Phytoextraction of Cadmium, Lead, and Zinc

A case study of *Salix Schwerinii* and *Salix Viminalis* and approaches of improving the method using arbuscular mycorrhizal fungi

Master Thesis

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Formålet med denne rapport er at bestemme om arterne *Salix schwerinii* og *Salix viminalis* er anvendelig til fytoekstraktion af kadmium (Cd), bly (Pb) og zink (Zn), samt undersøge om inokulering med arbuskulær mykorrhiza optimerer arternes optagelse af disse metaller. Jord er blevet udtaget fra en kendt tungmetalforurenet grund til en række potteforsøg, hvori arternes optagelse, translokation og oprensningstid af Cd, Pb og Zn er blevet undersøgt. Metalkoncentration i stiklingerne viste, at arterne var bedst til at optage og translokere Cd og Zn i deres overjordiske væv, og at en inokulering af arterne optimerede deres metaloptagelse i rødderne. Den estimerede oprensningstid af metallerne viste, at *Salix viminalis* kræver mindst tid til at reducere jordens metalkoncentration til det danske jordkvalitetskriterium. Det kan ud fra projektets resultater konkluderes, at *Salix schwerinii* er bedre til at optage og translokere de tre metaller i det overjordiske væv, men at *Salix viminalis*, grundet sin højere biomasseproduktion, er mere effektiv til at reducere metalkoncentrationen i jorden. Inokulering med arbuskulær mykorrhiza optimerede arternes metaloptagelse og har potentiale til at gøre arterne mere anvendelig til fytoekstraktion.

Contents

Preface

The following master thesis is written by a thesis student of the Environmental Engineering Cand. Scient. Techn. program at Aalborg University. The purpose of the project is to achieve knowledge and insight on the topic: Phytoextraction of Heavy Metals. The project are directed towards researchers, students and others with interests in biological- and environmental science. It is advised however that the reader have slight acquaintance with the topic beforehand.

The thesis include a theoretical and practical part in which the topic will be assessed. The theoretical part focuses on the extent on the problem: heavy metal contamination of soil, and how the problem are being handled in Denmark both legislative and practical, whilst introducing the reader to the remediation technique phytoextraction. The practical part consists of an environmental analysis of the two salix species *Salix schwerinii* and *Salix viminalis* applicability for phytoextraction, and whether it is feasible to enhance these species metal uptake through inoculation with the arbuscular mycorrhizal fungi *Rhizophagus irregularis*. The environmental analysis is performed under Danish conditions to assess whether the technique can be applied in Denmark.

The Harvard Referencing System is used for citation and references throughout the report.

Aalborg University, June 10, 2017

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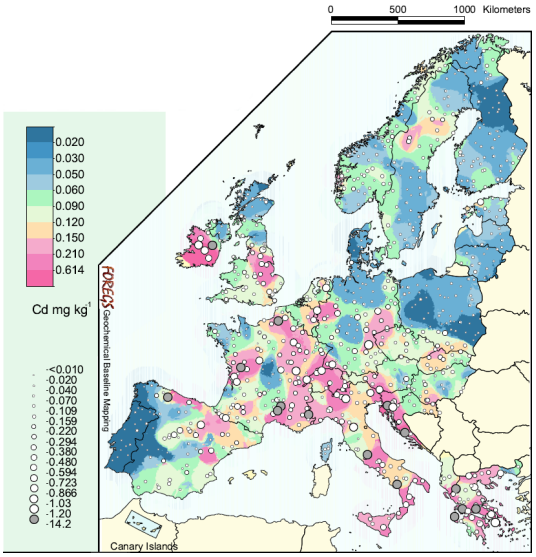
1. Introduction

Heavy Metal contaminated soils

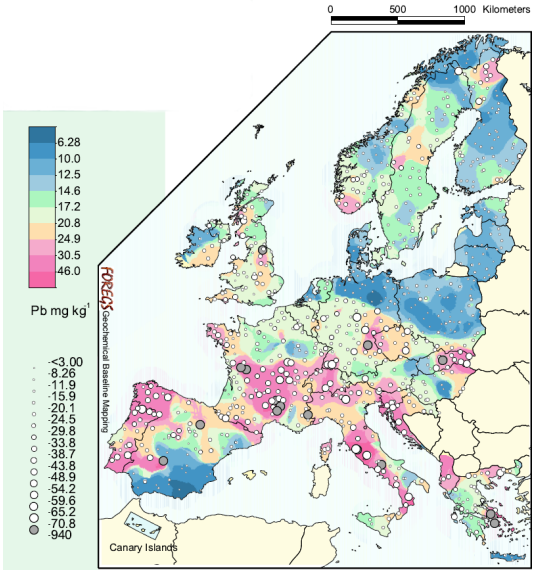
Heavy metals (HMs) are metallic elements with densities of ≥ 5 g/cm^3 that occur in the environment through natural and anthropogenic sources, such as weathering of rocks and industrial activities. The contribution of HMs to the environment from anthropogenic sources has in numerous sites far exceeded the natural background level (Maribo and Andersen, 2011). HMs are unlike organic contaminants non-biodegradable, and thereby accumulate in the environment (Ali, Khan, and Sajad, 2013; Tangahu et al., 2011). In Europe, high concentrations of HMs like cadmium (Cd), lead (Pb), and zinc (Zn) pose a serious problem in the agricultural sectors of many of the EU member states (Commission, 2016). These metals are found in the agricultural soil in median values of 0.090 mg kg^{-1}, 17.2 mg kg^{-1}, and 47 mg kg^{-1} respectively, and pose a risk to environmental and human health (fig. 1.1) (Forum of European Geological Surveys, 2017). The Commission of the European Communities estimated in 2006 that it would cost 17.3 billion euro annually to remediate all contaminated soil sites within the European Union (Tóth et al., 2016) In Denmark, the median concentrations of Cd, Pb, and Zn are estimated to be 0.050 mg kg^{-1}, 15.9 mg kg^{-1}, and 34 mg kg^{-1} respectively, and while the values are low in terms of most EU member states, the concentrations of these metals still pose a risk of causing adverse health effects (Forum of European Geological Surveys, 2017).

The accumulation of HMs in soil causes deterioration of the soil quality, reduction of soil biodiversity, lowers soil yield, and generally pose a potential risk of contaminating adjacent areas and the food chain (Commission, 2016; Lai et al., 2009). These elements occur in the food chain, as organisms absorb the metals through direct contact, inhalation, or ingestion of contaminated soil and food sources (Wuana and Okieimen, 2011). In organisms HMs accumulate in the body tissues, and the concentration increases as the metals pass through the trophic levels of the food chain (Ali, Khan, and Sajad, 2013; Jaishankar et al., 2014). However, some HMs, such as Iron (Fe), Manganese (Mn), and Zn are required by organisms

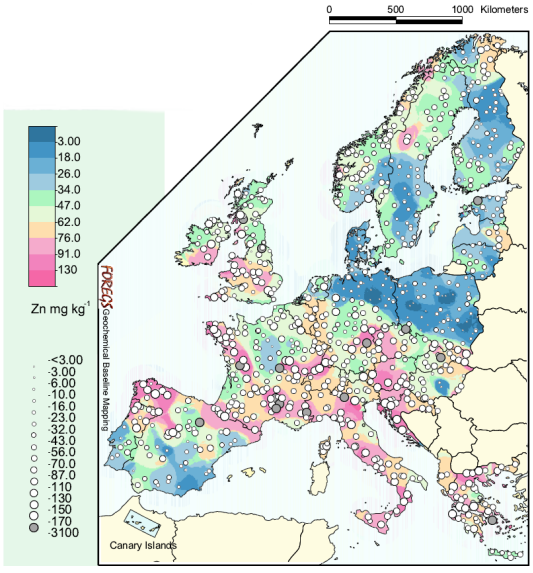
in minute quantities as micronutrients for physiological and biochemical functions. Metal concentrations above the threshold limit can cause adverse health effects in even low concentrations, such as endocrine disruption, neurological damage, and oxidative stress of cells (Ali, Khan, and Sajad, 2013; Tangahu et al., 2011).



(a) The median concentrations of Cd in the agricultural soils of the Europe



(b) The median concentration of Pb in the agricultural soils of Europe



(c) The median concentrations of Zn in the agricultural soils of Europe

Figure 1.1: The median concentrations of the HMs Cd, Pb, and Zn in the agricultural soils of the European Union (Forum of European Geological Surveys, 2017)

Danish soil legislation

Increased input of HMs from intensified industrialization and urbanization from the beginning of the 20^{th} century has caused numerous Danish soil sites to be contaminated with HMs. In 1990 the Danish Ministry of Environment began to address the problem by introducing the waste deposit act (WDA). The objective of the act was to register all landfills prior 1976 (Miljøstyrelsen, 2000). In 1995, the Danish quality criteria for contaminated soil sites was implemented in the Danish soil legislation (table 1.1). The purpose of the criteria was to enable comprehensive assessments of contaminated sites and the associated risks (Miljø- og Fødevareministeriet, 2016b). The criteria forms the basis for the prioritizing and decisions concerning the remediation and investigation of contaminated soil sites (Miljø- og Fødevareministeriet, 2017).

Table 1.1: Modified version of the Danish quality criteria for soil and groundwater containing some of the HMs present in the official list (Miljøstyrelsen (Danish EPA), 2015)

Substance	Soil quality criteria [mg/kg]	Cut off criteria [mg/kg]	Groundwater quality criteria [μg/l]
Cadmium (Cd)	0.5	5	0.5
Copper (Cu)	500	1000	100
Lead (Pb)	40	400	1
Nickel (Ni)	30	30	10
Zinc (Zn)	500	1000	100

In 2000, the WDA was replaced with the contamination soil act (CSA). The purpose of the act was to improve the prior legislation and include new initiatives, concerning the prevention of further soil contamination and protection of human health and the environment (Miljøstyrelsen, 2000; Natur- og Miljøklagenævnet, 2016). The regions are through the CSA, obligated by the government to register and remediate soil sites with or suspected of containing soil contamination. The registration is based on a charting system that categorize the sites in two knowledge levels that describes the severity and nature of contamination (Maribo and Andersen, 2011). An area is designated as knowledge level 1, if there is suspicion or knowledge of activities on-site or on adjacent sites that could have caused contamination on the area. Areas designated as knowledge level 2 are sites where contamination have been registered in such concentrations or nature that the contamination pose an risk of causing hazardous effects on environmental and human health (Miljø- og Fødevareministeriet, 2015).

The CSA forms the basis of the regions administration of individual contaminated soil sites. Since the implementation of the CSA, there have been ongoing

alterations to the act (Miljø- og Fødevareministeriet, 2016a). In 2014, the CSA was altered to include the protection of surface waters and environment from leaching and migration of contaminants from contaminated soil sites. The purpose of the alteration was for the CSA to be in compliance with the prioritization and management of international protected areas and surface waters (Aisopou et al., 2016; Auken, 2013).

