with pig manure.

G.1 Seeding procedure

Soil preparation

Cu and Zn were supplied in the form of the sulphate compounds $CuSO_4$ and $ZnSO_4 \cdot 7H_2O$ respectively. The content of Cu in Zn in the respective sources were calculated in order to know how much of the compounds that needs to be weighed in order to add 60 mg Cu per kg soil and 150 mg Zn per kg soil. Reference is made to the previous experiment (*F.1 Seeding procedure*) for detailed calculations. Each plastic pot was filled with 0.6 kg soil.

Amount of $CuSO_4$ that must be weighed in order to get 60 mg of Cu per kg soil:

$$\frac{60\,\frac{mg\ Cu}{kg\ soil}}{0.3981} \cdot 0.6\,\frac{kg\ soil}{pot} = 90.43\,\frac{mg}{pot} = 0.0904\,\frac{g}{pot}$$

Amount of $ZnSO_4 \cdot 7H_2O$ that must be weighed in order to get 150 mg of Zn per kg soil:

$$\frac{150\,\frac{mg\ Zn}{kg\ soil}}{0.2274} \cdot 0.6\,\frac{kg\ soil}{pot} = 395.8\,\frac{mg}{pot} = 0.3958\,\frac{g}{pot}$$

Each pot was added the calculated amounts of Cu and Zn from the sulphate compounds according to the plan from design of experiment. The pot experiments were carried out with a duplicate determination. The actual weighed amounts are seen in *Table G-1*.

Table G-1: Actual weighed amounts for preparation of metal addition to the soil.

Run	$ZnSO_4 \cdot 7H_2O$ [g]	$CuSO_4$ [g]	Run	$ZnSO_4 \cdot 7H_2O$ [g]	$CuSO_4$ [g]
1-1	0	0	**1-2**	0	0
2-1	0	0	**2-2**	0	0
3-1	0	0.0908	**3-2**	0	0.0911
4-1	0	0.0901	**4-2**	0	0.0912
5-1	0.3951	0	**5-2**	0.3955	0
6-1	0.3960	0	**6-2**	0.3961	0
7-1	0.3955	0.9010	**7-2**	0.3959	0.0904
8-1	0.3959	0.0912	**8-2**	0.3952	0.0910

The chemicals were mixed with the soil in each pot as described in the previous experiment *F.1 Seeding procedure*. Some of the pots are seen in *Figure G-1*.

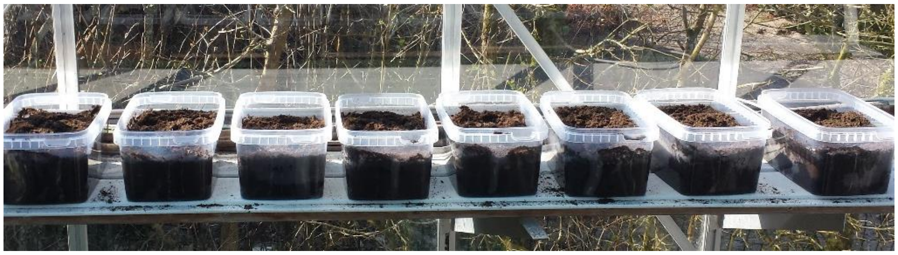

Figure G-1: Preparation of soil for cress experiment.

In addition to the metals Cu and Zn, some of the soils were supplied with pig manure according the plan from design of experiment, see *Figure G-2*.

Figure G-2: Pig manure was added some pots according to the plan from design of experiment.

The amount of added manure were calculated from the harmony rules and the soil surface area in the pots used for the experiment, illustrated in *Figure G-3*.

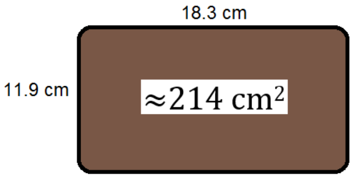

Figure G-3: Size of pots used for pot experiment and calculated area of soil surface.

