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Master Thesis

Combined Chemical Immobilization and Subsequent Re-mobilization of Metallic Pollutants in Enhanced Phytoextraction

for the attainment of the academic degree of

Master of Science

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Acknowledgement

During the process of planning, conducting the experiments and writing my master thesis, I certainly went through all of the typical stages involved in this process. From curiosity and excitement to disenchantment and even exhaustion but also from moments of desperation to joy, reverence and proud.

To handle the positive stages and successful parts of my work was rather easy and enjoyable, however, dealing with setbacks and failure in experiments was much harder to bear. Especially in these moments, there have been a lot of people I could address to. Indeed, there was plenty of assistance, advice and support. Finally, I figured out that problems, unscheduled complications and their overcome mostly improved my thesis and brought me at least some new insight or perspective.

I would like to express my gratitude to all the people who accompanied me during this process: To my supervisors Prof. W. Wenzel and Prof. P. Tlustoš for their valuable inputs, ideas and plenty of meetings we had. To my colleagues in the laboratories in Tulln as well as in Prague. To Stáňa (Stànislava Vondráčková) for her precious help in countless 'operational questions' in the laboratories and her experience. And finally, to my family and my friends who persistently kept an eye on me and helped me to cope with difficult situations.

Abstract

To improve phytoremediation (PR) efficiency, two common approaches have evolved in science: soil amendments are either used to immobilize pollutants thus decreasing the uptake by plants or to increase the pollutant availability for phytoextraction crops.

Based on the previous work of Iqbal et al. (2012), we combined these two PR strategies by using vermicompost (VC), lignite (Lig) and biochar (BC) as immobilization agent (with application rates of 45 and 90 g kg^{-1}) and elemental sulfur (S) as mobilization agent. In contrast to Iqbal et al. (2012), S was not applied with the immobilization agent but after a period of several weeks. For testing this new method, we used a highly Zn, Cd and Pb contaminated Gleyic Fluvisol in an incubation experiment with two S rates (0.5 and 1.5 g kg^{-1}) and a pot experiment with a S rate of 0.5 g kg^{-1} and *Zea mays*. Metal (Zn, Cd, Pb, Mn, Fe), phosphorus and S concentrations in the soil solution, $CaCl_2$ extraction and plant tissues were assessed and scans of the root systems were conducted over time.

During the immobilization period, the application of VC, BC and Lig significantly reduced the $CaCl_2$-extractable Zn and Cd concentrations in both experiments. When S was applied during the incubation experiment, $CaCl_2$-extractable concentrations of Zn increased by 8 to 41 times, those of Cd by 6 to 14 times and pH_{CaCl2} decreased significantly. However, S oxidation and mobilization of metals in the pot experiment were very limited: only in one Lig treatment $CaCl_2$ concentrations of Zn, Pb, Cd, Mn and sulfate increased significantly after S addition. The heterogeneous application of S in the pots, oxygen depletion due to root and microbial respiration and the use of organic amendments (lowering the redox potential) may have inhibited S oxidation.

The highest total Zn and Cd contents in the shoots of maize were measured in the VC treatments increasing PR efficiency by 100 % for Zn and 400 % for Cd in comparison with the control. However, rather the effective immobilization of pollutants and the provision of nutrients than the oxidation of S led to this increase.

Zusammenfassung

In der Phytosanierung (PR) werden Bodenzugabestoffe entweder dazu verwendet, um eine Immobilisierung von Schadstoffen zu bewirken und die Pflanzenaufnahme zu verringern oder, um eine Zunahme der Schadstoffverfügbarkeit zu erreichen.

Basierend auf den Ergebnissen von Iqbal et al. (2012) kombinierten wir diese beiden Ansätze und verwendeten Wurmkompost (VC), Weichbraunkohle (Lig) und Biokohle (BC) (in Raten von 45 und 90 g kg^{-1}) als Immobilsierungs- und Schwefel (S) als Mobilisierungmittel. Im Gegensatz zu Iqbal et al. (2012) führten wir S erst nach einer Zeit von mehreren Wochen zu. Um diese neue Methode zu bewerten, wurde ein mit Zn, Cd und Pb belasteter Fluvisol in einem Inkubationsversuch mit zwei verschiedenen S-Raten (0.5 und 1.5 g kg^{-1}) verwendet und ein Topfversuch mit einer S-Rate von 0.5 g kg^{-1} und *Zea mays* durchgeführt. Es wurden die Metall- (Zn, Cd, Pb, Mn, Fe), Phosphor- und S Konzentrationen im Bodenwasser, $CaCl_2$ Extrakt und Pflanzengewebe, wie auch Veränderungen in der Wurzelmorphologie über die Versuchsdauer hinweg untersucht.

Vor der S-Zugabe wurden in beiden Versuchen signifikant verringerte $CaCl_2$ extrahierbare Zn und Cd Konzentrationen in den VC, BC und Lig Behandlungen gemessen. Während die S-Zugabe in der Inkubation einen Anstieg der $CaCl_2$ extrahierbaren Zn und Cd Konzentrationen um das 8 bis 41- bzw 6 bis 14-fache bewirkte, fand die Metallmobilisierung im Topfversuch nur sehr eingeschränkt statt. Lediglich die $CaCl_2$ extrahierbaren Zn, Pb, Cd, Mn und Sulfatkonzentrationen in der Lig-Behandlung zeigten einen signifikanten Anstieg nach der S-Zugabe. Dabei könnte die inhomogene Einbringung von S, Sauerstoffzehrung durch Respiration der Wurzeln und Mikroben sowie die Zugabe von organischen Stoffen (durch Senken des Redoxpotentials) eine Verringerung der S-Oxidation im Topfversuch bewirkt haben.

Die höchsten Zn und Cd Gehalte wurden in den Maistrieben der VC-Behandlung gemessen, womit die Effizienz der PR um 100 % bzw 400 % erhöht werden konnte. Allerdings führte nicht die angestrebte S-Oxidation, sondern die erhöhte Nährstoffverfügbarkeit in den VC-Behandlungen zu dieser Verbesserung.

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List of Abbreviations

Abbreviation	Full Name of Item
BC	Biochar
C	Control
CEC	Cation exchange capacity
CPR	Combined phytoremediation
Demi-water	Purified water 'type I' with EC < 0.1 µS cm^{-1} at 25 °C
df	Degrees of freedom
DOC	Dissolved organic carbon
DW	Dry weight
EC	Electrical conductivity (mS cm^{-1})
extr.	extractable
Lig	Lignite (soft brown coal)
MIBRAG	Mitteldeutsche Braunkohlegesellschaft
n	Number of replicates
n.a.	Not available (used in tables or figures)
PR	Phytoremediation
rcf	Relative centrifugal force (x g)
RM-A	Repeated measurement ANOVA
SEM	Standard error of the mean
SSR	Soil solution ratio
VC	Vermicompost
WC	Water content
WHC	Water holding capacity (wt %)
wt%	Weight percent

1. Introduction

1.1 Phytoremediation in the Context of Future Challenges

Following the deliberations of the United Nations Environmental Programme (2012), the preservation of the world's ecosystems has to be considered as one of the most momentous challenges for sustaining nature's services and their contributions to human endeavour. Whilst often less regarded by public, soil is tightly connected to climate change mitigation, carbon storage and the water cycle. Beyond that, it is not only the spatial basis for human development but also the basis of our nutrition.

However, current global trends such as urbanization in agglomerations and splinter development in rural areas increased the sealing pressure on land. Also Austria and the Czech Republic are affected by this issue (European Commission, 2012) and other additional driving factors as population growth or food production play a vital role concerning soil degradation. Former as well as ongoing industrial activities such as smelter or tannery processes cause severe and persistent soil contaminations limiting the usage of these areas for productive, ecological or recreational purposes.

To 'reactivate' and remediate polluted soils for all of these mentioned usages, different strategies have been developed. Unfortunately, the most common techniques such as excavation or ex-situ clean-up measures are accompanied by considerable cost. Here, phytoremediation (PR) concepts can be regarded as suitable and cost-efficient alternatives (Adriano et al., 2004; Marques et al., 2009). By using natural processes as for instance the uptake of metals by plants and the subsequent translocation into above ground biomass not only economic but also ecological benefits can be achieved. Yet, their efficiency and reliability have to be improved to compete with the traditional approaches. By selecting suitable species, so-called hyperaccumulators exhibiting an “exceptional metal-accumulating capacity” (McGrath et al., 2001, p. 208), progresses have been made. However, some of them are rather fragile and difficult to cultivate on a large scale. Moreover, the addition of soil amendments improving the plant performance or enhancing the pollutant uptake is frequently studied to further improve the the potential of the PR application.

To illustrate the complex socio-economic environment of this relatively new remediation approach, the interactions of PR in a wider context are shown in Figure 1 using the DPSIR-approach (European Environmental Agency, 2007). Only if further improvements of the PR technique will be made, a frequent implementation into practise can be achieved. However, it should not be neglected that PR is only able to improve and recover soil state (i.e reduction of contaminants). Further ecological and economic aspects as well as people's awareness are at least equally important in a bigger framework and have to be considered as key factors for the success of practical PR applications.

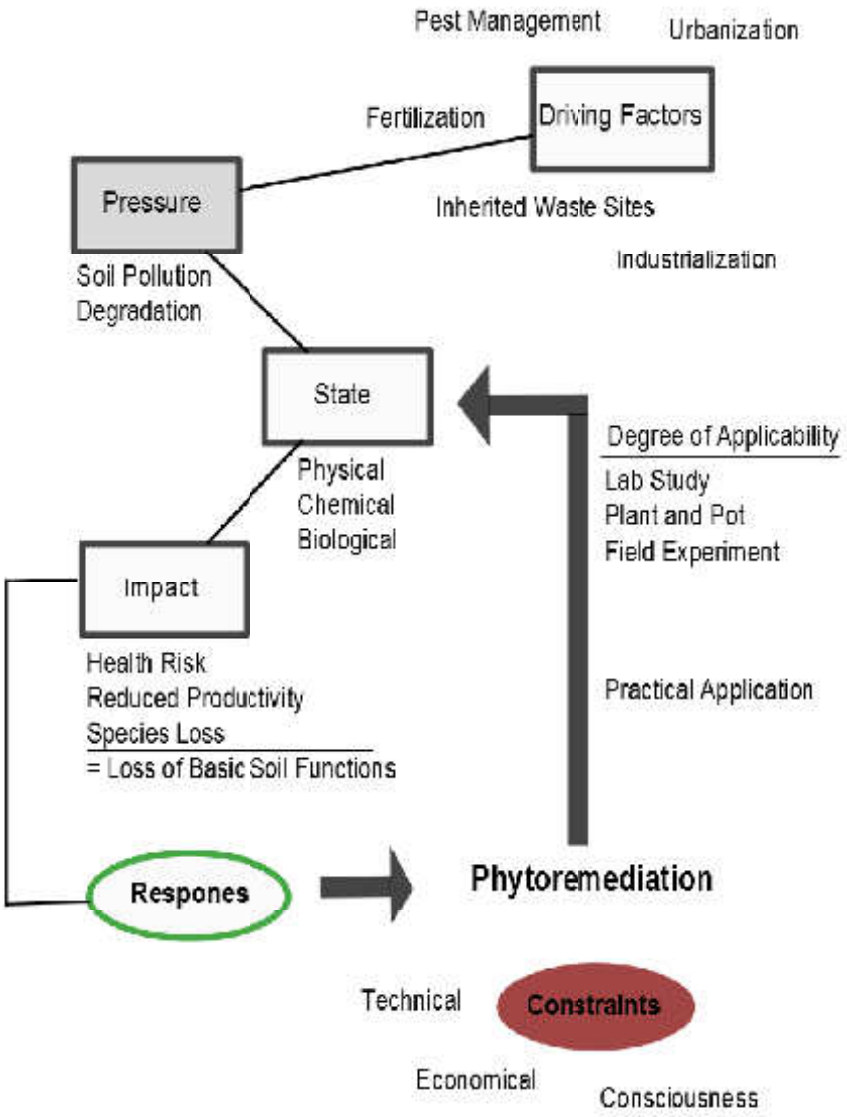

Figure 1: Social-economic and ecological context of the PR technique

1.2 Case Study in the Příbram Area

The area of Příbram is located approximately 70 km in the South-West of Prague in the Czech Republic and can be considered as one of the most contaminated areas in this country (Sichorová et al., 2004). The first historic record of a smelting work dates back to the 21 April 1311, which illustrates the long-term tradition of metal processing and associated soil degradation in this region (Kunicky and Vurm, 2011).