Conventional remediation methods

In 2016, approximately 33.000 soil sites in Denmark was registered by the Danish regions as contaminated or potentially contaminated (Danske Regioner, 2016). The majority of feasible conventional remediation methods in Denmark are limited as high implementation costs, and lack of documentation on the methods efficiency at Danish conditions blocks their implementation (Danish Enviromental Protection Agency, 2002).

In Denmark, conventional remediation methods of HM contaminated soil often involves either the isolation, immobilization, excavation, chemical extraction, electrokinetics or biological treatment of the contaminated soil (Miljøstyrelsen (Danish EPA), 2006). The implementation of on-site or off-site remediation methods often require the isolation of the contamination. Through the construction of vertical barriers or membranes, the contaminants are prevented from migrating into adjacent areas (Wuana and Okieimen, 2011).

Stabilization of contamination are used for reducing the mobility and immobilizing of the contaminants. The methods can be implemented on-site or at a treatment plant, and involve either chemical treatment or an amendment of the soils organic or inorganic composition. Primarily, organic and inorganic amendment of the soil involves the addition of clay, cement, or organic material to the soil. Chemical treatment of the soil generally involves addition of inorganic or alkaline solutions to the soil in order to amend the properties of the contaminant or soil. In the soil, the amendments causes the contaminants to bind through different movement impairing complexes, such as chemical adsorption or redox reactions to the soils other particles, reducing the mobility of the contaminant (Maribo and Andersen, 2011; Wuana and Okieimen, 2011).

Conventional excavation of contaminated soil as a remediation method, involves the physical removal and disposal of the soil to a specialized landfill or a central treatment plant. At landfills, the contaminated soil is often merely stored and thus the method solely shifts the contamination to another area (Tangahu et al., 2011). Remediation of the contaminated soil at treatment plants are often done through several processes. Initially the soil is often physically separated into the different soil fractions as contaminants tend to bind to the finer soil particles, such

as clay or silt (Miljøstyrelsen (Danish EPA), 2006). The separation process can be done on-site or at the treatment plant by either sieving the soil or using a soil washing plant. The contaminated soil fractions are then further processed through other methods such as chemical extraction, electrokinetic or biological treatment (Miljøstyrelsen (Danish EPA), 2006).

Chemical extraction of HM contaminated soil are primarily done by adding aqueous chemicals to a contaminated soil fraction. The aqueous chemical alters the physicochemical properties of the contaminant, causing the contaminant to desorb from the soil particles. The slurry of soil, contaminant, and chemical are then often separated through electrokinetic treatment (Miljøstyrelsen (Danish EPA), 2006; Wuana and Okieimen, 2011). Electrokinetic treatment uses electrical currents to separate the negative charged soil particles from positive charged metals. Electrokinetic treatment can function as a stand-alone remediation method, but are often used in connection with other methods like chemical extraction or biological treatment, due to its high implementation costs. In Denmark, electrokinetic treatment are rarely used in the treatment of soil contaminants as the method often requires multiple treatments of the soil (Miljøstyrelsen (Danish EPA), 2006).

Remediation methods that involves a biological treatment of the soil, are often used when the soils biological properties are preferred to be retained, such as in soils used for agriculture. Biological treatment are normally performed by either biologically amending the soil composition of bacteria and microfauna, or by using the biological processes of plants. Biological amendment of the soil differs widely, and typically involves the addition of selective bacteria cultures or microfauna that immobilizes, degrade or transforms the contaminant into non-toxic forms (U.S. EPA, 2006). Remediation of contaminants by using the biological processes of plants are known as phytoremediation. Phytoremediation utilizes plants capability to attract contaminants in the soil solution towards their roots. At the roots, contaminants are either immobilized within the root zone, reduced in mobility or absorbed at the root surface and accumulated in the plant (Tangahu et al., 2011). Immobilization in the root zone, prevents the contaminants from leaching to the groundwater or adjacent environments, and allows soil bacteria and microfauna to degrade or absorb the contaminants. The accumulation of contaminants in plants prevent leach and in general spread of the contaminant to the groundwater, and surrounding environment (U.S. EPA, 2006). Plants used for phytoremediation are in some cases inoculated with arbuscular mycorrhizal fungi (AMF), to increase their growth and tolerance against contaminants. AMF are soil microorganisms that forms symbiotic relations with terrestrial plants, and provides the plants with nutrients in exchange for carbohydrates (Buysens et al., 2016). This symbiotic relation promotes plant growth and increases the plants tolerance against contaminants through more selective nutrient uptake. In general, the plant species used for

phytoremediation are non-edible, to prevent the contamination of the food chain (Buysens et al., 2016). In Denmark, biological treatment are often used in the remediation of aqueous and soil environments contaminated with certain types of contaminants, such as organic pollutants, tar-oil contaminants (PAH), and HMs (Miljøstyrelsen (Danish EPA), 2006).

Phytoextraction

In order to ensure environmental and human health, HM contaminated soil sites needs to be decontaminated by reducing the HM concentrations to an acceptable concentration for the area or the natural background level. Today, several techniques exists for the remediation of HM contaminated soil. However, most conventional methods are costly and causes irreversible changes to the soil environment (Tangahu et al., 2011). This have led to an emerging interest for more biologically and environmentally friendly remediation techniques like phytoextraction of soil contaminants (Szemmelveisz, 2014). Phytoextraction is the uptake of soil contaminants by plant roots, and the translocation of these into either root or aboveground tissue (Ali, Khan, and Sajad, 2013; McCutcheon and Schnoor, 2003). Plant roots absorb metalloids and free metal ions from the soil solution at the root surface by transporting these across the plasma membrane of the root cells (Wuana and Okieimen, 2011) (fig.1.2). Once inside the root, metals are either sequestered in the root cells or transported by root pressure and the transpirational pull to aboveground tissue, primarily through the vascular tissue of the xylem (Ali, Khan, and Sajad, 2013; Bhargava et al., 2012).

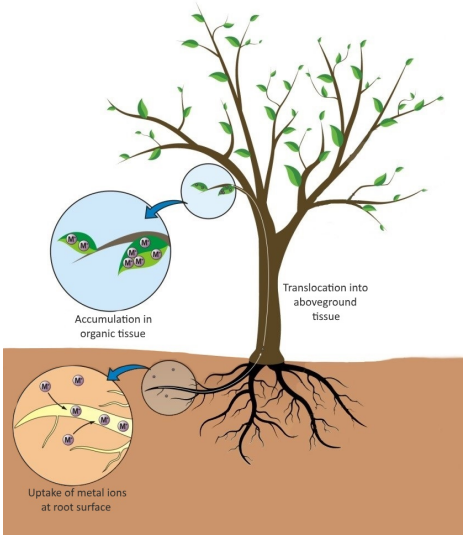


Figure 1.2: Principles of phytoextraction, modified from (Ayanda, 2015).

In plants cells HM are toxic at even low concentrations, and are capable of causing adverse effect for the plant. The toxicity of HMs often occur from the metals tendency to bond with the plant cells organic compounds like protein complexes and molecules. In the cells, these chelations may result in oxidative stress, membrane damage, and alterations of the plants fundamental processes by inactivating enzymes, disrupting membrane transport, and blocking the functional groups of essential molecules (Rascio and Navari-Izzo, 2011). Most plants deal with the toxicity of metals by bringing metal ions to their non-toxic forms through various regulating mechanisms, such as redox reactions and chelation of metal ions with specific organic ligands. Once in their non-toxic form, the metals are accumulated throughout the plant, primarily in the vacuoles (Ali, Khan, and Sajad, 2013; Bhargava et al., 2012).

Plants grown in metalliferous soils are generally grouped into the three categories metal excluders, accumulators and indicators in respective to their efficiency in accumulating and translocating metals into their aboveground tissue (Ali, Khan, and Sajad, 2013). Metal excluders are plants like *Cyperus articulatus* and *Pluchea dioscoridis* that efficiently limits the translocation of metals within them, thereby preventing higher concentrations of metals from accumulating in their aboveground tissue. Metal excluders have generally low potentials as phytoextractors, but are often used for soil stabilizing purposes in biological treatments of contaminated soil (Bhargava et al., 2012; Mganga, Manoko, and Rulangaranga, 2011). Metal accumulator are a general term for plants that are capable of accumulating metals into their aboveground tissue at even low soil concentrations. Metal accumulators are a broad group of plant species that varies greatly in their uptake of metals (Ali, Khan, and Sajad, 2013). A subgroup of metal accumulators species are classified as hyperaccumulators of certain metals. Hyperaccumulators are plants that are capable of accumulating metals in concentrations that far exceeds those present in the nearby non-accumulating species or surrounding soils (Bhargava et al., 2012). A plant is categorized as a hyperaccumulator, if the concentration of a metal within the species aboveground tissue are above the concentration criteria for the specific metal (table 1.2) (Branquinho et al., 2007). Hyperaccumulator species like *Alyssum bertolonii* and *Noccaea caerulescens* are widely studied, but have limited usage in phytoextraction of larger areas due to their often relative low biomass production (Robinson, Anderson, and Dickinson, 2015). Metal indicators are plants species that accumulate metals in their aboveground tissue in concentrations that reflects the surrounding soils. Indicators consist of both accumulator and hyperaccumulator species, and are generally used as indicators of metalliferous soils, and soil contamination.

Table 1.2: The hyperaccumulation criteria for seven of the most common soil contaminating HMs (Branquinho et al., 2007).

Metal	Concentration criteria [mg/kg]
Cadmium	100
Copper	1000
Chrome	1000
Nickel	1000
Lead	1000
Arsenic	1000
Zinc	10000

For phytoextraction a plant should ideally possess multiple traits, such as high biomass production, rapid growth, high metal tolerance, efficient translocation and accumulation of metals in aboveground tissue, and be easily cultivated (Ali, Khan, and Sajad, 2013). Studies in Denmark and China have shown that fast-growing trees like birch, poplar, and willow has a potential as phytoextractors of metal contaminants (Andersen et al., 2000; Wang et al., 2014). The potential in fast-growing trees lies in their extensive root systems, great biomass production, and easily harvesting with ensuing resprouting. The capability to resprout makes fast-growing trees ideal for phytoextraction, as it allows multiyear cycles of decontamination of the contaminated site (Bhargava et al., 2012). The harvested plant material can be used for industrialized purposes, such as energy production, furnitures or processed to recover precious metals, and thus reduce the cultivation and processing costs of the plants (Ali, Khan, and Sajad, 2013).

1.1 Case study: Lindholm Fjordpark

In 2016, Aalborg University was given permission by Aalborg municipality to investigate the physical and chemical composition of the fill soil of cadastral 7fq in Lindholm Fjordpark. The permission were given along with additional permit to excavate 220 kg of the fill soil from the cadastral for the analysis of the two salix species Salix schwerinii (*S. schwerinii*) and Salix viminalis tordis (*S. viminalis*) capabilities as phytoextractors.