The surface area of the pots is:

$$11.9\ cm \ \cdot 18.3\ cm = 218\ cm^2$$

However, the corners of the pots are round and the real soil surface in the pots is therefore a little smaller than the above calculated. The soil surface area is therefore assumed to be the above calculated area minus four times 1 cm^2 and hence 214 cm^2. The harmony rule states that maximum 140 kg nitrogen from a pig production may be applied per hectare. The content of nitrogen in the pig manure used in this experiment was determined to 1.739 kg/t. The maximum permitted amount applied to fields of this manure is therefore calculated as:

$$Maximum\ ton\ per\ hectare: \frac{140\ kg\ N\ per\ ha}{1.739\ kg/t} = 80.5\ t/ha$$

In order to calculate the amount of manure, which gives a realistic demonstration of the conditions on a real farmland reflected in the pot experiments the area of the soil surface in the pots were calculated to 214 cm^2. In order to convert 80.5 ton/ha to units suited for the pot experiment, the following calculation are made:

$$214\ cm^2 = 214\ cm^2 \cdot 10^{-8} \frac{ha}{cm^2} = 0.00000214\ ha$$

$$Amount\ per\ pot\ [t]: 80.5\,\frac{t}{ha}\cdot 0.00000214\ ha = 0.000172\ t$$

$$Amount\ per\ pot\ [g]: 0.000172\ t\cdot 10^{6}\,\frac{g}{t} = \underline{172\ g\ per\ pot}$$

The actual weighed amounts of manure for the treatments with manure are seen in *Table G-2*.

Table G-2: Actual weighed amounts of pig manure for the treatments with manure.

Run	Pig Manure [g]	Run	Pig Manure [g]
2-1	172.7	**2-2**	173.5
4-1	173.2	**4-2**	173.1
6-1	172.1	**6-2**	172.8
8-1	173.6	**8-2**	171.9

The manure were added by pouring it out on the soil surface before seeding.

Seeding

The cress seeds were purchased in the locale supermarket and are seen in *Figure G-4*. The cress were sown at a rate of 5 g per pot.

Figure G-4: Cress (Lepidium sativum) seeds used for the experiment.

All pots were watered with tap water. The amount was not noted down, but all pots received exactly the same amount of water. The pots after seeding are seen in *Figure G-5*.

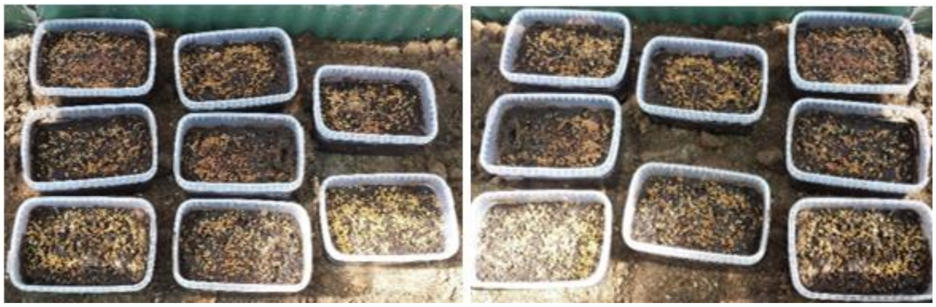

Figure G-5: Pot experiment with cress after seeding.

The cress were harvested after 25 days (see *Figure G-6*) and the soils were sampled at the same time.

Figure G-6: Pot experiment with cress 25 days after sowing.

The uptake of Cu and Zn was determined by analysis of the Cu and Zn concentration in the cress by ICP-OES as described in the following.

G.2 Cu and Zn in cress

The cress was harvested by cutting it just above the soil surface, as seen in *Figure G-7*.

Figure G-7: The cress was cut just above the soil surface and placed in aluminium trays for drying.

The cress was placed in aluminium trays for drying at 105°C to a constant weight was obtained.

Figure G-8: After drying the cress was milled to powder in a mortar.

After drying, the cress was milled to powder in a mortar as seen in *Figure G-8*. A small amount was accurately weighed in an autoclave bottle and added 20 mL of 7 M HNO_3 for digestion at 120°C for 30 minutes. After cooling to room temperature, the samples were filtered through ordinary filter paper and diluted to 50.00 mL with demineralised water.

G.2.1 Results

The measured concentration of Cu and Zn in the blanks are seen in *Table G-3*.

Table G-3: Measured concentration of Cu and Zn in blank samples.

Element	Blank 1 [mg/L]	Blank 2 [mg/L]	Blank 3 [mg/L]	Mean [mg/L]
Cu	0.005	0.002	0.003	**0.003**
Zn	0.000	0.000	0.000	**0.000**

The concentration of Cu and Zn in the cress are seen in *Table G-4* and *Table G-5* , respectively.

Table G-4: Measured concentrations of Cu in dried cress.