Due to mining and metallurgical processing activities, various soils are enriched with As, Cd, Zn and Pb (Sichorová et al., 2004). Besides the 'typical' contaminations in the vicinity of smelters or mines caused by atmospheric deposition and disposal of mine waste, the highest concentrations occur in alluvial soils transported by the Litavka river in the past. Here, floods occasionally washed away severely contaminated wastes and formed these sediments (Boruvka and Vacha, 2006).

Figure 2 displays a map created by Vaněk et al. (2005) showing the river Litavka and its surroundings. The sampling location of the soil used in this work is marked with P2 in the map.

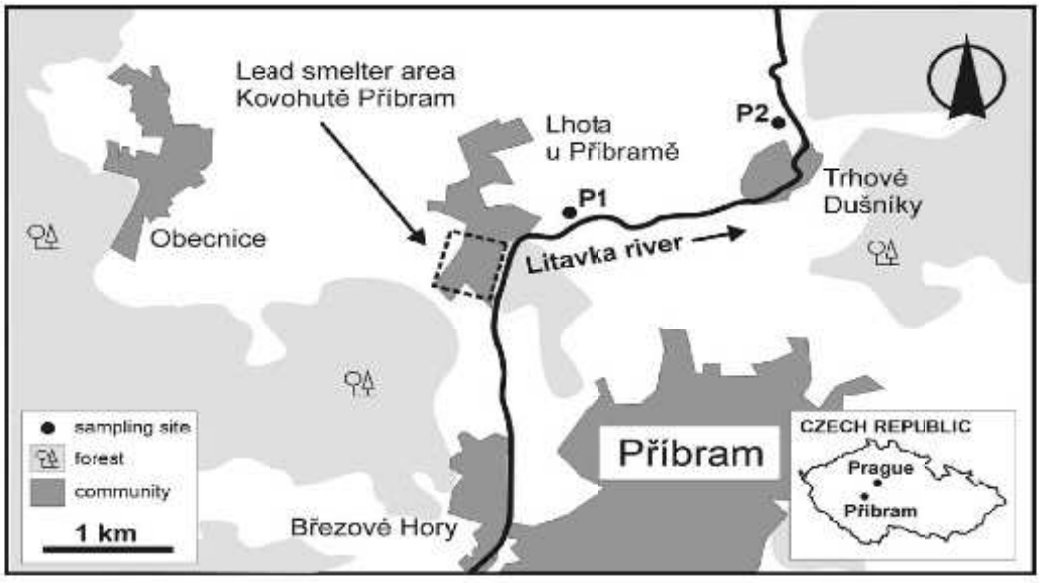

Figure 2: Area around Příbram and Litavka River. P2 marks sampling location from Litavka alluvium. Modified from Vaněk et al. (2005, p. 317).

According to the IUSS Working Group WRB (2006) the soil can be classified as Gleyic Fluvisol (eutric) and exhibits an Ah horizon with a thickness of 10 to 15 cm followed by several G horizons (Figure 3). Due to occasional flood events after extreme precipitations, Litavka soil is affected by periodically occurring anaerobic conditions (Boruvka and Vacha, 2006; Mikanova, 2006; Vanek et al., 2008). The most relevant pollutants are Pb, Cd and Zn occurring not only in high concentrations but also in a great horizontal and vertical variability (Vanek et al., 2008).

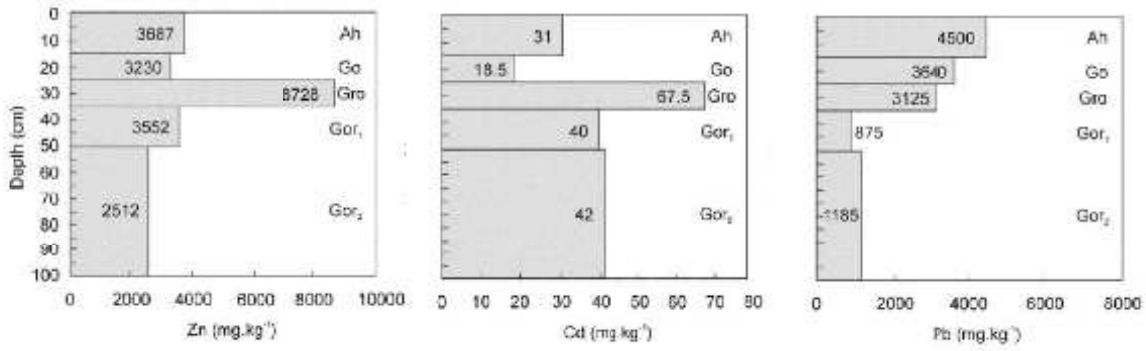

Figure 3: Total concentrations of Zn, Pb and Cd in Litavka soil. Modified from Vaněk et al. (2005, p. 320)

1.3 Combined Phytoremediation (CPR)

1.3.1 Distinction between Different PR Techniques

Generally speaking, bioremediation can be defined as "the use of living organisms to manage or remediate polluted soils" (Wenzel, 2008, p. 385) and further specified as "elimination, attenuation or transformation of polluting or contaminating substances by the use of biological processes" (Wenzel, 2008, p. 385). As emerging topic in both scientific communities and practice, different types of PR have been developed (Table 1). Common draw-backs of these approaches relate to the limitation to the root zone and the ability of plants to establish on contaminated sites. Moreover, the 'polluted' plant material has to be harvested and disposed properly.

Table 1: Overview of different PR technologies modified from United States Environmental Protection Agency (2000)

Technique	Principle	Media
Phytoextraction	Extraction and uptake of pollutants (often metals) into plant tissue	Soil, sediments and water
Phytostabilization	Mechanical (erosion) and physio-chemical immobilization of pollutants	Soil and sediments
Rhizofiltration	Adsorption and/or precipitation in the root zone (probably also uptake)	Ground and surface water
Phyto/ Rhizodegradation	Contaminant degradation and degradation (organic pollutants)	Soil, sediments, sludges and groundwater
Phytovolatilization	Extraction of pollutants and subsequent transformation into volatile chemical forms	Soil, sediments and groundwater

Since the focus of this research is a combination of **phytoextraction and phyto-immobilization** and for further convenience, the term '**combined phytoremediation**' will be used and abbreviated with **CPR**.

1.3.2 Suitable Strategies for Immobilization in Litavka Soil

According to Friesl (2006) and Hamon et al. (2002), an effective immobilization of metals can be achieved by:

- Addition of sorption sites thus reducing the available contaminant fraction in soil, e.g clays, red mud or iron oxides;
- Manipulation of the pH: for cations an increase in pH will likely result in a lower availability;
- Nutrient provision: some amendments will provide nutrients for the plants to overcome deficiencies and thus increase the biomass production;
- Change of soil physical and chemical properties such as water holding capacity (WHC) or cation exchange capacity (CEC);
- Transformation of relatively soluble metals to 'stable' precipitates, i.e. application of phosphor additives forming stable precipitates over a wide pH range.

For taking advantage of those mentioned processes soil amendments such as liming agents, phosphates, oxyhydroxides, organic materials and many more have been frequently used (Vangronsveld et al., 2009).

The main problem of Litavka soil relates to its extremely high contamination with Zn reaching a total concentration of about 6100 mg kg^{-1} (compare Chapter 2). This is a major inhibitor for plant establishment and plant growth leading to low biomass production and toxicity symptomes in former trials (unpublished). Therefore, finding suitable soil amendments able to efficiently immobilize Zn was considered as the key to success for the conducted experiments. Based on a comprehensive literature review of experiments dealing with highly Zn contaminated soils (Table 2), we decided to focus on the three 'carbon amendments' lignite, biochar and vermicompost. All of them performed well in former experiments and are easily available. Biochar possesses a very high specific surface area and according to this also a notable sorption capacity for binding pollutants. Additionally, also liming effects might occur (Beesley et al., 2011). As typical property of soft brown coals, lignite contains high amounts of humic substances and is often used as 'soil conditioner' and nutrient provider for plants, which have been reported to respond with

Table 2: Literature review of experiments using Zn-immobilzation amendments

Author	Experiment and location	Soil	Total Zn (mg kg^{-1})	Additive	Rate (g kg^{-1})	Reduction of Zn (%)*	Extractant
Gray et al. (2006)	Field Experiment in UK	Zn/Pb smelter derived soil	3970	Lime	n.a.	36	Ammonium nitrate
				Red mud	30	27	
				Red mud	50	65	
Janos et al. (2010)	Sequential extraction	Silty sand	233	Humate K	50	45	Acetic acid
				Lignite	50	13	
Pusz (2007)	Pot experiment	Gleyic silt from Cu smelter	294	Lignite	30	76	Ammonium nitrate
Brown et al. (2005)	Pot experiment	Mine waste, US.	18500	Triple super phosphate	20	98	Ammonium nitrate
				Phosphoric acid	ratio	99	
Siebielec and Chaney (2012)	Pot experiment	Sandy loam from military site	7200	Compost	various	75	Strontium nitrate
				Lime		99	
				Compost and lime		98	
				P with lime		91	
O'Dell et al. (2007)	Pot experiment	Cu and Zn bearing mine spoil	n.a.	Yard waste compost	300	94	Calcium chloride
Beesley et al. (2010)	Pot experiment	Multiply contaminated soil	249	Compost	125	51	Water extract
				Biochar	83	11	

* reduction of the extractable Zn concentration in comparison to the control treatment (in % rounded to two significant digits)

stimulated root and shoot growth (Karr, 2001). Moreover, complexation of metals at the surface of organic materials may decrease the pollutant availability (Pusz, 2007). Also vermicompost provides complexation of metals, but beyond that improves physical, chemical and biological properties of soil. Moreover, the addition of organic matter enhances soil fertility and plant growth. Further information about the amendments, their analyses and application rates are provided in Chapter 2.