Area

Lindholm Fjordpark is situated in the southwestern part of the city Nørresundby in Nordjylland (fig.1.3a). In 1913, cadastral 7fq was acquired by Dansk Andels Cementfabrik, during the increased industrial cement production in Aalborg and Nørresundby in the 1900s (Bender and Pedersen, 2006). Through the 1900s, the cadastral was used for sludge basins for filter dust until the 1970s, when the cadastral was acquired by the company F.L. Smidth (fig.1.3b) (Bender and Pedersen, 2006; Kirk, 2003). In 1978, F.L. Smidth decided to end the cement production on-site, and cadastral 7fq was later acquired by Aalborg municipality. Aalborg municipality decided in mid-1980s to extend the cadastral as part of the municipal plan of increasing the recreational value of the surrounding area (fig1.3c). In 1996, the cadastral was registered by Aalborg municipality as a landfill in accordance to the WDA after two orientational risk assessments in 1988 and 1996 documented HM and oil-tar (PAH) contamination on-site. Today the cadastral is categorized by the North Denmark Region in accordance to the current legislation as a knowledge level 2 area (Dansk Miljøportal, 2016; Kirk, 2003).

Orientational risk assessment

In 1988 and 1996, Aalborg municipality decided to perform orientational risk assessments of the fill soil on the cadastral, concerning the probability that the cadastral was contaminated (Kirk, 2003; Rasmussen, 1988). The risk assessments were based on soil samples drilled from the cadastral in the depths of 0.2-0.7 m under terrain (m u.t.) at three locations (Appendix A.1). The samples showed high concentrations of HMs, especially Cd, Pb, and Zn, along with tar-oil contamination (Kirk, 2003; Rasmussen, 1988). The extent of the contamination on cadastral 7fq is currently not limited to certain areas, and is estimated to occur on the whole cadastral. Based on the assessments, the fill soil of cadastral 7fq is considered to pose a risk to environmental and human health (Kirk, 2003).

Table 1.3: Geotechnical data provided by Aalborg municipality from the investigation of cadastral 7fq in 1988, all concentrations are in mg/kg dry matter (Rasmussen, 1988).

	pH	Lead	Cadmium	Chrome	Copper	Nickel	Zinc
A (drill site 1 and 2)	8.7	201	8.1	19	23	13	222
B (drill site 3)	8.1	8.1	0.14	16	9.6	14	76

(a) Lindholm Fjordpark and cadastral 7fq

(b) Cadastral 7fq, 1954

(c) Cadastral 7fq, 2015

Figure 1.3: Lindholm Fjordpark with cadastral 7fq in the southeastern corner. The cadastral area has changed considerably, when it in the 1900s was used for sludge basins by Dansk Andels Cementfabrik. Until it was converted into a natural recreation area by Aalborg municipality (Region Nordjylland, 2015; Styrelsen for Dataforsyning og Effektivisering, 2016).

2. Problem Statement

Are Salix schwerinii and Salix viminalis tordis suitable for phytoextraction of cadmium, lead, and zinc and is it feasible to enhance the uptake of these heavy metals through the inoculation of the arbuscular mycorrhizal fungi Rhizophagus irregularis.

To answer these questions, measurements of *S. schwerinii* and *S. viminalis T.* capability to accumulate and translocate Cd, Pb, and Zn, along with biometric traits, photosynthesis and biomass production will be assessed. Based on the results of the metal tolerance, phytoextraction, and mycorrhiza analysis, the applicability of *S. schwerinii* and *S. viminalis T.* as phytoextractors, and the possibility of increasing the species extraction rate through inoculation with *R. irregularis* will be discussed.

3. Methodology

In order to quantify how efficient *S. schwerinii* and *S. viminalis* are as phytoextractors, an analysis of the soil at cadastral 7fq are necessary. Several soil samples are extracted from cadastral 7fq, and are subsequently analyzed ex-situ in the lab. The soil assessment consists of several analyses of the soil texture, organic content, pH, and concentration of Cd, Pb, and Zn. The results of soil assessment are used to determine the area on the cadastral, at which the soil is applicable as a growth medium for the plants. For the phytoextraction assessment, cuttings of *S. schwerinii* and *S. viminalis* are planted in pots filled with either non-contaminated or HM contaminated soil from the cadastral. The reason are to measure the metals effects on the cuttings, and the cuttings phytoextraction capabilities with and without the presence of the arbuscular mycorrhizal fungi (AMF) *R. irregularis*. The analysis of the metals effect on the cuttings consists of periodical measurements of the cuttings height, shoots, leafs, and leaf surface area, along with the cuttings photosynthetic rate and biomass production. The measurements are used to assess if Cd, Pb, and Zn affects the salix species physical traits and photosynthesis. At the end of the growth period, the cuttings are harvested, and the root and shoot systems of the cuttings grown in contaminated soil with and without the presence of *R. irregularis* are used in the phytoextraction and mycorrhiza analysis. The cuttings root and shoot systems are used in the analyses to determine the species efficiency in accumulating and translocating Cd, Pb, and Zn in their belowground and aboveground tissue, and to assess if *R. irregularis* enhances their metal uptake. The results of these analyses are used to determine the time required by *S. schwerinii* and *S. viminalis* to remediate the soil on cadastral 7fq, and in the overall assessment of the two salix species suitability for phytoextraction.

3.1 Sampling stations

Soil samples were extracted in-situ at four sampling stations at cadastral 7fq (figure 3.1). The sampling area was delimited to be within the part of the cadastral that was formerly owned by Dansk Andels Cementfabrik. The stations (st.) 1 and 2 are located in two of the former sludge basins used for filter dust, and st. 3 are located near the center of the cadastral. St. 4 is on the ridge that separates the cadastral from Lindholm stream. The soil samples were excavated at each station in three different depths, and the samples was analyzed for the soil texture, organic material content, pH and concentration of Cd, Pb, and Zn.

Figure 3.1: Sampling stations on cadastral 7fq.

3.2 Soil assessment

The physical and chemical composition of the soil is essential for the assessment of the soils applicability for phytoextraction, and in the estimation of the time required to remediate an area. The soil texture, content of organic material, pH, and metal concentration was measured ex situ using samples extracted from cadastral 7fq, while the surface area, soil volume, and mass of the cadastral was determined by using QGIS data and satellite image.

3.2.1 Soil extraction

For the analysis of the soil, 44 soil samples were extracted from cadastral 7fq. The samples were extracted horizontally in the soil wall at each station in the depths of 0.15, 0.30, and 0.45 m under terrain (m u.t.) for the analysis of the soil texture, organic content, and pH, and to get a vertical profile of the soils Cd, Pb and Zn concentration. At st. 1, samples were not extracted at 0.45 m u.t, as a raising soil water level prevented sample extraction. The extraction of soil samples for the analysis of the Cd, Pb, and Zn concentrations were conducted without the use of metal equipment in order to prevent contamination of the samples. Instead of metal equipment, shovels of hardened plastic were used for the extraction of the soil samples. The samples were transferred into 100 ml blue cap bottles and stored at 5°C to reduce biological activity.

3.2.2 Soil texture, organic content and pH analysis

Soil texture

For the determination of the soil texture, soil samples were weighed, and subsequently dried at 105°C for 18 hours. The soil particle size distribution was then determined in compliance to Aalborg University (AAU) sieving standard in order to separate the clay particles from the other soil particles (Appendix B.1). Clay particles range in size from $\leq 2\mu$m, in order to determined the clay content, the sieving remnant with a diameter lower than 63μm, were collected from the base cover in accordance with Dierickx, (2009). The soil remnant, were then air dried for three weeks to determine the water content at air dry conditions. The water content was determine by introducing the samples to a Mettler Toledo HB43 halogen. The content of clay within the sample was then estimated as 10 times the content of water at air dry conditions in accordance to Møldrup, (2015). Subsequently, the clay content and distribution of other soil particles were then compared to a soil texture classification triangle, to determine the samples soil texture (figure 3.2).

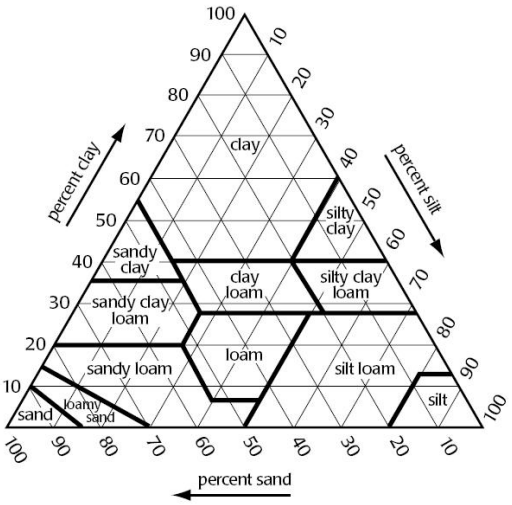


Figure 3.2: Soil texture classification triangle (Loll and Møldrup, 2000).

Organic content

The determination of the organic content was performed in compliance to [DS 204]. The samples were weighed, before introduced to an oven at 105°C for 18 hours in order to evaporate the water content. The dried samples were then placed in a desiccator to prevent condensation from the air until the samples had cooled down. Subsequently, the samples were then weighed, and the dry matter content was determined by using equation 3.1.

$$D = \frac{w - t}{s} \times 10^3 \tag{3.1}$$

Where:

D: is the dried matter content within the sample [g/kg]

w: is the combined weight of dried sample and tray [g]

t: is the weight of the tray [g]

s: is the amount of used sample [g]

The organic content were determined by incinerating the samples at 550°C in a furnace for 1 hour, in order to the remove the organic material. The samples were then placed in a desiccator, before weighed. The organic content was determined by using equation 3.2.

$$O = \frac{i - t}{s} \times 10^3 \tag{3.2}$$

Where:

O: is the amount of ignited organic material within the sample [g/kg]

i: is the combined weight of the tray and sample after ignition [g]

The loss of ignition was subsequently determined by using equation 3.3

$$I = D - O \tag{3.3}$$

Where:

- I: is the loss of ignition of the sample [g/kg]

pH analysis

For the determination of the soil pH, 10 g of each soil sample were dried at 105°C for 18 hours, before transferred into 100 ml BlueCap bottles. Subsequently, 25 ml of 0,01 M calcium chloride ($CaCl_2$) were added to the samples, before the samples were put in suspension for a hour by using an HS 501 digital shaking table. The samples were then allowed to settle, before the pH were determined by using a PHM210 standard pH meter. Measurements of soil pH using $CaCl_2$ solutions are 0.5 lower than samples measured in water, as $CaCl_2$ affect the release of H^+ ions from the soil. In order to bring the samples pH value to the same as measured in an aqueous solution, an additional value of 0.5 were added to the measured pH (Andersen et al., 2000; Note et al., 2017).