Run	Mass [g]	Cu ICP [mg/L]	Cu ICP corrected for blank [mg/L]	Cu in cress [mg/kg]	Cu mean of runs [mg/kg]
11A	0.6231	0.031	0.028	2.22	**2.05**
11B	0.7351	0.032	0.029	1.95	
12A	0.6135	0.028	0.025	2.01	
12B	0.7321	0.033	0.030	2.03	
21A	0.6154	0.029	0.026	2.09	**2.23**
21B	0.7510	0.037	0.034	2.24	
22A	0.6310	0.032	0.029	2.27	
22B	0.7423	0.038	0.035	2.34	
31A	0.6213	0.113	0.110	8.83	**8.40**
31B	0.7502	0.116	0.113	7.51	
32A	0.6201	0.118	0.115	9.25	
32B	0.7326	0.121	0.118	8.03	
41A	0.6187	0.116	0.113	9.11	**9.03**
41B	0.7498	0.131	0.128	8.51	
42A	0.6548	0.124	0.121	9.21	
42B	0.7531	0.143	0.140	9.27	
51A	0.6354	0.029	0.026	2.02	**2.01**
51B	0.7487	0.033	0.030	1.98	
52A	0.6345	0.028	0.025	1.94	
52B	0.7506	0.035	0.032	2.11	
61A	0.6387	0.031	0.028	2.17	**2.10**
61B	0.7654	0.039	0.036	2.33	
62A	0.6451	0.029	0.026	1.99	
62B	0.7461	0.032	0.029	1.92	
71A	0.6389	0.117	0.114	8.90	**8.14**
71B	0.7591	0.112	0.109	7.16	
72A	0.6287	0.11	0.107	8.48	
72B	0.7451	0.123	0.120	8.03	
81A	0.6348	0.128	0.125	9.82	**9.44**
81B	0.7482	0.134	0.131	8.73	
82A	0.6344	0.131	0.128	10.06	
82B	0.7760	0.145	0.142	9.13	

Table G-5: Measured concentrations of Zn in dried cress.

Run	Mass [g]	Zn ICP [mg/L]	Zn ICP corrected for blank [mg/L]	Zn in cress [mg/kg]	Zn mean of runs [mg/kg]
11A	0.6231	0.329	0.329	26.4	**23.8**
11B	0.7351	0.305	0.305	20.7	
12A	0.6135	0.306	0.306	24.9	
12B	0.7321	0.337	0.337	23.0	
21A	0.6154	0.405	0.405	32.9	**30.3**
21B	0.7510	0.419	0.419	27.9	
22A	0.6310	0.409	0.409	32.4	
22B	0.7423	0.415	0.415	28.0	
31A	0.6213	0.321	0.321	25.8	**24.7**
31B	0.7502	0.337	0.337	22.5	
32A	0.6201	0.336	0.336	27.1	
32B	0.7326	0.345	0.345	23.5	
41A	0.6187	0.403	0.403	32.6	**31.2**
41B	0.7498	0.428	0.428	28.5	
42A	0.6548	0.443	0.443	33.8	
42B	0.7531	0.451	0.451	29.9	
51A	0.6354	1.568	1.568	123.4	**128.5**
51B	0.7487	1.987	1.987	132.7	
52A	0.6345	1.673	1.673	131.8	
52B	0.7506	1.894	1.894	126.2	
61A	0.6387	1.784	1.784	139.7	**141.6**
61B	0.7654	1.999	1.999	130.6	
62A	0.6451	1.877	1.877	145.5	
62B	0.7461	2.248	2.248	150.7	
71A	0.6389	1.666	1.666	130.4	**131.1**
71B	0.7591	1.879	1.879	123.8	
72A	0.6287	1.749	1.749	139.1	
72B	0.7451	1.955	1.955	131.2	
81A	0.6348	2.120	2.12	167.0	**171.8**
81B	0.7482	2.390	2.39	159.7	
82A	0.6344	2.271	2.271	179.0	
82B	0.7760	2.814	2.814	181.3	

G.3 Cu and Zn in soil

The soil samples were dried in aluminium trays at 105°C to a constant weight was obtained as seen in *Figure G-9*.

Figure G-9: Drying of soil samples for determination of Cu and Zn concentrations.

After drying, the soil samples were digested exactly as described in the previous radish experiment.

G.3.1 Results

The concentration of Cu and Zn in the soil *before* pot experiments are seen in *Table G-6* and

Table G-7, respectively.

Table G-6: Measured concentrations of Cu in soil.