1.3.3 Sulfur Chemistry of Soils and Sulfur-Assisted Pollutant Mobilization

Sulfur (S) is usually considered as plant macro nutrient, however, its uptake and incorporation into plant tissue can reach similar amounts as phosphorus. For this reason, S ranks between macro and micro nutrients (Nutrient Stewardship, 2010) and has a notable effect on plant growth and also yield quality (De Kok et al., 2012; Kumar and Sharma, 2013; Sager, 2012). Plants take up S in form of sulfate, which can be considered as active pathway.

The major inputs of S in soils are atmospheric deposition, geogenic sources (e.g. gypsum or pyrite), organic matter decomposition and fertilizer application (Edwards, 1998; Sager, 2012; Scheffer et al., 2010). The most prominent output of S from soils can be seen in the leaching of sulphates. Generally speaking, two major forms have to be distinguished: organically bound S representing the bulk part and inorganic S. The processes of immobilization and mobilization connect these two essential S pools: immobilization is the uptake of S by microbes including the subsequent incooperation into organic matter (Edwards, 1998; He et al., 1994). Mobilization and mineralization reverse this process and convert organically bound S into available forms.

Another governing factor concerning S cycling in soils can be seen in the redox reactions of S components. Depending on the redox conditions (i.e aeration of soils) oxidized species (sulfates) or the reduced forms (hydrogen sulfides) are prevailing. However, the latter species can also be quickly volatilized, oxidized to elemental S or form precipitates with metals (Edwards 1998). Moreover, an often neglected retention mechanism in the movement of S in soils is adsorption. Besides the weaker non-specific adsorption at

double-diffusion layers, also specific adsorption at Al- and Fe-hydroxides occurs. In this ligand exchange reaction, OH^- ions are replaced by sulfates depending on the sulfate concentration in soil solution (Edwards, 1998).

Even if microbes only contain very small amounts of S, they contribute considerably to its fate in the cycle. By oxidation of elemental S, sulfuric acid can be produced leading to a locally restricted acidification in the rhizosphere (Iqbal et al., 2012). In this way, excessive leaching can be prevented which renders S oxidation to a suitable method for enhanced phytoextraction. Under aerobic conditions, this oxidation is accomplished by bacteria of the genus *Acidithiobacillus* (Hagedorn, 2010) and can be stated as follows (Eq. 1):

$$S^0 + 1.5\,O_2 + H_2O \rightarrow H_2SO_4 \qquad \text{(Eq. 1)}$$

Under occasional anaerobic conditions in the rhizosphere, S oxidizing bacteria may use Mn and Fe oxides as electron acceptors for S oxidation (Eq. 2), hence, leading to a reduction and dissolution of these oxides highly contributing to metal adsorption. As a result, highly increased Cd and Zn concentrations have been observed (Iqbal et al. 2012; Höfer 2013). The underlying equation can be stated as follows (Thamdrup et al., 1993):

$$S^0 + 3\mathrm{MnO_2} + 2\,H_2O \rightarrow SO_4^{2-} + 3\mathrm{Mn^{2+}} + 4\mathrm{OH^-} \qquad \text{(Eq. 2)}$$

In Figure 4, the most important pathways of S in soils are outlined and summarized in a simplified way, since they play a vital role in the analyses and interpretations of this thesis.

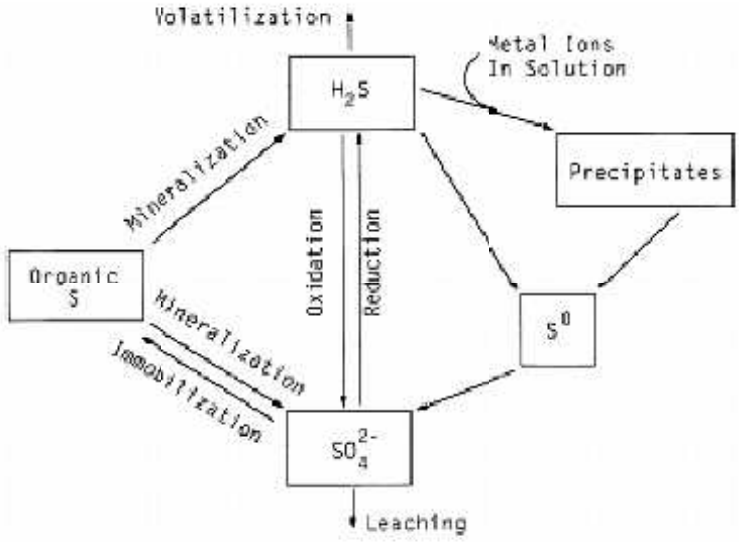

Figure 4: Simplified overview of S reactions in soils (Edwards 1998, p. 1)

1.4 Hypothesis and Research Questions

Since PR has emerged as a new technology for cleaning polluted soils, scientists tried to further improve its reliability and efficiency. Different methods and modifications evolved and a wide range of both mobilization and immobilization agents have been tested.

However, a combination of both methods (CPR) has only been investigated recently by Iqbal et al. (2012) using gravel sludge or red mud as immobilization agents and elemental S for optimizing the phytoextraction with S*alix smithiana*. In a pot experiment, not only plant uptake of metals but also biomass production could be improved. Höfer (2013) confirmed these findings in a succeeding research focussing on S oxidation processes in the rhizosphere of S*alix smithian*a by using different S application rates and a rhizobox design. Similar mobilization effects of S could be proved by Cui et al. (2004). Here, S application increased the Zn concentration in the shoots of maize by the factor 2.3.

However, in the proof-of-concept study of Iqbal et al. (2012), immobilization and mobilization additives were applied together and the authors suggested to further refine their concept by establishing a strictly separated immobilization period followed by mobilization, which has not been evaluated so far.

This thesis aimed at testing the CPR concept introduced by Iqbal et al. (2012) by the subsequent application of immobilization (vermicompost, lignite, biochar) and mobilization (elemental S) soil additives in a preliminary incubation experiment (without plants) and a pot experiment using *Zea mays* as phytoremediation crop.

Since we used highly contaminated Litavka soil in our experiments, the initial immobilization period aimed at reducing the bioavailability of pollutants (Zn, Cd, Pb) and supporting plant growth (e.g. by the provision of nutrients). After vigorous maize plants had established, the subsequent mobilization targeted an increase in the pollutant (i.e. Zn, Cd, Pb) uptake of maize.

The following general research questions were addressed:

- Does the combination of an immobilization period by either using lignite, vermicompost or biochar with the subsequent mobilization by elemental S lead to an increased efficiency and reliability of PR?
- Which effects can be observed concerning the response of *Zea mays*, pollutant availability, leachate composition and phytoextraction efficiency due to the subsequent application of the soil additives?

Moreover, the following detailed questions were investigated:

- Which of the amendments performs best in supporting plant establishment on the highly polluted Litavka soil?
- Does the S application lead to the desired steady and controlled acidification of soil?
- How do the different treatments respond towards a decrease of pH by adding S?
- How is the leachate composition affected by S application?
- Which plant toxicity symptoms can be observed?
- How does the S application influence root morphology?
- Which effects do the different treatments and the mobilization have on plant performance and phytoextraction efficiency?

2. Materials and Methods

2.1 Materials

2.1.1 Soil

While the origin of Litavka soil was described in Chapter 1.2, Table 3 shows its main characteristics according to Vondráčková et al. (2013), Vaněk et al. (2008) and our own measurements.

Table 3: Different soil characteristics of Litavka soil. Modified from Vondráčková (2013) and Vaněk et al. (2008). SEM is calculated from n = 3.

Soil Parameter	Units	Litavka Soil
Soil group [a]	-	Gleyic Fluvisol (eutric)
Sand	g kg^{-1}	550
Silt	g kg^{-1}	388
Clay	g kg^{-1}	62
pH_{CaCl2}	-	6.5 ± 0.02
EC [b]	(mS cm^{-1})	0.07
CEC	(mmol kg^{-1})	55
OC	g kg^{-1}	36 ± 1
Total N [b]	g kg^{-1}	2.53
Total S [b]	g kg^{-1}	0.37
$CaCO_3$	g kg^{-1}	0
Ca [c]	mg kg^{-1}	1860 ± 31
Mg [c]	mg kg^{-1}	160 ± 5
K [c]	mg kg^{-1}	192 ± 8
P [c]	mg kg^{-1}	9 ± 0.3
Cd $_{total}$	mg kg^{-1}	53.8 ± 0.9
Zn $_{total}$	mg kg^{-1}	6170 ± 42
Pb $_{total}$	mg kg^{-1}	3300 ± 85
Fe $_{total}$	mg kg^{-1}	21200 ± 146
Mn $_{total}$	mg kg^{-1}	2690 ± 16

a - according to IUSS Working Group WRB (2006)
b - obtained from own measurements (see Section 2.2.1)
c - plant-available concentrations of nutrients determined by Mehlich III
total - total concentrations of elements extracted by *Aqua Regia*.

2.1.2 Amendments

Table 4 shows the used amendments and their individual properties and origins. The additives are abbreviated as BC (biochar), Lig (lignite), VC (vermicompost) and S (suflur). For the unamended control treatment, the letter C is used.

Table 4: Short description and selected parameters of the used soil amendments

Parameter	BC	Lig	VC	S
Description	Pyrolysis of plant material	Soft brown coal from opencast mine	Earthworm derived compost	Elemental S
Input Material	Coconut shells	Sedimented organic substances	Horse manure	-
Supplier	ERSPOL Ltd., Telnice, Czech Rebublic	MIBRAG mbH, Profen, Germany	OEKOVERMES, Arrach, Germany	CentralChem, Prague, Czech Republic
pH_{H20}	7.56	3.38	5.49	-
EC (mS cm^{-1})	0.79	0.29	5.87	-
WC (g kg^{-1})	176	812	453	-
C (g kg^{-1})	647	629	247	-
N (g kg^{-1})	4.3	5.3	23.3	-
S (g kg^{-1})	1.6	28.7	11.2	-
Al (mg kg^{-1})	12000	3300	16200	-
Ca (mg kg^{-1})	3100	19700	27000	-
Fe (mg kg^{-1})	4130	8600	8400	-
K (mg kg^{-1})	470	47	12500	-
Mg (mg kg^{-1})	2200	< 70	< 300	-
Zn (mg kg^{-1})	8.3	2.8	231	-
Pb (mg kg^{-1})	-	< 2.0	-	-

Remark: A detailed description of the analysis can be found in Section 2.2.1

2.1.3 Experimental Plant

Originally, we intended to use *Salix smithiana* as experimental plant. However, due to low-quality cutting material, *Zea mays* from the variety Colisee was used instead. Colisee is a high yielding, undressed (i.e. without chemical treatment of seeds) variety, suitable for both silage and grain usage (KWS Saat AG, n.d.) and was approved in 2012 (Proplanta, 2013).

The use of maize for PR is well documented and may have various advantages (Wuana and Okieimen, 2010): it is an abundant, fast growing plant and able to accumulate considerable amounts of metals. Additionally, several valorizations can be chosen depending on the metal concentrations in the plant tissue: if legal thresholds are not exceeded it may be used as fodder, but also gasification and conversion to biogas is possible. Van Slycken et al. (2013) proved in their research that the biogas yields of maize harvested from a metal contaminated site did not differ to maize cultivated in normal sites. A suitable valorization is particularly important if we consider the long duration of PR processes which may last over several hundred seasons depending on soil and used additives (Neugschwandtner et al., 2008).