3.2.3 Heavy metal concentration in samples

The concentration of Cd, Pb and Zn in samples was determined in triplicates, and in accordance to a AAU modified version of [DS 259] (Appendix B.2). For the analysis of the Cd, Pb and Zn concentrations, the samples were transferred into ceramic crucibles, before dried in an oven at 105 °C for 12 hours. The samples were then placed in a desiccator for 30 min in order to prevent condensation from the air. The samples was subsequently homogenized by using an agate mortar and 1 g of each sample was transferred to 100 ml blue cap bottles. 20 ml of ultra pure 7M nitric acid (HNO_3) was then added to the samples, before the mixture was autoclaved at 120 °C for 30 min in order to open the samples. Samples with high humus content or consisting of dried plant material are before being introduced to an autoclave,

kept agitated in the HNO_3 for approximately 12 hours or until all organic material are dissolved. The samples were then diluted in 80 ml of ultra pure demineralized water, that had been filtered through an ELGA Purelab FLEX, before 9 ml of the samples was transferred to 15 ml Greiner centrifuge tubes and subsequently mixed with 1 ml of a 10 ppm yttrium (Y) standard. The samples were then shaken manually, before centrifuged at 4.000 rpm for 5 min. The centrifuged samples were introduced to an Inductively Coupled Plasma Mass Spectrophotometer (ICP-MS) in order to determine the concentration of Cd, Pb, and Zn in the sample. In the ICP-MS each sample is mixed with argon (Ar) under a steady flow, before it is nebulized in order to remove excessive H_2O and waste. The nebulized sample is then ionized with inductively coupled plasma and subsequently introduced to a mass spectrometer (Nielsen, 2016). The mass spectrometer is capable of determining the concentration of metals in a sample by sorting the mass of ratio of the ions. The Cd, Pb, and Zn concentrations measured by the ICP was used to determine the concentrations in mg/kg by using equation 3.4.

$$x = a \times \frac{d}{c} \tag{3.4}$$

Where:

x: is the metal concentration of the sample [mg/kg TS]

a: is the concentration of metals within the diluted sample [mg/l]

d: is the volume of the diluted sample [ml]

c: is the weight of dried sample [g]

3.2.4 Cadastral area, soil volume, and mass

Cadastral area

For the assessment of the surface area of cadastral 7fq, satellite images was used in combination with the geographic information system (GIS) software QGIS. The surface area was generated in QGIS by creating an outline of the soil surface of the cadastral.

Soil volume and mass

The soil mass is essential in the estimation of the time required by *S. schwerinii* and *S. viminalis* to remediate the soil of the sampling area at cadastral 7fq. In order to estimate the soil mass, the volume of the soil at cadastral 7fq was calculated. For

the calculation of the soil volume, the generated surface area was combined with the maximum depth of the soil samples of 0.45 m u.t. by using equation 3.5.

$$V = SA \times d \tag{3.5}$$

Where:

V: is the soil volume [m^3]

SA: is the surface area [m^2]

d: is the maximum depth of the samples [m]

The soil volume were then used to determine the bulk density, and subsequently the mass of the soil at the sampling area of cadastral 7fq. The bulk density was determined by using the samples of the organic content analysis (section 3.2.2) average weight fraction of mineral particles, water- and organic content (Klausen et al., 2016). The bulk density was estimated by using equation 3.6.

$$\rho_b = \frac{S_w}{S_v} \tag{3.6}$$

Where:

ρ_b: is the bulk dry soil density [g/cm^3]

S_w: is the dry soil weight [g]

S_v: is the soil volume [cm^3]

The soil mass was then estimated by using the soil volume and bulk density (equation 3.7).

$$M_s = P_b \times V_t \tag{3.7}$$

Where:

M_s: is the soil mass [t]

P_b: is the bulk dry soil density [t/m^3]

S_v: is the total soil volume [m^3]

3.3 Phytoextraction assessment

The capability to efficiently accumulate metals into aboveground tissue, and how a species are affected by these, are essential in the assessment of a plant species applicability as a phytoextractor. The assessment of *S. schwerinii* and *S. viminalis* are based on pot experiments, using soil excavated from cadastral 7fq. The species are analyzed for how Cd, Pb, and Zn affects their physical traits and photosynthesis, and their capability to accumulate and translocate these three metals. In the assessment of the species phytoextraction capabilities, both inoculated and non-inoculated cuttings are used, to asses if the AMF *R. irregularis* has potential of enhancing the species metal uptake.

3.3.1 Plant cultivation

For the assessment of *S. schwerinii* and *S. viminalis* suitability for phytoextraction, three 60 cm cuttings of both Salix species were planted for each of the analyses of the species metal tolerance, and phytoextraction in soil with and without arbuscular mycorrhiza. The cuttings was donated by Ny Vraa Bioenergy I/S, and were planted in 12 L pots filled with soil excavated from 0.15 m u.t at st. 1 and 2. The depth and stations, at which soil was extracted for the pots was selected based on the results of the soil analysis. The cuttings was weighed and subsequently planted respectively by immersing approximately 15 cm of the steam in the soil of each respective pot. In order to ensure that the cuttings were at the same growth stage during measurements of how the metals affect the species and the species phytoextraction, the cuttings for each analyses were planted at the same time. Due to a delay in the shipment of spores, the cuttings inoculated with *R. irregularis* were planted later, than the cuttings of the other analyses. The cuttings were then transferred to two Snijders Labs high specification plant growth chambers for a growth period of 47 days. The growth period of 47 days were based on providing the cuttings enough time to develop a sufficient root surface area, and accumulate Cd, Pb, and Zn from the contaminated soil. The chambers were set to run by a fixed day cycle over the growth period (fig. 3.3). The programmed environmental conditions of the day cycle corresponds approximately with the average day and night temperature, humidity, and light intensity in July month in Denmark (Andersen et al., 2000). For the prevention of differences between the used chambers and the pots placement within these, the pots were rotated within the chambers every 10^{th} day, and relocated between the chambers every 20^{th} day.

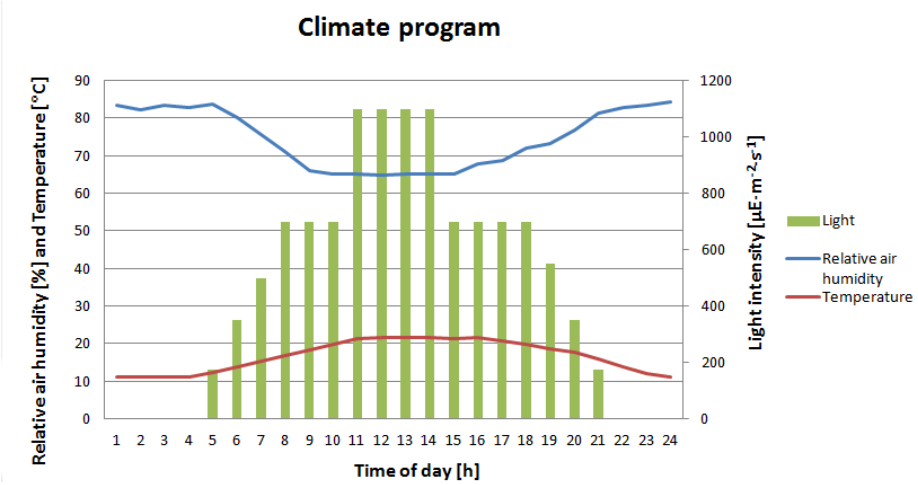


Figure 3.3: Day and night cycle based on the logged data from growth chambers

At the end of the growth period, the cutting was harvested by slowly removing the soil around the roots, before any remaining soil were removed with a brush and demineralized water. The cuttings root and shoot system were after the harvest separated and subsequently dried at 105°C for 39 hours in order remove the water content. The dried samples were then processed into smaller homogenized fragments by using a rubber mallet, before transfered to 250 ml blue cap bottles.

3.3.2 Heavy metals effect on plants

For the assessment of the possible inhibitory effects of Cd, Pb, and Zn on the growth and photosynthesis of *S. schwerinii* and *S. viminalis*, the cuttings of the HMs effect and phytoextraction analyses were compared in terms of biometrics, photosynthetic rate, and biomass production. The cuttings used to determine the metals effect on the species, were planted in non-contaminated soil from st. 2, to enable a comparison between cuttings grown in non-contaminated and contaminated soil (section 3.3.1).

Biometric measurements

Biometric measurements were carried out on the cuttings for the inhibition and phytoextraction analysis, during the growth period in order to assess any characteristic differences between the cuttings. Measurements of the cuttings height, number of shoots and leaves, and leaf surface area, were measured at three different dates separated by 10 days respectively. The measuring of height, number of shoots and leaves were carried out manually, while the leaf surface area were

assessed by using a combination of photographed images and the image processing tool ImageJ. 12 randomly selected leaves were photographed alongside a ruler for scale, and subsequently processed in ImageJ to create an outline of each leaf, before the leaf surface area was generated.

Photosynthetic rate

To asses the possible inhibiting effects of Cd, Pb, and Zn on the photosynthetic rate for *S. schwerinii* and *S. viminalis*, a Ciras-3 portable photosynthesis system was used to measure the rate of photosynthesis of each cutting. The Ciras-3 system, functions by having a leaf carefully inserted into a leaf chamber, and subsequently adding a steady air flow with a known humidity and CO_2 concentration to the chamber (fig.3.4). Environmental controls ensures parameters like temperature, light intensity, and the air flow humidity and CO_2 concentration are kept constant inside the chamber. The system uses the difference in humidity and CO_2 in the air that enters and leaves the chamber to calculate the photosynthetic rate.

Figure 3.4: Leaf inside the leaf chamber

The photosynthetic rate of *S. schwerinii* and *S. viminalis* were measured at CO2 concentration for atmospheric air and within the growth chamber of 400 ppm and 500 ppm respectively (Monroe, 2017). The environmental settings of the Ciras-3 systems are shown in table 3.1 . The CO_2 concentrations and environmental conditions were chosen to simulate semi-natural conditions, and the growth chambers programmed day cycle.

Table 3.1: Environmental settings for the Ciras-3 portable photosynthesis system.