Run	Mass [g]	Cu ICP [mg/L]	Cu ICP corrected for blank [mg/L]	Cu in soil [mg/kg]	Cu mean of runs [mg/kg]
11A	0.8461	0.050	0.043	**2.55**	**2.63**
11B	0.9132	0.059	0.052	**2.83**	
12A	0.8676	0.051	0.044	**2.54**	
12B	0.9543	0.057	0.050	**2.62**	
21A	0.8413	0.053	0.046	**2.73**	**2.95**
21B	0.9647	0.064	0.057	**2.95**	
22A	0.8746	0.058	0.051	**2.92**	
22B	0.9687	0.069	0.062	**3.20**	
31A	0.8317	1.097	1.090	**65.53**	**71.0**
31B	0.9870	1.428	1.421	**71.99**	
32A	0.8761	1.105	1.098	**62.66**	
32B	0.9873	1.665	1.658	**83.97**	
41A	0.8605	1.245	1.238	**71.93**	**73.0**
41B	0.9473	1.377	1.370	**72.31**	
42A	0.8106	1.184	1.177	**72.60**	
42B	0.9633	1.454	1.447	**75.11**	
51A	0.8476	0.054	0.047	**2.77**	**2.76**
51B	0.9650	0.056	0.049	**2.54**	
52A	0.8666	0.059	0.052	**3.00**	
52B	0.9414	0.058	0.051	**2.71**	
61A	0.8647	0.053	0.046	**2.66**	**2.78**
61B	0.9446	0.057	0.050	**2.65**	
62A	0.8761	0.058	0.051	**2.91**	
62B	0.9317	0.061	0.054	**2.90**	
71A	0.8743	1.136	1.129	**64.57**	**67.7**
71B	0.9413	1.305	1.298	**68.97**	
72A	0.8941	1.115	1.108	**61.96**	
72B	0.9317	1.408	1.401	**75.19**	
81A	0.8475	1.154	1.147	**67.67**	**71.7**
81B	0.9318	1.347	1.340	**71.90**	
82A	0.8333	1.201	1.194	**71.64**	
82B	0.9904	1.503	1.496	**75.53**	

Table G-7: Measured concentrations of Zn in soil.

Run	Mass [g]	Zn ICP [mg/L]	Zn ICP corrected for blank [mg/L]	Zn in soil [mg/kg]	Zn mean of runs [mg/kg]
11A	0.8461	0.142	0.142	8.39	**8.59**
11B	0.9132	0.158	0.158	8.65	
12A	0.8676	0.154	0.154	8.88	
12B	0.9543	0.161	0.161	8.44	
21A	0.8413	0.151	0.151	8.97	**8.90**
21B	0.9647	0.164	0.164	8.50	
22A	0.8746	0.178	0.178	10.2	

Run	Mass [g]	Zn ICP [mg/L]	Zn ICP corrected for blank [mg/L]	Zn in soil [mg/kg]	Zn mean of runs [mg/kg]
22B	0.9687	0.154	0.154	7.95	
31A	0.8317	0.148	0.148	8.90	**8.55**
31B	0.9870	0.154	0.154	7.80	
32A	0.8761	0.161	0.161	9.19	
32B	0.9873	0.164	0.164	8.31	
41A	0.8605	0.164	0.164	9.53	**9.23**
41B	0.9473	0.167	0.167	8.81	
42A	0.8106	0.159	0.159	9.81	
42B	0.9633	0.169	0.169	8.77	
51A	0.8476	2.654	2.654	157	**156**
51B	0.9650	2.987	2.987	155	
52A	0.8666	2.704	2.704	156	
52B	0.9414	2.970	2.970	158	
61A	0.8647	2.879	2.879	166	**165**
61B	0.9446	3.018	3.018	160	
62A	0.8761	2.819	2.819	161	
62B	0.9317	3.197	3.197	172	
71A	0.8743	2.668	2.668	153	**156**
71B	0.9413	2.968	2.968	158	
72A	0.8941	2.785	2.785	156	
72B	0.9317	2.910	2.910	156	
81A	0.8475	3.014	3.014	178	**164**
81B	0.9318	3.021	3.021	162	
82A	0.8333	2.874	2.874	172	
82B	0.8461	2.854	2.854	144	