2.2 Methods

2.2.1 Application Rates, Basic Procedures and Elemental Analysis

2.2.1.1 Soil and Amendment Preparation and Application Rates

Soil was collected from the sampling site (see Chapter 1) and air dried for 9 days at warm ambient spring temperature. Water content was below 10 % after drying. We sieved the soil (<2 mm), crushed bigger aggregates and homogenized it by mixing in plastic tubs.

The amendments were ordered from the respective supplier (Table 4) and also sieved (<2 mm) without any drying. They were kept in tight plastic bags to avoid a change in water content which is important for applying precise rates. Before sieving, BC and Lig were ground using pestle and mortar.

In both incubation and pot experiment the **same application rates of the amendments BC, Lig and VC** were used. All rates were calculated and applied to **soil dry weight**. To avoid confusion with the different S application rates, the immobilization agents are called **treatments**. In contrast to the S application, they were added at the beginning of the experiments as follows:

- C = plain soil, no immobilization agent added
- BC 1 = biochar with 45 g kg^{-1} application rate
- BC 2 = biochar with 90 g kg^{-1} application rate
- VC 1 = vermicompost with 45 g kg^{-1} application rate
- VC 2 = vermicompost with 90 g kg^{-1} application rate
- Lig 1 = lignite with 45 g kg^{-1} application rate
- Lig 2 = lignite with 90 g kg^{-1} application rate

In general, the application rates can be considered as high to very high. A field experiment using these rates would cause notable costs. However, due to former experiments and experiences gathered at the Department of Agro-environmental Chemistry and Plant Nutrition (Czech University of Life Science, Prague), these exceptional rates were necessary to alleviate the extreme pollutant concentrations of Litavka soil.

To identify suitable S application rates, we conducted a **preliminary incubation experiment** in which three different S rates were tested: 0.5, 1.5 and 2.5 g S kg^{-1}. The same methods as in the incubation experiment were applied (see Section 2.2.2). After three weeks of incubation, soil was extracted and metal (Zn, Cd, Pb) concentrations were determined using ICP-OES (VARIAN Visto Pro, Australia). The results of this experiment are not further discussed here. Consequently, the S rates were determined according to the results of the preliminary experiment. Table 5 summarises all application rates.

Table 5: Different additive rates used in incubation and pot experiment

Additive	Used in	Rates (g kg^{-1})	Time of addition	Action
BC, Lig, VC	Incubation and Pot	45 and 90	Start of experiment	Immobilization
Sulfur	Incubation	0, 0.5 and 1.5	After 22 days	Mobilization
	Pot	0.5	After 9 weeks	

2.2.1.2 Electric Conductivity and pH Measurement

Electric conductivity was measured according to EN 13038 (2011): sub-samples of soil and amendments were mixed with demi-water at a ratio of 1:5 (w v^{-1}). After 10 min of shaking and a short settlement time, EC was measured (HANNA INSTRUMENTS HI 991300, Germany). Similar, pH was measured (WTW pH 315, Germany), but with a ratio of 1:2.5 (w v^{-1}) and after 2 h of equilibration.

2.2.1.3 CNS and Neutron Analysis of Amendments

Elemental analysis of C, N, S was made using a CNS analyser (THERMOSCIENTIFIC Flash 2000, Germany). Prior to analysis, sub-samples of the amendments and soil were pulverised (FRITSCH pulverisette 0, Germany). Additionally, the total Al, Cd, Ca, Fe, K, Mg, Mn, S and Zn concentrations of the amendments were measured by an external laboratory using neutron activation analysis. The data were processed according to Kubesová & Kucerá (2012).

2.2.2 Incubation Experiment

The design of the incubation experiment was adapted from Vondráčková et al. (2013). 20 g of soil was mixed with the respective treatment (Table 5) in an acid-washed 250 ml shaking bottle. After thorough blending with a spatula, demi-water was added to obtain a water content of 60 wt% of the maximum water holding capacity, similar to other incubation experiments in literature (Iqbal et al., 2012; Vondráčková et al., 2013).

Each treatment was replicated three times and incubated for 0, 7, 14 and 21 days without S application. After 22 days, elemental S (CENTRAL CHEM, Czech Rebublic) was added in three rates (0 g kg^{-1}, 0.5 g kg^{-1} and 1.5 g kg^{-1}) and further incubated until the extraction at 28 and 40 days, respectively. When adding elemental S, also thorough mixing was conducted. The temperature was kept constant at 25 °C throughout the whole experiment (BMT Venticell, Germany). Furthermore, we aerated the samples every three days to avoid the formation of a bottom carbon dioxide layer, thus providing enough oxygen needed for S oxidation. For every time-treatment-sulfur combination different samples were prepared. By doing so, the whole amount of soil could be extracted thus avoiding inhomogeneities.

Figure 5 exemplifies the experimental design for one treatment. The same procedure was applied for all other treatments. The statistical analyses are described in Section 2.2.5.

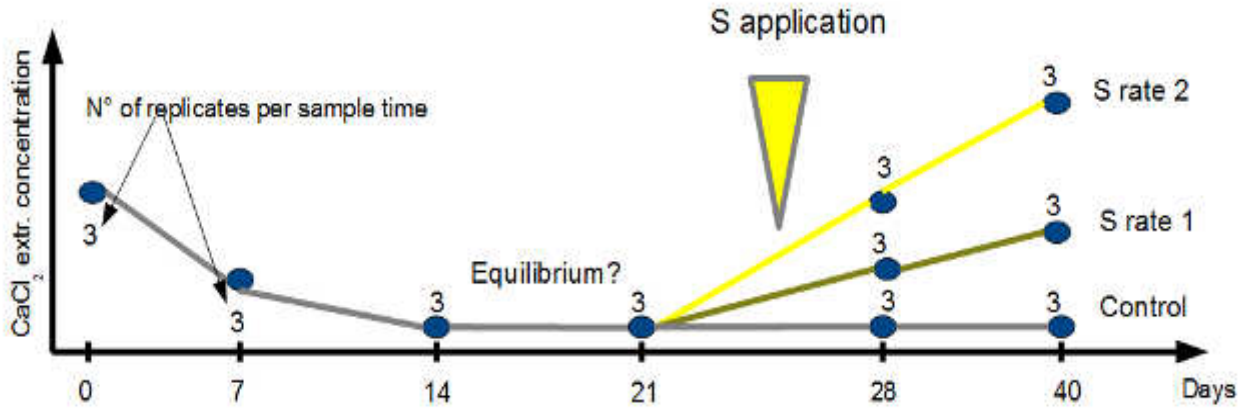

Figure 5: Experimental design and schematic representation of the expected results of the incubation experiment shown for one treatment.

At every time step, the respective samples were extracted using 0.01 M $CaCl_2$ (LACHNER, Czech Republic) with a SSR of 1:2.4 (Vondráčková et al. 2013). $CaCl_2$ of low ionic strength is frequently used as extractant due to its low cost and reliable results, since it contains "more or less the same ionic strength as the average salt concentration in many soil solutions" (Houba et al., 2000, p. 1).

Samples were shaken for 6 h (GFL 3006, Germany) and finally centrifuged with 978 x g relative centrifugal force for 10 min (HETTICH Universal 30RF, Germany). Afterwards, pH (HANNA INSTRUMENTS HI 991300, Germany) and metal (Zn, Cd, Pb) concentrations using ICP-OES (VARIAN Visto Pro, Australia) were measured.

2.2.3 Pot Experiment

2.2.3.1 Experimental Design

For the pot experiment 49 pots (with a volume of 5 L) were set up; each of them contained 4 kg of dried soil. The added rates for immobilization were the same as in the incubation experiment. However, it should be noted that C (i.e. control) refers to pots that contained no immobilization agent but maize plants and (later in the experiment) S as mobilization agent. Therefore, C was used to investigate how the plants and metals (Zn, Cd, Pb, Mn, Fe) behaved without immobilization amendments. Furthermore, only one S rate (e.g. 0.5 g S kg^{-1} soil) was used for every pot.

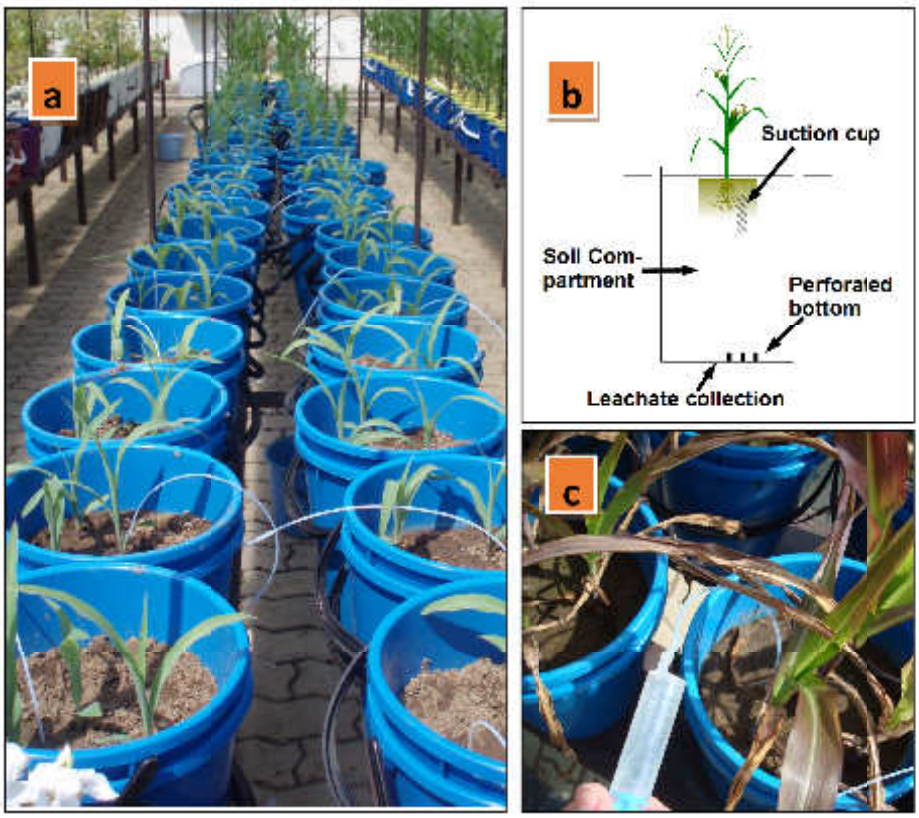

Figure 6: a) Experimental pots at the Czech University of Life Sciences, Suchdol, Prague; b) Schematic illustration of pot set-up and c) Photograph of soil solution sampling using suction cups.

Figure 6 shows the experimental set up in the sheltered facilities of the department. The expendable roof was removed on sunny days and used as protection during night.

To provide an overview concerning the growth period, different analyses and sampling dates (e.g. harvests), Figure 7 illustrates the sequence of sampling and analyses. Since we conducted three harvests, a high number of replicates was necessary: for each treatment seven pots were set up (resulting in a total of 49 pots). Two of them were harvested at the 1st and 2nd harvest date, while the remaining three were harvested at the final (= 3rd) harvest. Each replicate was equipped with two plastic pots: one in which the plant was growing (with a pierced bottom) and a second outer pot for collecting leachate samples (compare Figure 6 b). The replicates for the last harvests were equipped with suction cups (RHIZOSPHERE RESEARCH PRODUCTS, Netherlands), which were inserted in the vicinity of the roots in a depth of 2 to 7 cm.