Light intensity [μE·m^{-2}·s^{-1}]	Relative humidity [%]	Temperature [°C]
350	80	14
700	70	17
1100	65	21.5

Biomass production

In order to the determine the biomass production, the harvested cuttings were weighed and compared to their initial weight, before the root and shoot system was separated. The biomass production were determined by using equation 3.8.

$$\Delta M = m_g - m_i \tag{3.8}$$

Where:

ΔM: is the change in biomass from the growth period [g]

m_g: is the weight of the cuttings after the growth period [g]

m_i: is the initial weight of the cutting [g]

3.3.3 Phytoextraction and mycorrhiza

For the assessment of *S. schwerinii* and *S. viminalis* capability as phytoextractors of Cd, Pb, and Zn, and *R. irregularis* potential of enhancing the species uptake of these metals, inoculated and non-inoculated cuttings were planted in contaminated soil from st. 1 (section 3.3.1). The cuttings that was used to analyze *R. irregularis*, was inoculated with a spore solution before the cuttings were planted in the soil. The spore solution were made by diluting 1 ml axenic culture of *R. irregularis* in 500 ml demineralized water. The axenic culture were acquired from the company Agronutrition, and the cuttings were placed in the spore solution for 10 min, before each of them were planted in their respective pot (Brundrett et al., 1996). In order to ensure the mycorrhization of the cuttings root system, each of the cuttings were during the growth period, watered respectively every 14^{th} day with 20 ml of the *R. irregularis* solution (Brundrett et al., 1996). The roots of the inoculated cuttings were after the harvest examined through a SMZ-168 Motic microscope for the presence of *R. irregularis*. The inoculated and non-inoculated cuttings were then processed into dried samples (section 3.3.1), and subsequently determined for their concentrations of Cd, Pb, and Zn in accordance to section 3.2.3.

Root concentration factor

Using the concentrations of Cd, Pb, and Zn in the dried root samples it was possible to determine the root concentration factor (RCF) of *S. schwerinii* and *S. viminalis*. The RCF describes the roots capability of accumulating metals from the surrounding soil, and was determined by using equation 3.9 (Kang et al., 2010).

$$RCF = \frac{C_R}{C_S} \tag{3.9}$$

Where:

RCF: is the root concentration factor

C_R: is the concentration of Cd, Pb, or Zn in the root [mg/kg TS]

C_S: is the concentration of Cd, Pb, or Zn in the soil medium [mg/kg TS]

Bioconcentration factor

To assess the efficiency of *S. schwerinii* and *S. viminalis* as phytoextractors, the bioconcentration factor (BCF) was determined by using equation 3.10. The BCF indicates how efficiently *S. schwerinii* and *S. viminalis* are in accumulating metals into their aboveground tissue from the soil (Ali, Khan, and Sajad, 2013). For most plants, the BCF is $\leq$1, and increments in the value indicates an increase in the species uptake and accumulation (Bhargava et al., 2012).

$$BCF = \frac{C_P}{C_S} \tag{3.10}$$

Where:

BCF: is the bioconcentration factor

Translocation factor

In order to estimate how efficient *S. schwerinii* and *S. viminalis* translocate Cd, Pb, and Zn from the root to shoots system, the transloaction factor (TF) of each of the metals was determined by using equation 3.11 (Ali, Khan, and Sajad, 2013). TF values $\geq$1 indicates an efficient translocation of metal between a species belowground and aboveground tissue (Rezvani and Zaefarian, 2011).

$$TF = \frac{C_P}{C_R} \tag{3.11}$$

Where:

TF: is the Translocation factor

C_P: is the concentration of Cd, Pb, or Zn in the stem and shoots [mg/kg TS]

Remediation time

The two salix species extraction rate of Cd, Pb, and Zn are essential for the estimation of the time required by the species to remediate the soil in an area. The extraction rate of both species were estimated by using equation 3.12 (Algreen, Trapp, and Rein, 2014).

$$k = -(BCF \times \frac{\Delta M_y}{M_s}) \tag{3.12}$$

Where:

k: is the extraction rate [years]

ΔM_y: is the change in biomass per year [kg m^{-2}]

M_s: is the soil mass [kg m^{-2}]

Using the estimated extraction rate of Cd, Pb, and Zn, the time required by *S. schwerinii* and *S. viminalis* to reduce the concentrations of the three metals to the Danish soil quality criteria was determined by using equation 3.13(Algreen, Trapp, and Rein, 2014).

$$t = \frac{ln(\frac{C_{(0)soil}}{C_{(t)soil}})}{k} \tag{3.13}$$

Where:

t: is the remediation time [years]

$C_{(0)soil}$: is the current soil concentration of metal [mg/kg]

$C_{(t)soil}$: is the soil quality criteria for the metal [mg/kg]

4. Results

4.1 Soil analysis

4.1.1 Soil texture, organic content, and pH analysis

The particle distribution, organic content, and pH of the soil samples from each sampling station and depth are presented in table 4.1. There are a clear difference in the distribution of soil particles at each station, and most notable are the difference between st. 2 and 3. Based on the distribution, the soil texture at st. 1 can be classified as sandy clay loam, while it at st. 2 are sandy loam soil. The soil texture at st. 3 and 4 can be categorized as a clay and sandy clay respectively (fig.3.2)(Loll and Møldrup, 2000). Soil pH at st. 1 and 2 lies within the neutral range, while the soil pH at st. 3 and 4 ranges between 8.9 to 12.5, indicating strong alkaline conditions.

Table 4.1: Soil particle distribution and pH of the soil samples extracted from the stations at cadastral 7fq, the samples are presented in percent based on the weight of the particle fraction

St.	Depth m u.t.	Clay $\leq 2\mu$m (%)	Silt 2-63μm (%)	Sand 63-200μm (%)	Coarse sand 200-2000μm (%)	Organic content (%)	pH
1	0.15	27.4	0.86	58.50	9.93	3.31	7
	0.30	26.7	0.19	60.88	9.06	3.17	7.2
2	0.15	17.2	1.53	61.29	17.72	2.26	7.1
	0.30	13.9	0.69	61.62	22.16	1.64	7.1
	0.45	16.1	0.90	59.52	22.04	1.44	7.1
3	0.15	43.6	0.22	43.68	9.11	3.39	9.5
	0.30	47.9	0.31	37.97	9.42	4.41	12
	0.45	48.9	0.99	38.16	8.13	3.82	12.5
4	0.15	37.9	1.11	50.38	8.30	2.31	9.1
	0.30	40.8	0.89	41.78	13.81	2.72	8.9
	0.45	38	1.33	44.47	12.63	3.56	9

4.1.2 Heavy metal concentration in soil samples

The concentration of Cd, Pb, and Zn measured in the fill soil samples from cadastral 7fq are shown in table 4.2. Generally, the measured concentrations were lowest for Cd, and highest for Zn at all sampling depths. There are notable variations in the concentrations of Cd, Pb, and Zn among the four sampling stations, with st. 4 having the highest concentrations of all the HMs, followed by st. 3. The concentration of Cd and Pb were far above the Danish soil quality criteria for almost all of the sampling stations, with exception of st. 2. In accordance to the Danish quality criteria, the soil at st. 2 can be categorized as non-contaminated (Miljøstyrelsen (Danish EPA), 2015).

Table 4.2: Vertical profile of the mean Cd, Pb, and Zn concentrations in the soil from the different depths

St.	Depth m u.t.	Cd [mg/kg dm]	Pb [mg/kg dm]	Zn [mg/kg dm]
1	0.15	10.80 (±0.23)	220.86 (±3.44)	323.09 (±4)
	0.30	3.05 (±0.54)	28.23 (±6.55)	101.55 (±11.47)
2	0.15	0.06 (±0.01)	4.39 (±0.03)	18.72 (±1.97)
	0.30	0.06 (±0.03)	4.46 (±0.80)	20.35 (±7.56)
	0.45	0.11 (±0.01)	6.70 (±0.67)	26.28 (±1.60)
3	0.15	10.65 (±0.45)	86.96 (±1.32)	302.45 (±4.36)
	0.30	11.56 (±0.62)	216.20 (±14.41)	338.83 (±5.02)
	0.45	10.10 (±0.09)	365.76 (±16.31)	544.70 (±11)
4	0.15	15.76 (±0.23)	320.64 (±0.77)	432.22 (±2)
	0.30	12.31 (±0.60)	282.34 (±0.67)	403.58 (±6.79)
	0.45	16.76 (±0.81)	367.03 (±17.17)	481.82 (±16.29)
Soil quality criteria		0.5	40	500

Surface area, soil volume and mass

The surface area of the sampling area at cadastral 7fq was determined to be 55.000 m^2 (5.5 ha), and by combining the surface area with the maximum depth of extracted samples of 0.45 m u.t., a soil volume of 25.000 m^3 was estimated. The total mass of soil at the sampling area that requires to be remediated was determined to be 23.000 t.

4.2 Phytoextraction Analysis

4.2.1 Heavy metals effect on plants

Biometric measurements

The measured biometric traits of *S. schwerinii* and *S. viminalis* are plotted in figure 4.1. There are a clear difference in the measured traits between the cuttings that have grown in contaminated and non-contaminated soil. The height and amount of leaves were generally higher for the cuttings of both *S. schwerinii* and *S. viminalis* grown in non-contaminated soil, than for the cuttings grown in HM contaminated soil. The amount of shoots varies slightly between the two species, with *S. viminalis* grown in non-contaminated soil producing the highest amount of new shoots during the growth period. The average leaf surface area were significantly highest for *S. viminalis* in non-contaminated soil, followed by the cuttings of *S. schwerinii* grown in both contaminated and non-contaminated soil.

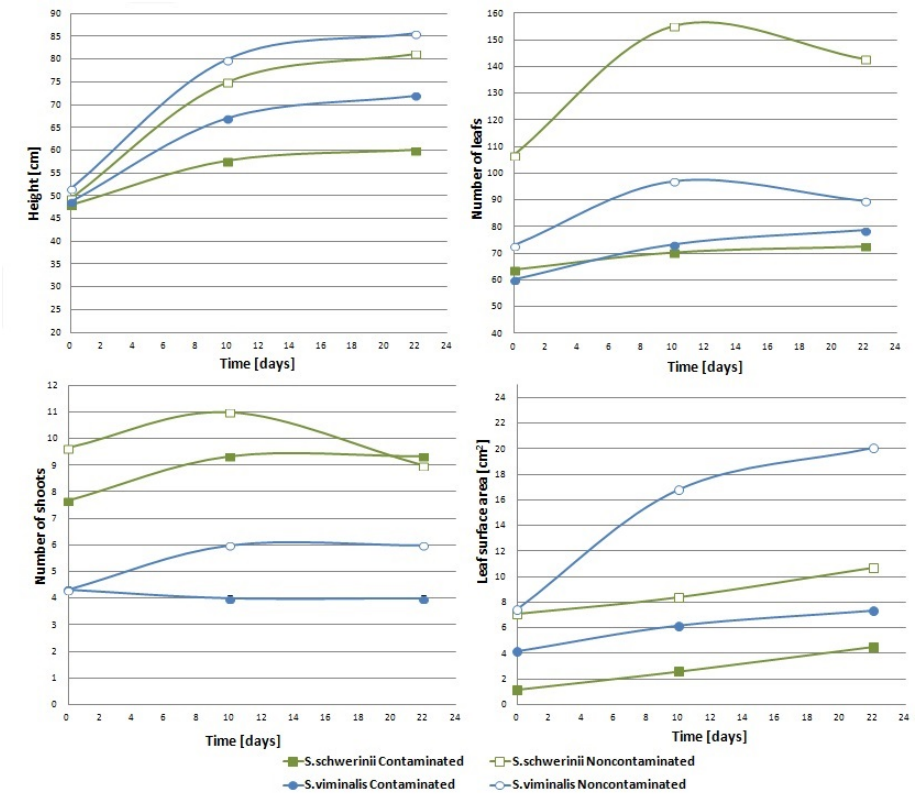
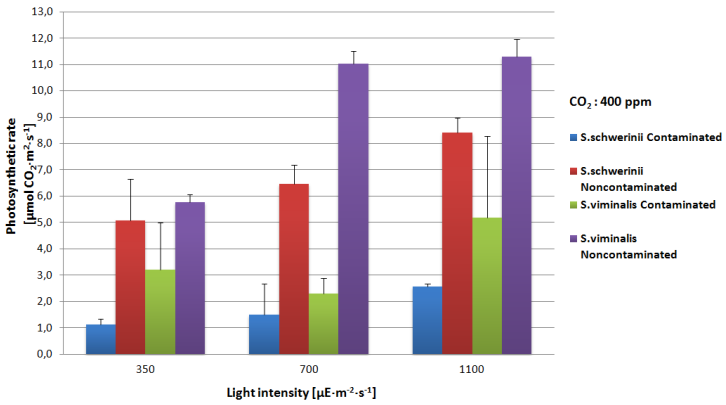