G.4 Statistical analysis - MATLAB

The full factorial radish experiment was analysed using MATLAB. In this analyse the function named *genff2n* are used as previously described. The MATLAB script of the full factorial analysis are presented below. The uptake of Cu and Zn are used as the response variables.

```
clear
clc
close all

factors = [0 0 0; 150 60 150];
factor_names = {'Pig Manure', 'Cu', 'Zn'};

% Cu in Cress
y=[2.05; 2.23; 8.40; 9.03; 2.01; 2.10; 8.14; 9.44;]; %Run[1 2 3 4 5 6 7
8]

m=genff2n(factors);
dm=m.dmb;
cm=m.cmb;

fme= (y'*dm)/4;
fie= (y'*cm)/4;

fprintf('Main effects:\n');
fprintf('%.3f\n', fme);
```

```
fprintf('Interaction effects:\n');
fprintf('%.3f\n', fie);

figure
maineffectsplot(y,dm, 'varnames', factor_names)
title('Main Effect - Cu in Cress','Fontsize',24,'FontWeight','bold')

figure
interactionplot(y,dm, 'varnames', factor_names)
title('Interaction Effect - Cu in Cress','Fontsize',24,'Font-
Weight','bold')

%% Zn in Cress
y=[23.8; 30.3; 24.7; 31.2; 128.5; 141.6; 131.1; 171.8;]; % Run[1 2 3 4 5
6 7 8]

m=genff2n(factors);
dm=m.dmb;
cm=m.cmb;

fme= (y'*dm)/4;
fie= (y'*cm)/4;

fprintf('Main effects:\n');
fprintf('%.3f\n', fme);

fprintf('Interaction effects:\n');
fprintf('%.3f\n', fie);

figure
maineffectsplot(y,dm, 'varnames', factor_names)
title('Main Effect - Zn in Cress','Fontsize',24,'FontWeight','bold')

figure
interactionplot(y,dm, 'varnames', factor_names)
title('Interaction Effect - Zn in Cress','Fontsize',24,'Font-
Weight','bold')
```

H Pig manure vs. cattle manure

This appendix covers the procedure as well as the obtained raw data results of the cress experiment with pig manure versus cattle manure.

H.1 Seeding procedure

Soil preparation

Cu and Zn were supplied in the form of the sulphate compounds $CuSO_4$ and $ZnSO_4 \cdot 7H_2O$ respectively as in the previous experiments. The content of Cu in Zn in the respective sources were calculated in order to know how much of the compounds that needs to be weighed in order to add 60 mg Cu per kg soil and 150 mg Zn per kg soil. Reference is made to the previous experiment (*F.1 Seeding procedure*) for detailed calculations. Each plastic pot was filled with 0.6 kg soil. The amounts are seen in *Table H-1*.

Table H-1: Calculated amounts for metals addition in pot experiments.

	Cu [mg/kg]	**Zn [mg/kg]**	**$CuSO_4$ per 600 g soil [g]**	**$ZnSO_4 \cdot 7H_2O$ per 600 g soil [g]**
Pot 1	25	75	0.0377	0.1979
Pot 2	75	175	0.1130	0.4617
Pot 3	100	225	0.1507	0.5937

The pot experiments were carried out with a duplicate determination. The actual weighed amounts are seen in *Table H-2*.

Table H-2: Actual weighed amounts for metals addition in pot experiments.

Replicate 1	**$CuSO_4$ [g]**	**$ZnSO_4 \cdot 7H_2O$ [g]**	**Replicate 2**	**$CuSO_4$ [g]**	**$ZnSO_4 \cdot 7H_2O$ [g]**
Control 1-1	0.0380	0.1983	**Control 1-2**	0.0384	0.1973
Control 2-1	0.1127	0.4618	**Control 2-2**	0.1130	0.4611
Control 3-1	0.1505	0.5935	**Control 3-2**	0.1508	0.5935
Pig manure 1-1	0.0379	0.1984	**Pig manure 1-2**	0.0383	0.1982
Pig manure 2-1	0.1132	0.4621	**Pig manure 2-2**	0.1127	0.4623
Pig manure 3-1	0.1510	0.5941	**Pig manure 3-2**	0.1512	0.5945
Cattle manure 1-1	0.0383	0.1977	**Cattle manure 1-2**	0.0375	0.1980
Cattle manure 2-1	0.1133	0.4626	**Cattle manure 2-2**	0.1129	0.4620
Cattle manure 3-1	0.1511	0.5939	**Cattle manure 3-2**	0.1503	0.5933

The chemicals were mixed with the soil in each pot as described in the previous experiment *F.1 Seeding procedure*. In addition to metals, some of the soils were supplied with pig manure and cattle manure as seen in *Table H-2* and seen in *Figure H-1*.

Figure H-1: Cattle manure and pig manure for treatment of cress compares to a control receiving no manures.

Since the pig manure and pot size used in this experiment was the same as used in the previous, the same amount of pig manure is used. The used amount of cattle manure was calculated in the same way from the content of N and the harmony rule:

$$Maximum\ ton\ per\ hectare: \frac{140\ kg\ N\ per\ ha}{2.406\ kg/t} = 58.188\ t/ha$$

The soil surface area in the pots were calculated to 214 cm^2, and 58.2 t/ha and are converted in the following:

$$214\ cm^2 = 214\ cm^2 \cdot 10^{-8}\frac{ha}{cm^2} = 0.00000214\ ha$$

$$Amount\ per\ pot\ [t]: 58.188\frac{t}{ha} \cdot 0.00000214\ ha = 0.000125\ t$$

$$Amount\ per\ pot\ [g]: 0.000125\ t \cdot 10^6\frac{g}{t} = \underline{125\ g\ per\ pot}$$

The actual weighed amounts of the manure for the treatments with manure are seen in *Table H-3*.