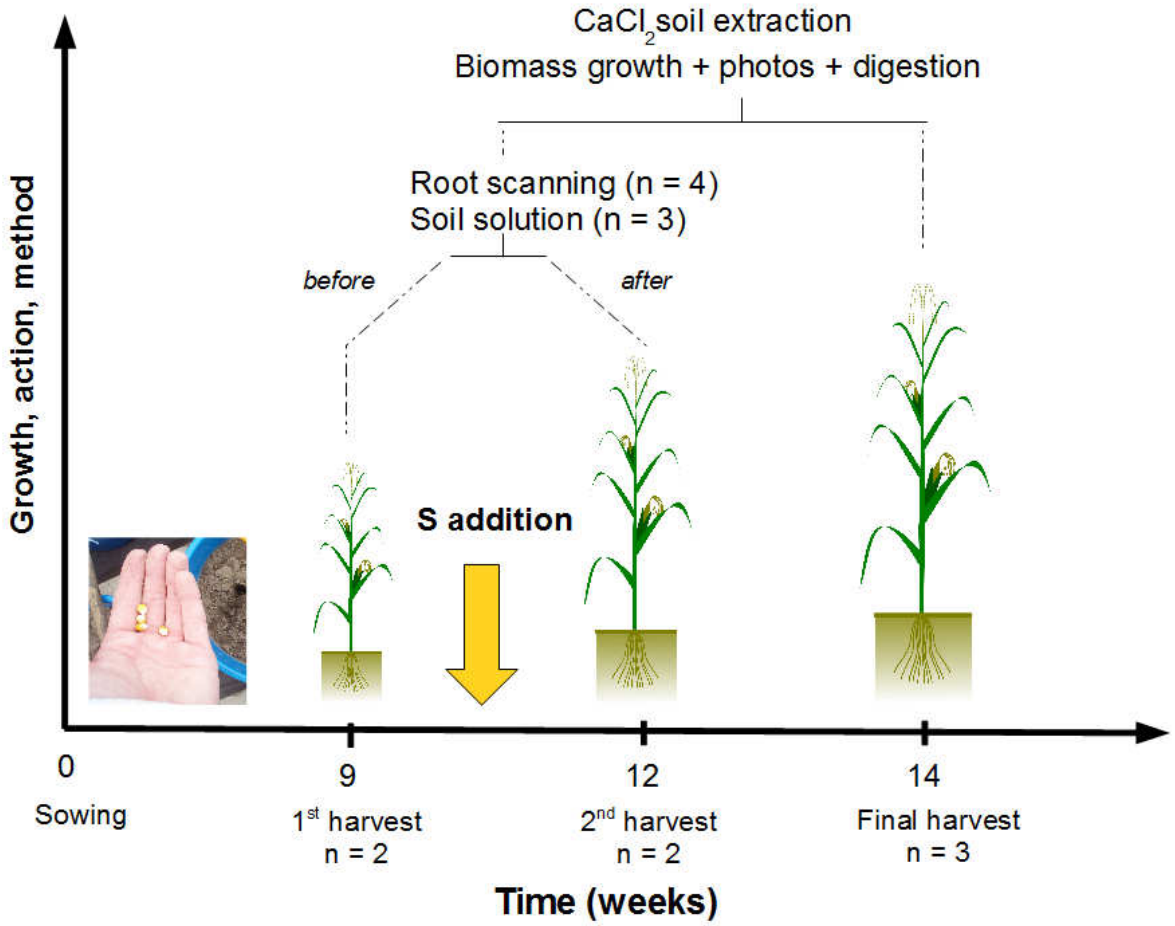

Figure 7: Experimental overview of different analyses and harvests in the pot experiment

The experiment started at 27 June 2013 and the final harvest was at 03 October 2013.

2.2.3.2 Implementation of Pot Experiment and Analyses

2.2.3.2.1 Pot Preparation and Fertilization

Before the air-dried soil was filled into pots, nutrients were applied as follows: 0.5 g N (in form of NH_4NO_3, LACHNER, Czech Republic), 0.16 g P and 0.4 g K (both in form of K_2HPO_4, LACHNER, Czech Rebublic). The fertilizer application was repeated after one month of growth to counter deficiencies. However, at the 2nd time we applied only 0.5 g of N (NH_4NO_3) and 0.16 g of P (H_3PO_4, LACHNER, Czech Republic). Another application of K was omitted to prevent further mobilization of cations.

2.2.3.2.2 Planting, Watering and Thinning of Maize

On the 27 June 2013, we inserted five maize seeds in each pot. The pots were regularly watered in the morning. After ten days of growth, we thinned the plants and removed two specimen to obtain three plants of similar height in each pot.

2.2.3.2.3 Nutrient Extraction with $CaCl_2$

Three weeks after growth, soil was sampled for nutrient extraction (compare Section 3.2.1). In Table 6, the methods are summarized. We used ICP-OES (VARIAN Vista Pro, Australia) for measuring the concentrations of P and Fe, while FAAS (VARIAN AA280 FS, Australia) was used for Ca, K and Mg. For measuring DOC and N forms a segmented flow analyser (SKALAR San++ system, Netherlands) was used.

Table 6: Overview of extraction methods used for the assessment of nutrient availability

Extractant	Fraction	Ratio (w v^{-1})	Chemicals	Weight of Soil (g)	Shaking (h)	Centrifuge Filtration	Reference
$CaCl_2$	Plant available	1:10	0.01 M $CaCl_2$	10	2	5 min, 2716 rcf [a]	Standard method
H_2O	Soluble P	1:5	Demi water[b]	10	2	10 min 10864 rcf [a]	Pierzynsky (2000)
Olsen	Soluble and exchangeable P	1:20	0.5 M $NaHCO_3$	2	0.5	Filtration	Pierzynski (2000)

[a] rcf refers to relative centrifugal force (x g) [b] quality of demi-water is specified in the list of abbreviations

2.2.3.2.4 Application of Elemental Sulfur

Elemental S with a rate of 0.5 g kg^{-1} was applied to the pots two days after the 1st harvest (i.e. 9 weeks after sowing). Five holes with a depth of 8 cm were drilled in the top soil of each pot and the respective amount of S was applied in form of a suspension of S and demi-water (~20 mL per hole). Prior to application, elemental S and demineralized water were mixed in a graduated cylinder and put into an ultrasonic bath (KAREL ELEKTRO, Czech Republic) for 5 min. After the application, the holes were closed again.

2.2.3.2.5 Soil Solution Sampling

We conducted two soil solution samplings (before S application and 19 days after S) and one leachate sampling (27 days after S application). After the collection, the samples were stored in vials, immediately cooled (4 °C) and analysed within 12 h. At the 1st and 2nd soil solution sampling, pH (WTW pH315, Germany), metal (Cd, Zn, Pb, Mn, Fe) and P concentrations using ICP-OES (VARIAN Vista Pro, Australia) were measured. In the leachate samples, the same characteristics and also Cl^-, SO_4^{2-}, oxalate and citrate concentrations were measured using ion chromatography (DIONEX Ion Pac AS14A, USA).

2.2.3.2.6 Harvests, Drying, Digestion and Analysis of Biomass

As already described, three different harvests were executed. Since root scanning was only performed for the 1st and 2nd harvest, the procedure differed from the final harvest.

Maize was cut above the soil surface using a sharp knife and samples were separated into roots and shoots. For the **final harvest**, it was sufficient to collect and store the separated roots and shoots of all three maize plants which had grown together in a pot. Prior to this, all soil particles were gently washed off using demi-water and washed in an ultrasonic bath (KAREL ELEKTRO, Czech Republic).

At the **1st and 2nd harvest**, two pots from each treatment were harvested. However, it was necessary to separate the three root samples for each pot to perform both scanning and digestion of roots. Therefore, the root system of one plant per pot (this equals two root systems per treatment) was washed and dried for the digestion and two root systems per

pot (this equals four per treatment) were used for root scanning (Figure 8). This procedure was necessary since roots had to be stored in ethanol prior to scanning which would have influenced the metal concentrations in the root tissue. The detailed procedure for root scanning is described in Section 2.2.3.2.7. All plant samples for plant tissue analysis were dried for 72 h at 60 °C (BMT Venticell, Germany) and milled (RETSCH SM 100, Germany).

Finally, the the digestion of plants was performed using a microwave digestion system (MILESTONE Ethos 1, Italy). For roots, 0.1 g and for shoots 0.5 g of dried biomass were digested in 8 ml 65 % HNO_3 (ANALYTIKA spol sro, Czech Republic) and 2 ml 30 % H_2O_2 (ANALYTIKA spol sro, Czech Republic). For each sampling set, one blank and one reference material sample (Oriental Tobacco Leaves, CTA-OTL-1) were included. We measured metal (Zn, Cd, Pb, Mn, Fe), S and P concentrations in the plant digests using ICP-OES (VARIAN Vista Pro, Australia).

Besides roots and shoots, representative soil samples from each pot (composed of several sub-samples from the top, middle and bottom of pots) were collected and dried for the subsequent extraction (compare Section 2.2.4.2.8).

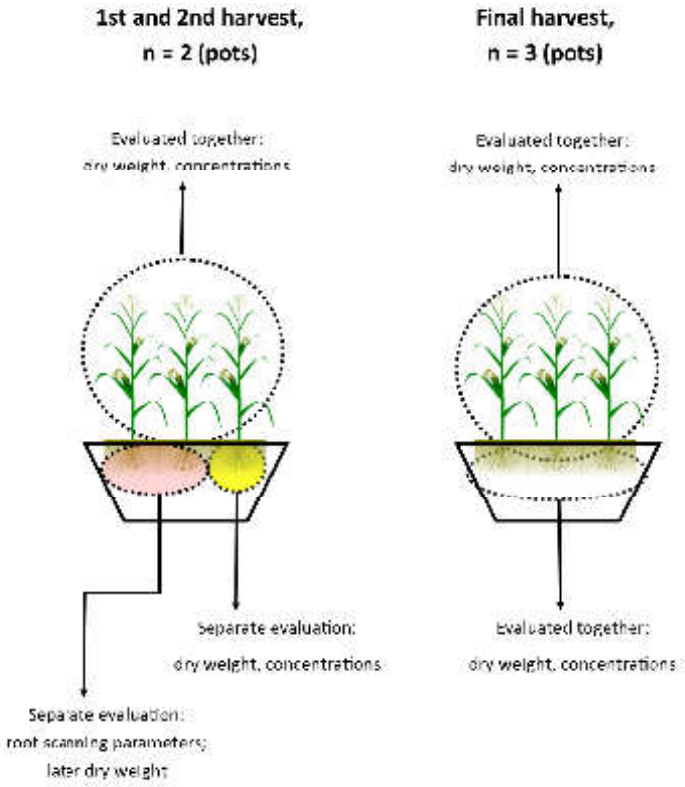

Figure 8: Different sampling procedures for the three harvests. 'Dry weight' refers to the weighting of biomass while 'concentrations' refers to plant tissue concentrations of metals (Cd, Pb, Zn, Mn, Fe), P and S.