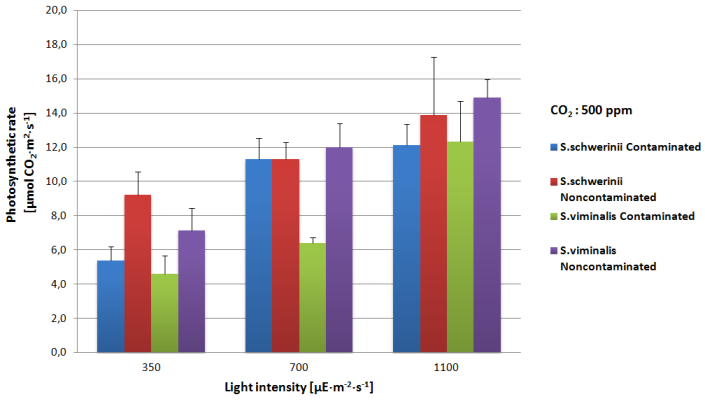
Figure 4.1: The different biometric measurements as a function of time. The measurements are based on the measured mean values of the cuttings biometric traits throughout the growth period

Photosynthetic rate

The photosynthetic rate of *S. schwerinii* and *S. viminalis* at 400 ppm and 500 ppm CO_2 are presented in figure 4.2. The highest photosynthetic rates was measured for the cuttings grown in non-contaminated soil. The photosynthetic rate of *S. schwerinii* and *S. viminalis* are significantly higher at elevated CO_2 concentrations, and most notable are the increased photosynthetic rates of *S. schwerinii* grown in contaminated soil at light intensity of 700 and 1100 μE·m^{-2}·s^{-1}.



(a) The photosynthetic rate of the two salix species at 400 ppm CO_2 concentration



(b) The photosynthetic rate of both salix species at 500 ppm CO_2 concentration

Figure 4.2: The photosynthetic rate of *S. schwerinii* and *S. viminalis* as a function of light intensity. At CO_2 concentrations of 400 and 500 μmol mol^{-1}, and the light intensities 350, 700, and 1100 μE·m^{-2}·s^{-1}. The error bars indicate $\pm$ standard deviations (SD) of triplicated samples

Biomass production

The total amount of biomass produced by *S. schwerinii* and *S. viminalis* during the growth period are shown in figure 4.3. The average biomass production of *S. viminalis* were generally higher than for *S. schwerinii* in contaminated soil. The biomass production are significantly higher for the cuttings grown in non-contaminated soil than for those cuttings grown in HM contaminated soil, with *S. schwerinii* having the highest biomass production. There are a slight difference in the biomass production of *S. schwerinii* between the cuttings grown with and without the presence of *R. irregularis*.

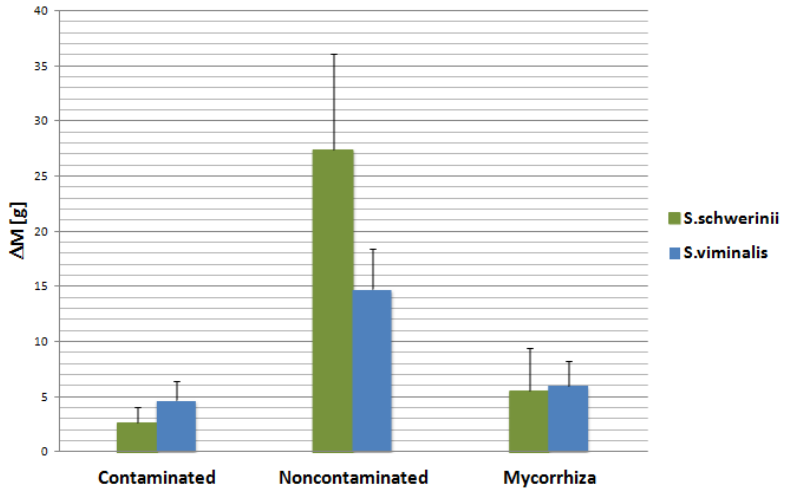


Figure 4.3: The total biomass production of *S. schwerinii* and *S. viminalis* during the growth period. The measurements are based on the mean initial and end weight of the cuttings used for the HM effects on salix species, phytoextraction, and mycorrhiza analyses. The error bars represents SD

4.2.2 Phytoextraction

The measured concentrations of Cd, Pb, and Zn in the soil and plant samples of the phytoextraction analysis are presented in table 4.3. The concentration of Cd, Pb, and Zn in the initial and ending soil samples shows a reduction in the soil metal concentration. Generally, the reduction of the initial concentration were highest for Zn, and lowest for Cd. There are a clear difference between the concentrations of Pb and Zn in the root and shoot systems for both *S. schwerinii* and *S. viminalis*. The variations in the metal concentrations between the two species root and shoot systems, indicates a difference in the species uptake, distribution, and accumulation of the three metals.

Table 4.3: The concentration of Cd, Pb, and Zn in the soil and plant samples of the phytoextraction analysis. The measurements are based on triplicates, and are presented as averaged values [mg/kg dry matter].

Species	Sample	Cd [mg/kg dm]	Pb [mg/kg dm]	Zn [mg/kg dm]
S. schwerinii	Initial soil	9.37 (±0.77)	155.02 (±18.86)	261.45 (±22)
	End soil	8.71 (±0.16)	145.66 (±14.99)	234.67 (±9.68)
	Root system	0.73 (±0.05)	2.11 (±0.64)	85.15 (±16.63)
	Shoot system	0.78 (±0.17)	0.14 (±0.08)	108.80 (±36.41)
S. viminalis	Initial soil	10 (±0.73)	181.96 (±21.73)	283.20 (±30.56)
	End soil	9.03 (±1.16)	166.17 (±33.20)	256.99 (±38.62)
	Root system	0.9 (±0.24)	11.39 (±6.18)	78.41 (±2.91)
	Shoot system	0.43 (±0.05)	0.14 (±0.03)	94.17 (±11.46)

Root concentration, bioconcentration, and translocation factor in non-inoculated samples

The determined RCF, BCF, and TF values of Cd, Pb, and Zn for *S. schwerinii* and *S. viminalis* are shown in table 4.4. The RCF and BCF of the three metals varies, and indicates a difference in the metal uptake of the two salix species. The TF of Cd and Zn were in general higher than for Pb. The difference between the TF values of the three metals are most notable for *S. schwerinii*, and indicates a more efficient transport of Cd and Zn between the belowground and aboveground tissue than for Pb.

Table 4.4: The calculated RCF, BCF, and TF values of Cd, Pb, and Zn for the two salix species. The measurements are based on the mean values of the soil and plant samples concentration of Cd, Pb, and Zn (table 4.3).

Species	Biological Factor	Cd	Pb	Zn
S. schwerinii	RCF	0.08	0.01	0.36
	BCF	0.09	0.01	0.46
	TF	1.07	0.07	1.26
S. viminalis	RCF	0.10	0.07	0.31
	BCF	0.05	0.01	0.38
	TF	0.5	0.01	1.20

Remediation time

The time *S. schwerinii* and *S. viminalis* requires to reduce the concentration of Cd, Pb, and Zn in the fill soil at cadastral 7fq is presented in table 4.5. There is a significant difference in the remediation time of the three metals for both of the salix species. *S. viminalis* showed to have a lowest remediation time of Pb and Zn, whereas *S. schwerinii* had the most efficient reduction time of Cd.

Table 4.5: The estimated reduction time of Cd, Pb, and Zn for *S. schwerinii* and *S. viminalis*

Species	Metal	Time of remediation [years]
S. schwerinii	Cd	178.000
	Pb	7.784.000
	Zn	1050
S. viminalis	Cd	240.000
	Pb	6.683.000
	Zn	920

4.2.3 Mycorrhiza

The concentrations of Cd, Pb, and Zn in the samples of the inoculated cuttings are shown in table 4.6. In general, the initial and ending soil samples shows an increase in the concentration of the three metals in the soil that surrounded the cuttings root system. The increment in the soil metal concentration were higher for Zn, than for any of the other metals. The concentration of Cd, Pb, and Zn was significantly higher in the roots, than in the shoot systems. The metal concentration ratio between the root and shoot systems, indicates a low translocation rate between the two systems.

Table 4.6: The measured concentrations of Cd, Pb, and Zn [mg/kg dry matter] in the soil and inoculated plant samples of the mycorrhiza analysis.

Species	Sample	Cd [mg/kg dm]	Pb [mg/kg dm]	Zn [mg/kg dm]
S. schwerinii	Initial soil	10.87 (±1.5)	214.92 (±61.67)	303.09 (±47.36)
	End soil	11.26 (±1.51)	222.84 (±62.43)	332.67 (±55.85)
	Root system	1.42 (±0.61)	22.14 (±17.06)	93.53 (±17.65)
	Shoot system	0.52 (±0.05)	0.23 (±0.03)	85.45 (±1.45)
S. viminalis	Initial soil	10.76 (±0.94)	183.02 (±21.61)	284.28 (±38.82)
	End soil	10.72 (±0.49)	203.10 (±17.7)	297.64 (±16.24)
	Root system	2.06 (±0.61)	32.29 (±10.3)	96.08 (±8.99)
	Shoot system	0.54 (±0.02)	0.20 (±0.03)	80.80 (±20.68)

Root concentration, bioconcentration and translocation factor

The inoculated samples of *S. schwerinii* and *S. viminalis* RCF, BCF, and TF values of Cd, Pb, and Zn are presented in table 4.7. The RCF value of the three metals differ between the two salix species, with *S. viminalis* having the highest measured RCF values of the three metals. There is no difference between *S. schwerinii* and *S. viminalis* BCF values of Cd, Pb, and Zn. The TF values of Cd and Zn measured for both *S. schwerinii* and *S. viminalis* clearly indicates a difference between the three metals translocation from root to shoot system.