Table H-3: Actual weighed amounts of pig manure for the treatments with manure.

	Pig Manure [g]	Run	Cattle Manure [g]
1-1	171.2	**1-1**	125.4
2-1	172.9	**2-1**	125.6
3-1	172.8	**3-1**	124.9
1-2	172.6	**1-2**	126.1
2-2	171.5	**2-2**	125.7
3-2	173.1	**3-2**	125.7

The manure were added by pouring it out on the soil surface before seeding.

Seeding

As in the previous experiment, similar seeds of cress from the local supermarket were used in this experiment. The cress were sown at a rate of 5 g per pot.

All pots were watered with tap water. The amount was not noted down, but all pots received exactly the same amount of water. The pots after seeding are seen in *Figure H-2*.

Figure H-2: Pot experiment with cress after seeding. The treatments were control, pig manure and cattle manure, respectively.

The cress was growing fast in a greenhouse and are seen 25 days after seeding in *Figure H-3*.

Figure H-3: Pot experiment with cress 25 days after seeding.

The analysis procedure are described in the following.

H.2 Cu and Zn in cress

The cress was harvested and prepared for analysis of Cu and Zn concentrations in the same way as described in the previous cress experiment. The cress after harvest are seen in *Figure H-4*.

Figure H-4: Harvested cress prepared for drying at 105°C.

Each treatment was analysed in duplicate. Some samples prepared for the ICP analysis are seen in *Figure H-5*.

Figure H-5: Samples prepared for ICP-OES analysis of Cu and Zn concentration.

Some blanks were prepares with HNO_3 and treated as the samples.

H.2.1 Results

The measured concentration of Cu and Zn in the blanks are seen in *Table H-4*.

Table H-4: Measured concentration of Cu and Zn in blank samples.

Element	Blank 1 [mg/L]	Blank 2 [mg/L]	Blank 3 [mg/L]	Average [mg/L]
Cu	0.001	0.004	0.003	0.003
Zn	0.000	0.000	0.000	0.000

The concentration of Cu and Zn in the 25 days after growth are seen in *Table H-5* and *Table H-6*, respectively.

Table H-5: Concentration of Cu in cress after pot experiments.

Pot	Mass [g]	Cu ICP [mg/L]	Corrected for blank [mg/L]	Cu in cress [mg/kg]	Mean Cu [mg/kg]
Control 1A	0.6986	0.056	0.053	3.79	**3.72**
Control 1B	0.9721	0.074	0.071	3.65	
Control 2A	0.7266	0.111	0.108	7.43	**7.74**
Control 2B	0.9745	0.160	0.157	8.06	
Control 3A	0.7280	0.133	0.130	8.93	**9.35**
Control 3B	0.9879	0.196	0.193	9.77	
Pig manure 1A	0.7379	0.103	0.100	6.78	**6.53**
Pig manure 1B	0.9931	0.128	0.125	6.29	
Pig manure 2A	0.7784	0.136	0.133	8.54	**8.17**
Pig manure 2B	1.0009	0.159	0.156	7.79	
Pig manure 3A	0.7521	0.253	0.250	16.6	**16.76**
Pig manure 3B	0.9881	0.337	0.334	16.9	
Cattle manure 1A	0.7199	0.096	0.093	6.46	**6.49**
Cattle manure 1B	0.9590	0.128	0.125	6.52	
Cattle manure 2A	0.7917	0.110	0.107	6.76	**6.25**
Cattle manure 2B	0.9853	0.116	0.113	5.73	
Cattle manure 3A	0.7282	0.229	0.226	15.5	**15.51**
Cattle manure 3B	0.9940	0.311	0.308	15.5	

Table H-6: Concentration of Zn in cress after pot experiments.