2.2.3.2.7 Root Scanning

Root scanning was conducted with the Epson STD 4800 optical scanner system, which is a modified commercially available scanner. For the image acquisition and analyses the software WinRhizo (REGENT INSTRUMENTS, version 2007, Canada) was used. In their comprehensive sensitivity analysis for the WinRhizo software, Bouma et al. (2000) recommended a sample preparation with 24 h staining and an image analysis setting with 400 dpi using an automatic threshold. However, we used 400 dpi resolution and an automatic threshold, whereas the roots were not stained since, even without this procedure, a good contrast could be achieved. Furthermore, at the time of scanning, it was still uncertain if the scanned roots would be needed for further analyses.

After harvest, roots were washed several times in thin sieves (<2 mm and <1 mm) to avoid the loss of thin roots. Subsequently, roots were soaked in demi-water for 20 min. Then, the three root systems of each pot were separated. Finally, the roots were cut into small pieces, stored in 30 % ethanol solution and kept at 4 °C.

We decided to scan the whole root system rather than taking sub-samples to avoid statistical errors. If root systems were too large for one scan, we conducted several scans and aggregated the results. After scanning, roots were dried for 72 h at 60 °C (BMT Venticell, Germany) and weighted. According to Lenger et al. (2010) the specific root length was calculated (i.e total root length divided by the dry weight of roots). Because dry weight of root systems differed largely (depending on the treatment), the calculation of the specific root length made the results more comparable. Finally, the dry weights of the scanned roots and the other remaining roots (kept for digestion) were aggregated to obtain the root dry weight per pot.

2.2.3.2.8 $CaCl_2$ Extraction of Dried Soil

All soil samples (from the three harvests) were extracted using 0.01 M $CaCl_2$ with a SSR of 1:10 (w v^{-1}) followed by 6 h of shaking and centrifugation for 10 min with 978 x g relative centrifugal force. Besides pH (WTW pH315, Germany), metal (Cd, Zn, Pb, Mn, Fe), S and P concentrations were measured using ICP-OES (VARIAN Vista Pro, Australia).

For measuring sulfate, nitrate and oxalate concentrations, another 0.01 M $CaCl_2$ extraction was performed, however, shaking time was reduced to 30 min according to Ketterings et al. (2011). S was measured again by ICP-OES and sulfate, nitrate and oxalate by ion chromatography (DIONEX Ion Pac AS14A, USA).

2.2.4 Statistical Analyses

2.2.4.1 Incubation Experiment

The results of the incubation experiment (i.e. pH_{CaCl_2}, $CaCl_2$-extractable concentrations of Cd, Zn, and Pb) at different time steps were interpreted as repeated measurements even if theoretically the sampling rows were independent. This procedure allowed us to include the factor time and its influence on our statistical model.

The so called 'repeated measurement ANOVA' (abbreviated as RM-A) was used to elicit significant influences of the factors 'treatment', 'time' and 'S-rate'. RM-A distinguishes between two different factor types. The first one is called **within-subject factor** and refers to 'time', while the second is called **between-subject factor** referring to 'treatment' and 'S-rate' as factors.

However, due to the design of the incubation not all factor combinations were present at each time step: for instance S rates 1 and 2 only existed for sampling day 28 and 40. Therefore, more sophisticated statistical analyses were not possible for the whole data set and a separation of the data was necessary (Figure 9). Basically, two blocks were formed: the first one consists of sampling days 0 to 21 prior to S application and the second one covers sampling days 28 to 40 (with S rates of 0.5 and 1.5 g kg^{-1}). This procedure guarantees that every factor combination is present in each analysed subset.

In contrast to the normal ANOVAs, RM-A requires sphericity, which can be seen as extension of the homogeneity of variances and defined as follows: “The sphericity assumption is that all the variances of the differences are equal (in the population sampled)” (Baguley, 2013, s.p.). Whenever a violation of sphericity is present, the more conservative 'Greenhouse-Geisser correction' can be used to obtain an adjusted p-value.

Since in the experiment only three replicates were used and as the Mauchly test of sphericity can have misleading results, we decided to use the Greenhouse-Geisser value as standard routine for every RM-A (Rasch et al., 2006).

After the normal statistical analyses with IBM SPSS (2007), a Bonferoni-Post Hoc test was conducted to find out which treatments and which S rate had a significant influence on metal concentrations or pH value.

Finally, we conducted regressions of pH against Cd, Zn or Pb concentrations, respectively. For this statistical evaluation, the respective module of SigmaPlot (2013) was used.

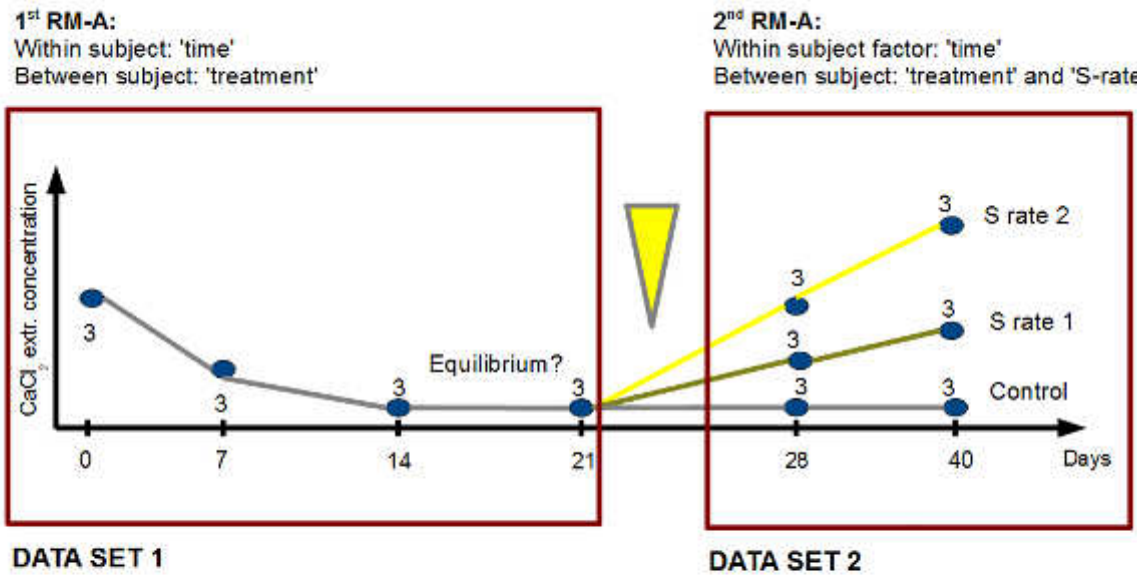

Figure 9: Separation of the experimental data of the incubation experiment to execute two RM-As.

2.2.4.2 Pot Experiment

Besides regressions similar to the incubation experiment, one-way ANOVAs (in case of homogeneity of variances, tested with Levene) or Kruskal-Wallis Tests (in case of violation of variance homogeneity requirement) were performed in the pot experiment.

3. Results

3.1 Incubation Experiment

3.1.1 pH Measurement

The different treatments had a distinguished influence ($p < 0.01$) on soil pH_{CaCl_2} before and after S addition (Figure 10, Table 7 and Table 8). For the C and VC treatments without S addition, pH ranged between 5.5 and 6.0 during the whole experiment whereas BC showed a higher and Lig a lower pH ($p < 0.05$, Figure 11).

Apart from a small peak (measured at day 7), the C, BC1, Lig1, VC1 and VC2 treatments without S addition exhibited a relatively stable pH over the whole experiment (Figure 10). Neglecting these initial and some further minor fluctuations, a quasi-equilibrium state of soil pH after 21 days can be assumed.

Adding S (0.5 and 1.5 g kg^{-1}) decreased soil pH ($p < 0.01$; Table 8). In every treatment, the higher S rate was consistently connected with a lower pH. Proton activity changes (between no-S and S rate 2) were approximately 70 % higher in BC1, Lig2 and VC1 compared to BC2 and VC2 (Table 9). Lig1 showed the largest increase in proton activity.

Repeated measurement ANOVAs proved that time (as within-subject factor) significantly influenced pH ($p < 0.01$, Table 8). Furthermore, interactions between time*treatment, time*S-rate and the combination of all three occurred ($p < 0.01$, Table 8). Besides treatment and S rate as between-subject factors, their interactions proved to be significant as well ($p < 0.01$, Table 8).

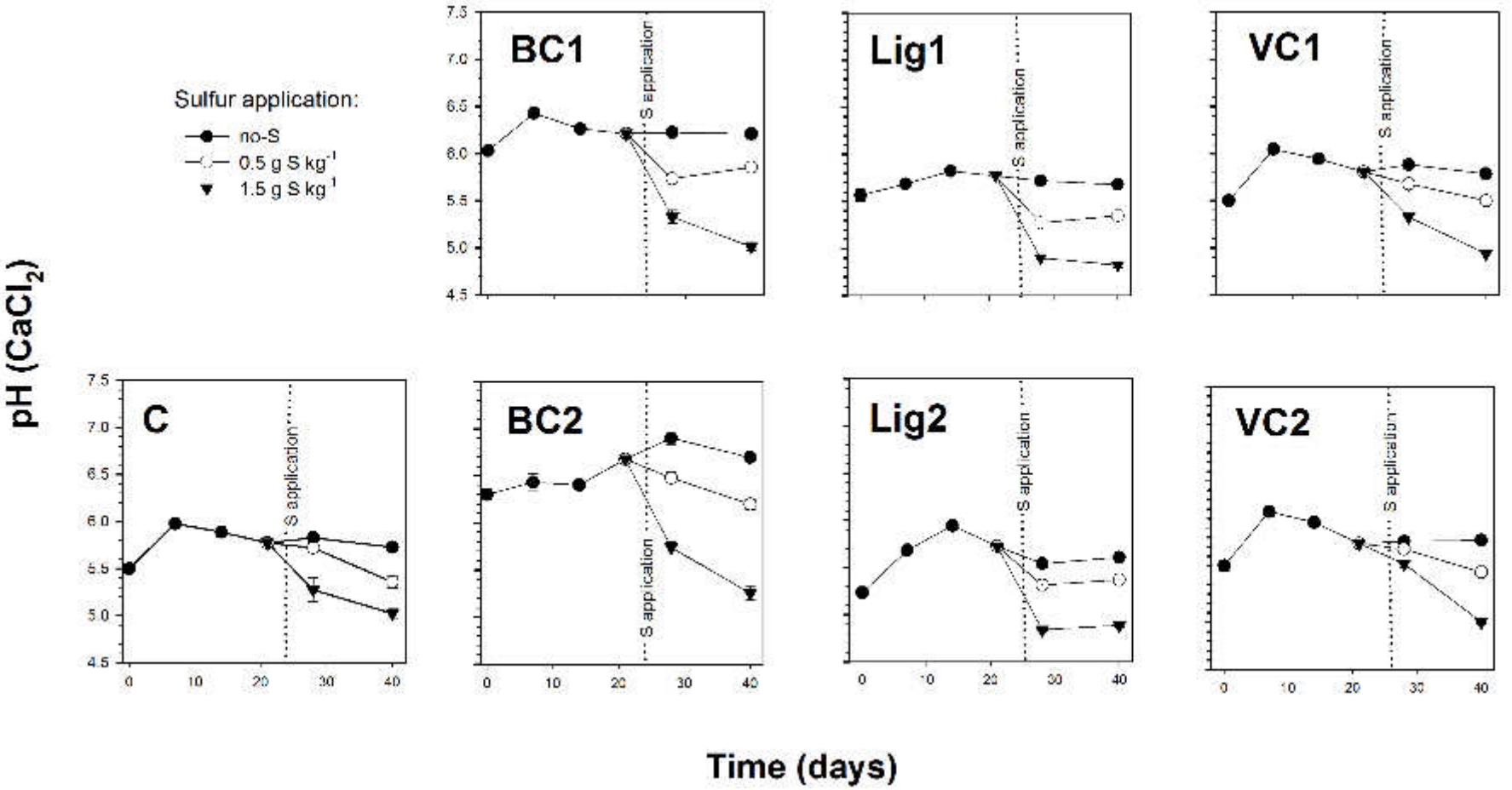

Figure 10: pH values for different time steps (0, 7, 14, 21, 28 and 40 days), treatments (C = control, BC = biochar, Lig = lignite and VC = vermicompost) and S rates (no-S, 0.5 g S kg^{-1} and 1.5 g S kg^{-1}; added at day 22). Error bars present SEM (n = 3).