Table 4.7: The calculated RCF, BCF, and TF of the three metals for the inoculated cuttings of *S. schwerinii* and *S. viminalis*.

Species	Biological Factor	Cd	Pb	Zn
S. schwerinii	RCF	0.12	0.09	0.28
	BCF	0.05	0.01	0.26
	TF	0.40	0.01	0.93
S. viminalis	RCF	0.19	0.16	0.32
	BCF	0.05	0.01	0.27
	TF	0.28	0.01	0.86

Remediation time

The estimated time required for the *R. irregularis* inoculated cuttings of *S. schwerinii* and *S. viminalis* to clean the soil at cadastral 7fq for Cd, Pb, and Zn are shown in table 4.8. The time required for each of the metals concentration to reach the criteria for non-contaminated soil varies considerably for both salix species. In general, *S. viminalis* showed the highest efficiency in reducing the metals concentration towards the Danish quality criteria.

Table 4.8: The time required to reach the Danish soil quality criteria for Cd, Pb, and Zn, using *R. irregularis* inoculated cuttings of *S. schwerinii* and *S. viminalis*.

Species	Metal	Time of remediation [years]
S. schwerinii	Cd	222.000
	Pb	5.693.000
	Zn	1090
S. viminalis	Cd	184.000
	Pb	5.136.000
	Zn	1000

5. Discussion

5.1 Soil assessment

The composition of the fill soil at the samplings stations was found to vary in the distribution of soil particles, and indicates a difference in the soil texture at the cadastral (table 4.1). An areas composition of particles can vary naturally by weathering and erosion (Lyles and Tatarko, 1986). However in this case, the difference in soil texture among the sampling stations can be assumed to be related to anthropogenic activities due to the history of the cadastral. The found soil textures sandy clay loam and sandy loam at st. 1 and 2 respectively indicate that changes in the soil texture occur among the former sludge basins. These results corresponds with the findings of Rasmussen, (1988) from the sludge basins at the cadastral.

Rasmussen, (1988) state that the fill soil at the cadastral partly consists of calcium carbonate ($CaCO_3$) and calcium oxide (CaO). $CaCO_3$ and CaO could explain the variations in soil pH at the cadastral (table 4.1) as these inorganic compounds in the presence of water forms calcium bicarbonate ($Ca(HCO_3)_2$) and calcium hydroxide $Ca(OH)_2$, which are strongly alkaline (Kouzu et al., 2008). In comparing the estimated clay content with the measured soil pH it can be suggested that the highest concentrations of $CaCO_3$ and CaO at the cadastral can be found at the sampling stations with the highest clay content (table 4.1). Taylor et al., (2015) describes that the soil capacity to adsorb inorganic compounds increases with a decrease in particle size. A high adsorption of $CaCO_3$ and CaO in the clay at st. 3 and 4, could explain why strong alkaline conditions are found at these stations comparing to the other sampling stations on the cadastral. A comparison between the findings by Rasmussen, (1988) and this study shows that the soil pH at st. 1 and 2 have visibly been reduced through out the years. Loll and Møldrup, (2000) describes that, the soils efficiency in retaining soil water and thereby reduce soil leaching, decreases with an increase in soil particle size. The reduction of soil pH at st. 1 and 2 might therefore be explained by a gradual leaching of the the soils content of $CaCO_3$ and CaO, induced by these stations higher composition of coarser soil particles.

The measured concentrations of Cd and Pb in the fill soil at sampling station 1, 3, and 4 are all above the quality criteria for contaminated soil (table 4.2). Therefore the only sampling station at cadastral 7fq that fulfills the criteria for non-contaminated soil are st. 2. The concentrations of Cd, Pb, and Zn at st. 2 are supported by the findings of Rasmussen, (1988), and corresponds with the observations made at the cadastral. Considerable more vegetation was observed at st. 2 compared to the other sampling stations at the cadastral which indicates less growth inhibition at this part of the cadastral (fig. 5.1).

(a) The vegetation at sampling station 2 **(b)** Vegetation at sampling station 3

Figure 5.1: Vegetation comparison between two of the sampling stations at cadastral 7fq.

The measured concentrations of Cd, Pb, and Zn in the fill soil from st. 1 indicate that metals at this station are somewhat soluble, and are being transported within the groundwater towards the topsoil. Metal solubility and movement in soil have not been measured in this report, due to administrative restrictions to prevent contamination of research equipment. However, findings from other studies describe that nutrients e.g. metals and phosphorous in the soil solution generally are mobilized towards the upper soil layers due to biological activity in the topsoil (Schachtman, 1998; Taylor et al., 2015). Metal ions have a strong affinity to the negatively charge clay particles, and tends to adsorb through ion exchange or specific adsorption to the surface of clay (Taylor et al., 2015). The adsorption onto clay particles reduces the leach of metals, and allow metal precipitation in the soil. The high measured clay content at st. 3 and 4 therefore can explain why the highest measured concentrations of Cd, Pb, and Zn in the fill soil was found at these stations compared to the other sampling stations.

5.2 Phytoextraction assessment

5.2.1 Heavy metals effect on plants

The purpose of the analyses was to assess the possible inhibiting effects of Cd, Pb, and Zn on the growth and photosynthesis of *S. schwerinii* and *S. viminalis*. The biometric measurements showed a significant reductive effect by the presence of Cd, Pb, and Zn on the growth of both of the salix species (fig. 4.1). Studies on HM toxicity in plants describes that signs of Zn toxicity generally become visible at aboveground tissue concentrations of >300 mg kg^{-1} Zn (Broadley et al., 2007). In this study the measured Zn concentrations in the aboveground tissue of *S. schwerinii* and *S. viminalis* did not exceed 108.80 mg kg^{-1} Zn (table 4.3). Thereby it is assumed that Zn is not the cause of growth reduction in the two salix species. Visible signs of metal toxicity was observed during measurements in form of reduced height, leaf growth, and the development of chlorosis on individual leaves. These are both signs of Cd and Pb toxicity as both metals affect various fundamental processes within the plants like nutrient uptake and photosynthesis (Li et al., 2012). Study made by Sandalio et al., (2001) on Cd toxicity states that elevated Cd concentrations decrease plant uptake of Zn, and found that Cd reduces the surface area of the leafs. A reduction of the leaf surface area corresponds with the findings of this study. The average leaf surface area of *S. schwerinii* and *S. viminalis* at the end of the growth period was inhibited by 6.2 cm^2 and 12.7 cm^2 respectively, when compared to the references grown in non-contaminated soil (fig. 4.1). Sharma and Dubey, (2005) describes that high concentration of Pb inhibits the chlorophyll synthesis and nutrient uptake by blocking essential enzyme activity. An inhibition of the chlorophyll synthesis, and nutrient deficiency affects the cuttings chlorophyll production and can lead to chlorosis (Sandalio et al., 2001; Sharma and Dubey, 2005).

Chlorosis and reduction of the leaf surface area are both parameters that affects the photosynthesis of plants. The measured photosynthesis rate of *S. schwerinii* and *S. viminalis* in this study (fig. 4.2) clearly indicated an inhibition of the photosynthesis rate at 400 ppm CO_2. The relative highest reduction in the rate was observed at 400 ppm CO_2 in *S. schwerinii* at 350 $\mu E \cdot m^{-2} \cdot s^{-1}$ and in *S. viminalis* at 700 $\mu E \cdot m^{-2} \cdot s^{-1}$ were the rate was reduced 5-fold for both species compared to the reference cuttings. A comparison between the photosynthesis rates measured at 400 ppm and 500 ppm CO_2 indicate that the inhibition of the photosynthesis rate are lessen at elevated CO_2 concentrations. In general the measured photosynthesis rates at 500 ppm CO_2 are higher than at 400 ppm CO_2, which could be due to the elevated CO_2 concentration (Silvola and Ahlholm, 1992). However, this does not explain why the inhibition of *S. schwerinii* and *S. viminalis* photosynthesis rate

are lessen at 500 ppm CO2 than at 400 ppm. Therefore in future studies it could relevant to examine if there is any correlation between elevated CO_2 concentration and the reduction of metal inhibition of photosynthesis rate. Since photosynthesis is essential for the plant growth, its inhibition can be assumed to be the principal factor of the growth reduction of *S. schwerinii* and *S. viminalis* (Sadava et al., 2009).

5.2.2 Phytoextraction and mycorrhiza

The growth soil of the non-inoculated and inoculated cuttings of *S. schwerinii* and *S. viminalis* were measured before and after the growth period for any variations in the concentrations of Cd, Pb, and Zn (table 4.3 and 4.6). The samples of the inoculated cuttings growth soil showed elevated concentrations of all three metals at the end of the growth period. The elevated metal concentrations can be explained by how the soil samples were extracted during the harvest, as samples were taken solely of the soil surrounding the cuttings roots. Therefore these soil samples are not representing the general metal concentration of growth soil in the pots, but rather the local soil concentration surrounding the roots of the inoculated cuttings. Other studies on phytoextraction describes that soluble metals in the soil solution are gradually drawn towards the roots by the plants active and passive adsorption of water (Ali, Khan, and Sajad, 2013). A gradually movement of the growth soils metal concentration towards the soil surrounding the cuttings roots can therefore explain why an elevation in the samples metal concentration are measured at the end of the growth period (table 4.6). The samples of the non-inoculated cuttings growth soil were extracted more thoroughly, as the soil for each sample were taken from different areas in the growth pots. These samples are therefore more representative for the general concentration of Cd, Pb, and Zn in the growth soil. The determined concentrations of the three metals in the soil samples of the non-inoculated cutting at the end of the growth period showed a reduction of the initial metal concentration (table 4.3). The reduction of the initial soil samples metal concentration suggests an adsorption of the three metals and can be assumed to be caused by the cuttings.