Pot	Mass [g]	Zn ICP [mg/L]	Corrected for blank [mg/L]	Zn in cress [mg/kg]	Mean Zn [mg/kg]
Control 1A	0.6986	1.497	1.497	107	**106**
Control 1B	0.9721	2.056	2.056	106	
Control 2A	0.7266	1.950	1.950	134	**138**
Control 2B	0.9745	2.778	2.778	143	
Control 3A	0.7280	3.268	3.268	224	**231**
Control 3B	0.9879	4.692	4.692	237	
Pig manure 1A	0.7379	2.673	2.673	181	**175**
Pig manure 1B	0.9931	3.335	3.335	168	
Pig manure 2A	0.7784	3.433	3.433	221	**205**
Pig manure 2B	1.0009	3.793	3.793	189	
Pig manure 3A	0.7521	8.298	8.298	552	**545**
Pig manure 3B	0.9881	10.65	10.65	539	
Cattle manure 1A	0.7199	2.529	2.529	176	**181**
Cattle manure 1B	0.9590	3.556	3.556	185	
Cattle manure 2A	0.7917	2.168	2.168	137	**129**
Cattle manure 2B	0.9853	2.372	2.372	120	
Cattle manure 3A	0.7282	7.923	7.923	544	**561**
Cattle manure 3B	0.9940	11.50	11.50	578	

H.3 Cu and Zn in soil

The soil was treated as previously described by drying and digestion with 7 M HNO_3. Some soil samples prepared for drying are seen in *Figure H-6*.

Figure H-6: Drying of soil was done at 105°C for about 24 hours.

The soil samples were analysed as previously described.

H.3.1 Results

The concentration of Cu and Zn obtained by ICP-OES analysis are presented in *Table H-7* and *Table* H-8, respectively.

Table H-7: Concentration of Cu in soil after pot experiment with cress and different manures.

Pot	Mass [g]	Cu ICP [mg/L]	Corrected for blank [mg/L]	Cu in soil [mg/kg]	Mean Cu [mg/kg]
Control 1A	0.7207	0.892	0.889	61.7	57.2
Control 1B	0.8843	0.935	0.932	52.7	
Control 2A	0.7258	0.848	0.845	58.2	66.2
Control 2B	0.9386	1.395	1.392	74.2	
Control 3A	0.7781	4.206	4.203	270	249
Control 3B	1.0588	4.83	4.827	228	
Pig manure 1A	0.8737	0.558	0.555	31.8	33.3
Pig manure 1B	0.9967	0.697	0.694	34.8	
Pig manure 2A	0.7938	1.604	1.601	101	105
Pig manure 2B	0.8808	1.91	1.907	108	
Pig manure 3A	0.7695	4.78	4.777	310	296
Pig manure 3B	0.9244	5.219	5.216	282	
Cattle manure 1A	0.7772	0.565	0.562	36.2	37.6
Cattle manure 1B	0.9288	0.730	0.727	39.1	
Cattle manure 2A	1.0020	2.098	2.095	105	114
Cattle manure 2B	0.8381	2.062	2.059	123	
Cattle manure 3A	0.7700	4.480	4.477	291	307
Cattle manure 3B	0.9794	6.318	6.315	322	

Table H-8: Concentration of Zn in soil after pot experiment with cress and different manures.

Pot	Mass [g]	Zn ICP [mg/L]	Corrected for blank [mg/L]	Zn in soil [mg/kg]	Mean Zn [mg/kg]
Control 1A	0.7207	1.823	1.823	126	135
Control 1B	0.8843	2.531	2.531	143	
Control 2A	0.7258	2.216	2.216	153	171
Control 2B	0.9386	3.567	3.567	190	
Control 3A	0.7781	8.285	8.285	532	508
Control 3B	1.0588	10.25	10.250	484	
Pig manure 1A	0.8737	1.821	1.821	104	105
Pig manure 1B	0.9967	2.103	2.103	105	
Pig manure 2A	0.7938	3.109	3.109	196	213
Pig manure 2B	0.8808	4.044	4.044	230	
Pig manure 3A	0.7695	9.700	9.700	630	603
Pig manure 3B	0.9244	10.65	10.650	576	
Cattle manure 1A	0.7772	1.590	1.590	102	99.1
Cattle manure 1B	0.9288	1.782	1.782	95.9	
Cattle manure 2A	1.0020	4.374	4.374	218	235
Cattle manure 2B	0.8381	4.225	4.225	252	
Cattle manure 3A	0.7700	8.979	8.979	583	574
Cattle manure 3B	0.9794	11.08	11.080	566	

I Cation Exchange Capacity

The CEC was determined in the agricultural soils with pig and cattle manure, as well as in the forest and planting soil.

I.1 Materials

The following chemicals and materials were used in order to determine the CEC of soil:

- Shaking incubator
- Centrifuge
- ICP-OES
- Dried soil samples
- Demineralised water
- 2 M HCl
- Tri-ethanol amine solution
    - 90 ± 1 mL tri-ethanol amine are diluted with water to 1 L and the pH are adjusted to 8.1 ± 0.05 by adding 145 ± 5 mL 2M HCl. This solution are diluted to 2 L with water, and stored with a cap.
- Barium chloride solution
    - 244 ± 2g $BaCl_2 \cdot H_2O$ are dissolved in water and filled up to 1.0 L.
- Buffered barium chloride solution
    - Equal volumes of the previous described solutions are mixed.
- Magnesium sulphate solution
    - 6.2 ± 0.05 g $MgSO_4 \cdot 7\ H_2O$ are dissolved in water and filled up to 1.00 L.