Table 7: RM-A for the time steps 0 to 21 days. Significance level is p < 0.05. While non-significance is indicated by bold letters, significant values are underlined.

Within subject:	pH			Cd			Zn			Pb		
Greenhouse-Geisser	df	F	sig.	df	F	sig.	df	F	sig.	df	F	sig.
Time[a]	2.16	216.09	0.00	2.31	11.09	0.00	2.39	10.23	0.00	1.80	63.40	0.00
Time*Treatment[c]	12.93	14.76	0.00	13.87	5.33	0.00	14.34	6.19	0.00	10.82	3.28	**0.07**
Error	30.12			32.36			33.47			25.24		
Between subject												
Intercept	1.00	817815.64	0.00	1.00	22628.47	0.00	1.00	26561.65	0.00	1.00	1155.90	0.00
Treatment[b]	6.00	289.75	0.00	6.00	553.59	0.00	6.00	860.05	0.00	6.00	13.99	0.00
Error	14.00			14.00			14.00			14.00		

[a] Within subject factor (four measurements at 0, 7, 14 and 21 days; n = 21 for each measurement)

[b] Between subject factor (n = 12 for each treatment)

[c] n = 3 for each treatment at the respective measurement day

Table 8: RM-A for the time steps 28 to 40 days. Significance level is p < 0.05. While non-significance is indicated by bold letters, significant values are underlined.

Within subject test:	pH			Cd			Zn			Pb		
Greenhouse-Geisser	df	F	sig.	df	F	sig.	df	F	sig.	df	F	sig.
Time[a]	1.00	135.09	0.00	1.00	595.27	0.00	1.00	445.96	0.00	1.00	247.77	0.00
Time*Treatment	6.00	16.89	0.00	6.00	9.66	0.00	6.00	2.24	**0.06**	6.00	3.74	**0.05**
Time*S Rate	2.00	29.56	0.00	2.00	276.75	0.00	2.00	232.41	0.00	2.00	134.88	0.00
Time*Treatment*S Rate[d]	12.00	4.61	0.00	12.00	6.00	0.00	12.00	2.13	0.04	12.00	1.76	**0.09**
Error	42.00			42.00			42.00			42.00		
Between subject test												
Intercept	1.00	715445.34	0.00	1.00	4231.27	0.00	1.00	1840.31	0.00	1.00	1300.31	0.00
Treatment[b]	6.00	336.94	0.00	6.00	32.34	0.00	6.00	9.39	0.00	6.00	4.92	0.01
S-Rate[c]	2.00	1324.24	0.00	2.00	712.67	0.00	2.00	508.26	0.00	2.00	213.06	0.00
Treatment*S Rate	12.00	19.89	0.00	12.00	11.94	0.00	12.00	4.21	0.00	12.00	5.86	0.00
Error	42.00			42.00			42.00			42.00		

[a] Within subject factor (two measurements at 28 and 40 days; n = 63 for each measurement)

[b] Between subject factor treatment (n = 18 for each treatment)

[c] Between subject factor S-Rate (n = 42 for each S rate)

[d] n = 3 for each treatment at the respective measurement day and s-rate

Figure 11: pH_{CaCl2} after 40 days of incubation with three S rates. Significance ($p < 0.05$) between treatments is indicated by different letters (Boniferroni Test). Error bars indicate SEM (n = 3).

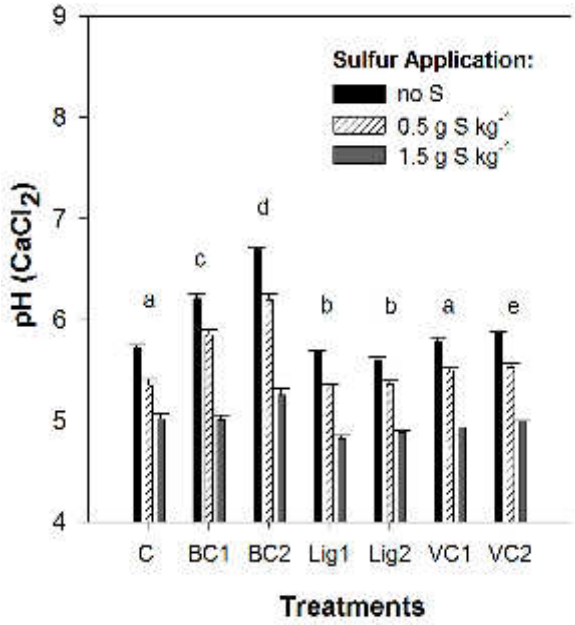

Table 9: pH values and proton activity changes ($\Delta [H^+]$ in mol L^{-1}) due to the application of different S rates calculated within each treatment at day 40 of incubation.

	pH ($CaCl_2$)			$\Delta [H^+]$ in mol l^{-1} between No-S and	
Treatment	**No-S**	**S rate 1**	**S rate 2**	**S rate 1**	**S rate 2**
C	5.73	5.36	5.02	25.2e-7	7.60e-6
BC1	6.21	5.86	5.01	7.74e-7	9.08e-6
BC2	6.69	6.20	5.25	4.33e-7	5.38e-6
Lig1	5.68	5.35	4.83	23.8e-7	12.8e-6
Lig2	5.61	5.37	4.89	17.9e-7	10.5e-6
VC1	5.79	5.50	4.94	15.3e-7	9.85e-6
VC2	5.87	5.53	5.00	16.0e-7	5.57e-6

3.1.2 Zinc

$CaCl_2$-extractable Zn concentrations remained stable in the treatments without S application over the whole incubation period (Figure 12). In every treatment, a strong increase of $CaCl_2$-extractable Zn concentrations could be observed after elemental S was added. Time independent tests (between-subject) showed significant differences in the Zn extractability between treatments, S rate and their interactions ($p < 0.01$, Table 8). However, the interactions of time*treatment (as within-subject factor) were not significant.

Before S application, we measured the highest $CaCl_2$-extractable Zn concentrations in the C treatment (61 – 76 mg kg^{-1}). In contrast, BC treatments showed about 55 % (BC1) and 75 % (BC2) lower $CaCl_2$-extractable Zn concentrations, VC2 about 45 % and Lig (both rates) and VC1 about 30 % ($p < 0.05$, Figure 13).

When 0.5 g S kg^{-1} were added (S rate 1), the $CaCl_2$-extractable Zn concentrations in the control still exceeded those of other treatments reaching 254 ± 17.7 mg kg^{-1} after 40 days of incubation. Only when 1.5 g S kg^{-1} (S rate 2) were applied, $CaCl_2$-extractable Zn concentration of BC1, BC2 and Lig1 surpassed the concentration in C while Lig2 and both VC treatments still displayed lower concentrations.

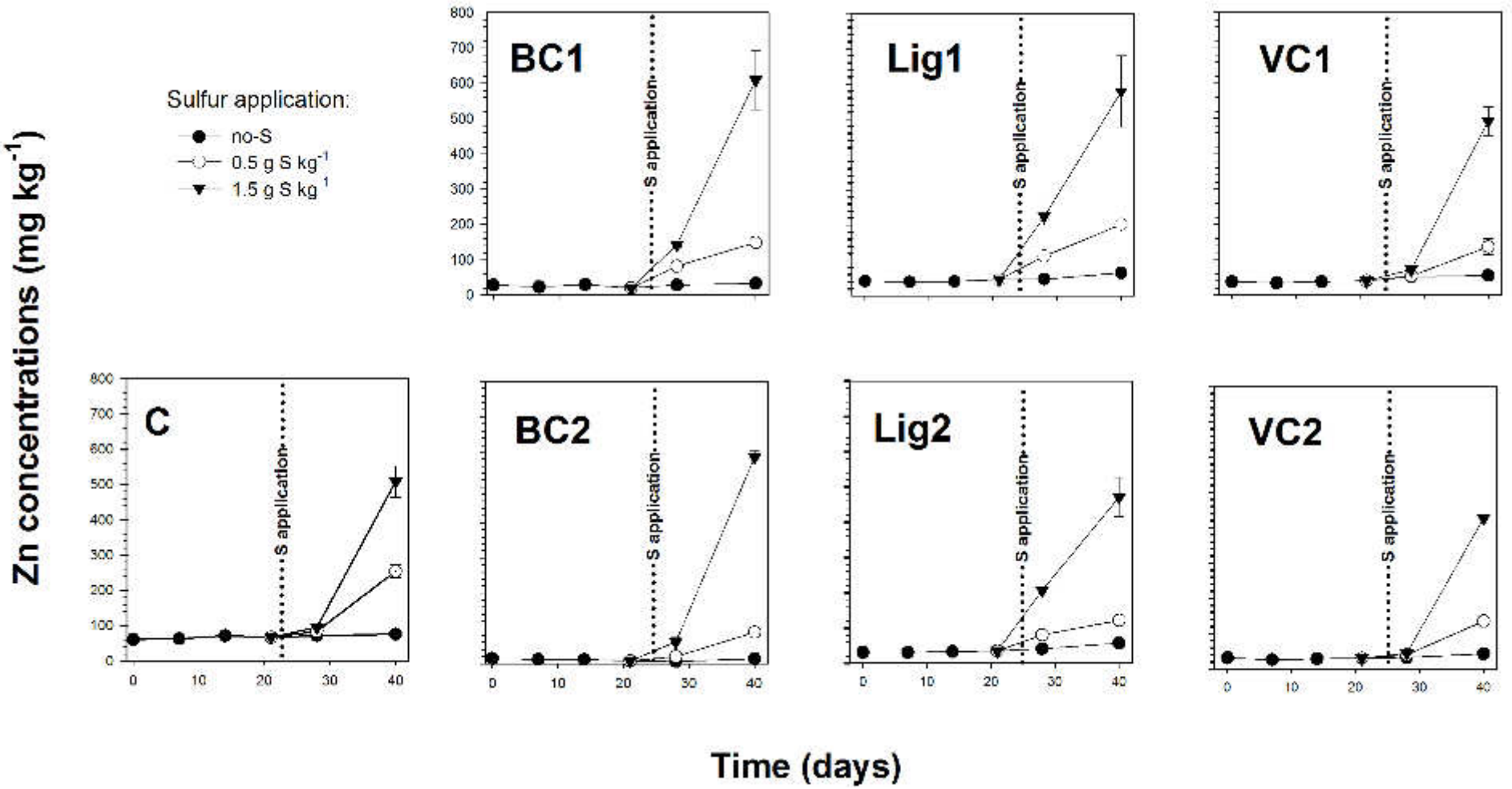

Figure 12: $CaCl_2$-extractable Zn concentrations (mg kg^{-1}) for different time steps (0, 7, 14, 21, 28 and 40 days), treatments (C = control, BC = biochar, Lig = lignite and VC = vermicompost) and S rates (no-S, 0.5 g S kg^{-1} and 1.5 g S kg^{-1}; added at day 22). Error bars present SEM (n = 3).