The belowground and aboveground tissue of the non-inoculated cuttings of *S. schwerinii* and *S. viminalis* metal concentration indicates a difference between the species translocation of Cd, Pb, and Zn (table 4.3). The concentrations of the three metals in *S. schwerinii* indicates that the species prefer to translocate Cd and Zn into its aboveground tissue whilst Pb generally are kept within the root. These findings corresponds well with the calculated TF values for *S. schwerinii* (table 4.4), and the results of other studies of *S. schwerinii* for phytoextraction (Meers et al., 2007). The belowground and aboveground tissue of *S. viminalis* concentrations of Cd, Pb, and Zn suggest that the species have a relative higher selective metal transport than *S.*

schwerinii as the species concentration of Cd and Pb were found to be highest in the belowground tissue. The calculated RCF and BCF of Cd and Pb supports these findings, and indicates that *S. schwerinii* are more efficient in accumulating Cd and Pb in its belowground tissue than in the aboveground tissue (table 4.4). However according to literature the calculated BCF value of Cd for *S. viminalis* are relatively low compared to cuttings grown in field studies in low to moderate contaminated soil (Algreen, Trapp, and Rein, 2014). Zhao, Lombi, and McGrath, (2003) describes that BCF decreases with elevated high metal concentrations, as the plants metal transport between the root and shoot may become saturated and thereby lessen the plants metal uptake. Therefore it is likely that the relative low calculated BCF values are affected by the high metal concentration in the soil (table 4.3).

The measured concentrations of Cd, Pb, and Zn in the *R. irregularis* inoculated cuttings of *S. schwerinii* and *S. viminalis* showed elevated metal concentrations in the species belowground tissue compared to the non-inoculated cuttings (table 4.6). The elevation in the belowground tissue indicate that a inoculation with *R. irregularis* may increase *S. schwerinii* and *S. viminalis* adsorption of Cd, Pb, and Zn. Yang et al., (2016) found that an inoculation of legume trees with AMF increased the adsorption of Pb in the species belowground tissue, and decreased the metal transport between the trees root and shoot system. The findings of Yang et al., (2016) corresponds well with the results of this study. The calculated BCF values of the three metals for the inoculated cuttings of *S. schwerinii* and *S. viminalis* suggest a decrease in the two species adsorption of Cd, Pb, and Zn in their aboveground tissue (table 4.7). The decrease in the inoculated cuttings BCF values are likely related to the saturation of metal transport between the cuttings belowground and aboveground tissue, caused by the enhanced metal uptake by *R. irregularis*Giasson et al., (2005).

The time required by *S. schwerinii* and *S. Viminalis* to reduce the concentrations of Cd, Pb, and Zn to the Danish soil quality criteria was found to be too high for the species to be applicable in practice for soil remediation purposes (table 4.5). Since the calculation of the reduction time is dependent on the cuttings biomass production and BCF, any inhibition of these will increase the time it takes to reduce the soil metal concentration to the quality criteria (table 1.1). Algreen, Trapp, and Rein, (2014) describes similar reduction times for poplar and *S. Viminalis* grown on a contaminated soil site in Denmark and found that the reduction time decreases at lower metal concentrations and higher BCF. This could indicate that the reduction times of Cd, Pb, and Zn for the salix species would gradually improve as the concentration of the three metals in the growth soil lessens. The reduction times of Cd and Zn for the inoculated cuttings of *S. schwerinii* and *S. viminalis* was found to be higher than for the non-inoculated cuttings (table 4.8). The higher reduction time are likely related to the reductive effect of *R. irregularis* on the cuttings BCF

values (table 4.7).

6. Conclusion

Det kan udfra rapportens resultater konkluderes, at *S. schwerinii* og *S. viminalis* ikke er anvendelig til fytoekstraktion af Cd, Pb, and Zn. Dette skyldes, at disse arters oprensningstid af de tre metaller er for lang tid til at kunne anvendes i praksis i forhold til de traditionelle metoder. En sammenligning af de to arter viser at *S. schwerinii* generelt havde den bedste optagese af de tre metaller i dens overjordiske væv. Men at *S. viminalis* grundet sin høj biomasse var bedre til at reducere omkringliggende jords metalkoncentraion.

For at kunne se om det er muligt at optimere arternes optagelse og oprensningstid af de tre metaller blev stiklinger af begge arter inokuleret med den arbuskulære mykorrhiza *R. irregularis*. Det blev fundet at en inokulering med *R. irregularis* optimerede arternes rodoptagelse af de tre tungmetaller, men at det resulterede i at arternes metaltransport til de høstbare dele blev reduceret.

Da forsøgene er udarbejdet i potter under optimale forhold og ikke ude på matriklen, forventes det, at resultaterne afspejler det bedst mulige udfald. Man kan derfor formode, at oprensningenstiden af de tre metaller vil stige under naturlige forhold. Der ud over er arternes vækstperiode begrænset til 47 dage, hvilket ikke ville være gældende, hvis forsøget skulle udføres i praksis. Det vil derfor anbefales at der til fremtidige undersøgelser af arterne vil tillagt en længere vækstperiode, således at arternes kan have mulighed for bedre at udvikle rodvækst.

Selvom at resultaterne viser, at de to arter ikke egner sig til fytoekstraktion, ses det dog, at de er velegnet til at stabilisere tungmetallerne i jorden omkring deres rodsystem. Det kan derfor anbefales, at der ved forurende grunde, hvor traditionel oprensning ikke er mulig grundet økonomiske omkostninger, kan plantes disse arter for at mindske spredning af forureningen.

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A. Geotechnical data

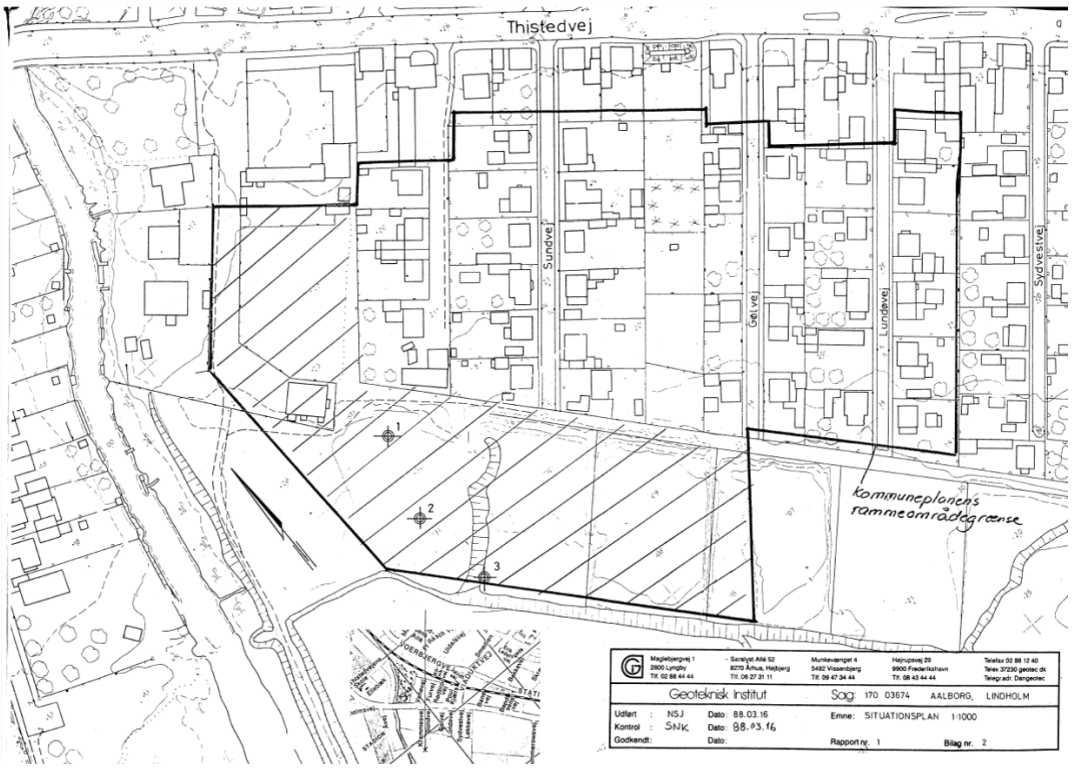

Figure A.1: Drill site locations on cadastral 7fq from the investigation of the area in 1988 (Rasmussen, 1988)

B. Extended Methodology

B.1 Sieving analysis

The soil particle size distribution was determined by using a Retsch As 200 basic sieve shaker. Each sample was weighed, before dried in an oven at 105°C in order to remove the water content. The sample were then observed for particle size, to determine the mesh size of the sieves used for the sieve shaker. The soil particles of the sample were estimated to be under 2 mm, and the meshes width were selected to range from 2 mm to 63μm. The sieves were assembled onto each other to form a sieving tower, with the highest mesh width on top and the mesh width declining toward the base cover. The sample were then transferred to the sieving tower from the top, before the tower was introduced to the sieving shaker for 20 min. The soil remnant within each sieve were then weighed, and subsequently compared with the soil particle size values (table B.1) to determine the distribution of soil particles. The soil particle distribution were determined in percentage by using equation B.1.

Table B.1: Classification of soil texture classes in accordance to particle size Loll and Møldrup, (2000)

Diameter [mm]	Particle size class
2-1	Very Coarse sand
1-0.5	Coarse sand
0.5-0.250	Medium sand
0.250-0.125	Fine sand
0.125-0.063	Very fine sand
0.063-0.002	Silt
$\leq$0.002	Clay

$$S_p = \frac{s_r}{s} \times 100 \tag{B.1}$$

Where:

S_p: is the soil particle distribution [%]

s_r: is the weight of the remnant soil particles in the sieve [g]

s: is the amount of used sample [g]

B.2 Metal concentration in dried samples [DS 259]

For the assessment of the samples metal concentration, the samples were dried at 105°C for 12 hours, to remove the samples water content. The samples were then placed in a desiccator for 30 min to prevent the samples from absorbing water from the surrounding air. Subsequently, the dried samples were then homogenized by using an agate mortar, and 1 g of sample was weighed, before transferred to a 100 ml blue cap bottle. The samples were then mixed with 20 ml of 7M HNO_3, before autoclaved at 120°C for 30 min, in order to open the samples. Samples of dried plant material or high humus content are before being introduced to an autoclave, kept agitated in the HNO_3 for approximately 12 hours or until all organic material are dissolved. Subsequently, the samples were diluted in 100 ml of demineralized water and then allowed to settle, before the samples were introduced to an atomic absorption spectrophotometer (AAS). The AAS determines the concentration of metals within a sample by bringing the sample to gaseous state using an flame atomizer. The atomized sample are then introduced to an adsorption spectrophotometer. The spectrophotometer reads the adsorption of the particles within samples by using the different wavelengths of light, these wavelengths enables the spectrophotometer to analyze the concentration of elements within the sample. The concentrations determined by the AAS were then used to determine the samples concentration of metals by using equation B.2

$$x = a \times \frac{d}{c} \tag{B.2}$$

Where:

x: is the metal concentration of the sample [mg/kg TS]

a: is the concentration of metals within the diluted sample [mg/l]

d: is the volume of the diluted sample [ml]

c: is the weight of dried sample [g]

C. Growth Chamber Data

The climate chambers logged data are due to its size attached to the project as an excel document, and is therefore not presented in the project.