I.2 Procedure

CEC was determined in the planting soil, the soil form the forest as well as the soil from the fields supplied with pig manure and cattle manure, respectively. About 1.25 g (W) of the dried soils were accurately weighed into 60 mL centrifuge tubed as seen in *Figure I-1*. The mass of the tubes including sample was determined (M_1).

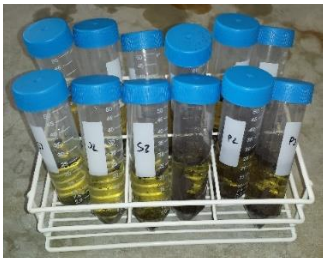

Figure I-1: Soil samples weighed and mixed with buffered barium dichloride solution.

The soils were mixed with 25 mL buffered barium dichloride solution and shaken for 1 hour as seen in *Figure I-2*.

Figure I-2: Shaking incubator used for shaking of samples in the saturation and displacement procedure.

The samples were centrifuged at 2000 RPM for 15 minutes as seen in *Figure I-3*.

Figure I-3: The samples were centrifuged several times in a centrifuge at 2000 RPM for 15 minutes.

After centrifuging the supernatant were discarded as seen in *Figure I-4*.

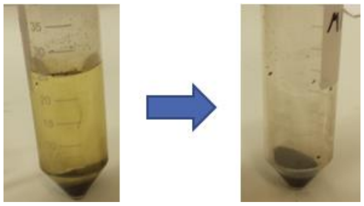

Figure I-4: After centrifugation the supernatant were discarded.

The centrifuging of the plating soil were problematic due to floating of organic particles, as seen in *Figure I-5*. Therefore, the supernatant were decanted by using a pipette. This resulted in discard of small amounts of the weighed planting soil particles.

Figure I-5: The centrifuging of the plating soil were problematic due to floating of organic plant material. Therefore, the supernatant were decanted by using a pipette.

The sample cake were broken up by adding 50 mL buffered barium dichloride solution and the tubes were left for stand overnight. The samples were centrifuged as above and the supernatant were discarded. 50 mL of demineralised water were added and shaken for 2 to 3 minutes by hand to break up the sample cake. The solution was centrifuged again and the tubes and contents were weighed (M_2). 25.0 mL of magnesium sulphate solution were added and the solutions were shaken for 2 hours as described above. The tubes were centrifuged and the supernatant were filtered into flask, as seen in *Figure I-6*.

Figure I-6: Filtration of magnesium sulphate supernatant.

The concentration of magnesium in the supernatant from the displacement procedure were analysed by ICP-OES.

I.3 Results

The concentration of magnesium in the residual solution from the displacement procedure determined by ICP (x) were converted using the following formula:

$$\left[\frac{608 - \frac{x(100 + M_2 - M_1)}{100}}{121.6}\right] \cdot \left[\frac{1000}{W}\right] \, meq/kg$$

The weighing data, concentration from ICP and the calculated CEC are seen in *Table I-1*.

Table I-1: Data for the calculation and results of CEC in soil.

Soil	Tube [g]	Tube and sample [g] (M_1)	Sample [g]	(M_2) [g]	ICP [mg/L]	Concentration Mg [mg/L] (x)	CEC [meq/kg]
Cattle 1	9.216	10.468	1.251	10.873	5.619	562	**288**
Cattle 2	9.228	10.478	1.249	10.842	5.535	554	**345**
Cattle 3	9.178	10.434	1.257	10.792	5.233	523	**542**
Pig 1	9.233	10.483	1.250	10.942	5.477	548	**380**
Pig 2	9.176	10.430	1.254	10.922	5.325	533	**478**
Pig 3	9.224	10.476	1.252	10.952	5.445	545	**400**
Forest 1	9.220	10.479	1.259	11.344	5.124	512	**596**
Forest 2	9.222	10.474	1.251	11.212	5.271	527	**506**
Forest 3	9.198	10.451	1.252	11.101	5.301	530	**489**
Planting 1	9.182	10.437	1.256	14.369	3.191	160	**2896**
Planting 2	9.188	10.439	1.251	14.718	3.122	156	**2926**
Planting 3	9.202	10.452	1.249	14.837	3.721	186	**2724**