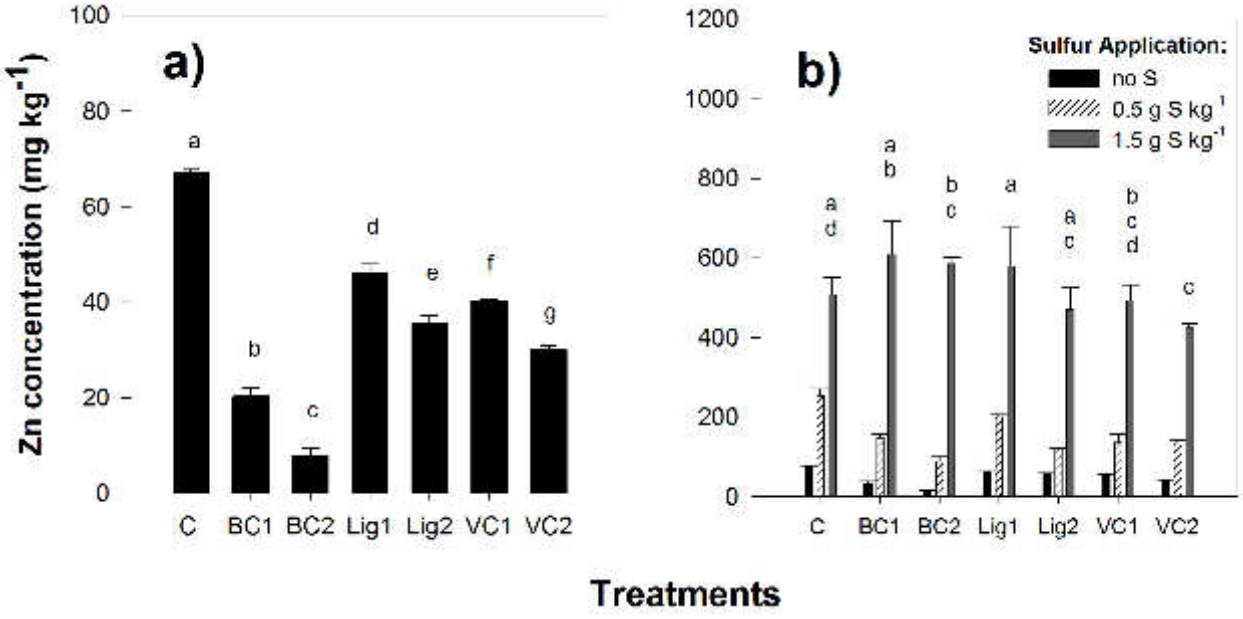

Figure 13: $CaCl_2$-extractable Zn concentrations (mg kg^{-1}) after a) 21 days of incubation before S was added and b) 40 days of incubation with three S rates. Significance ($p < 0.05$) between treatments is indicated by different letters (Boniferroni Test). Error bars indicate SEM (n = 3).

3.1.3 Cadmium

The behaviour of Cd resembles Zn, however, with two orders of magnitude lower in its $CaCl_2$-extractable concentrations (Figure 14). The treatments without S application did not show a noteworthy change in their $CaCl_2$-extractable concentrations of Cd, but a strong increase after S addition was visible (depending on the used S rate).

Results of the RM-As showed significant differences between measurement days (i.e. time), treatments and S rates as well as their interactions ($p < 0.01$, Table 8).

During immobilization (i.e. before S application), $CaCl_2$-extractable Cd concentrations were about 50 % lower in BC1, Lig2 and VC2, 70 % lower in BC2 and 40 % lower in Lig1 and VC1 treatments in comparison to the control ($p < 0.05$, Figure 15). While C still showed the highest extractable Cd concentration when S rate 1 (0.5 g kg^{-1}) was administered, both BC treatments and Lig1 exceeded C when S rate 2 (1.5 g kg^{-1}) was used.

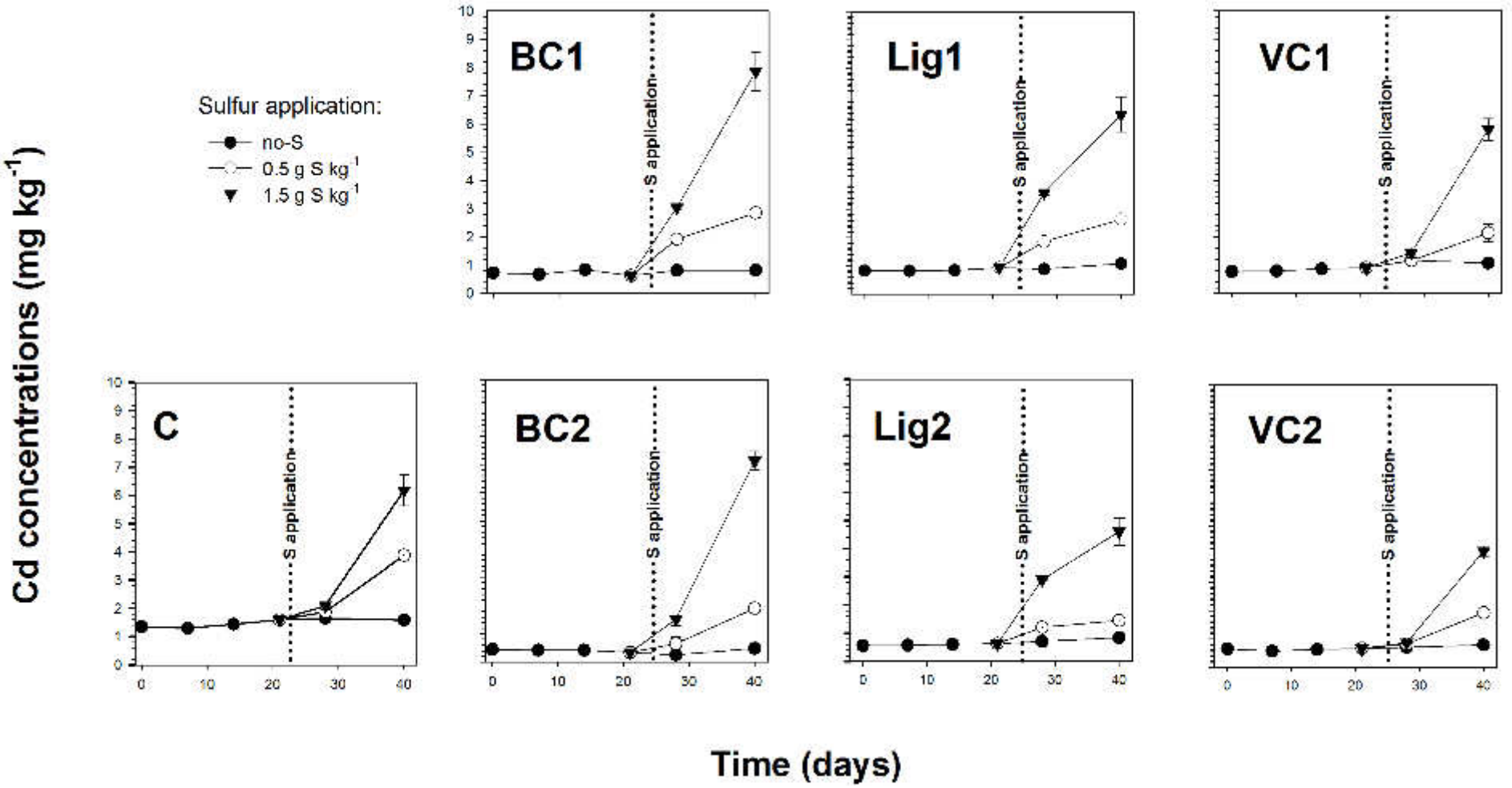

Figure 14: $CaCl_2$-extractable Cd concentrations (mg kg^{-1}) for different time steps (0, 7, 14, 21, 28 and 40 days), treatments (C = control, BC = biochar, Lig = lignite and VC = vermicompost) and S rates (no-S, 0.5 g S kg^{-1} and 1.5 g S kg^{-1}; added at day 22). Error bars present SEM (n = 3).

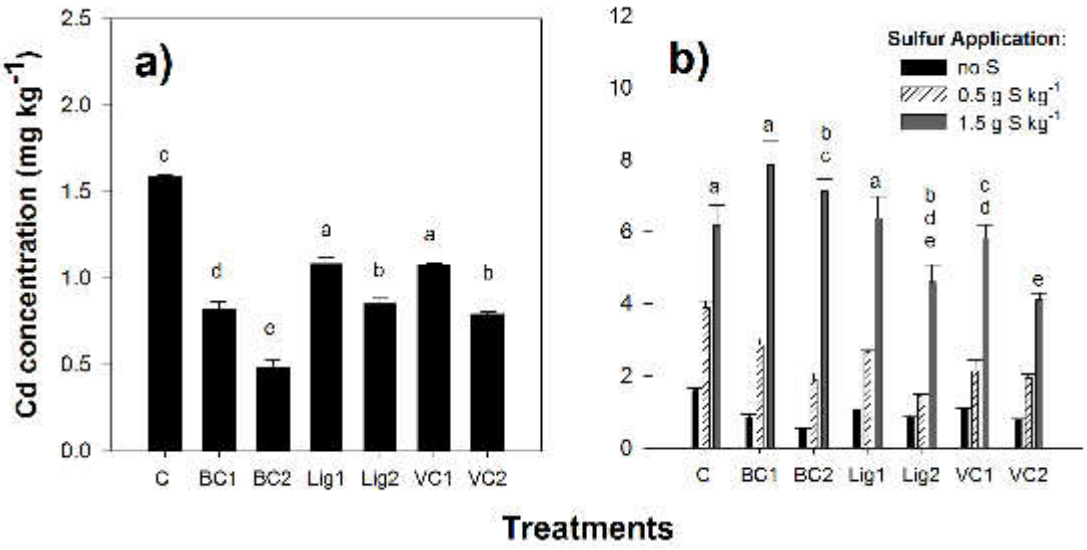

Figure 15: $CaCl_2$-extractable Cd concentrations (mg kg^{-1}) after a) 21 days of incubation before S was added and b) 40 days of incubation with three S rates. Significance ($p < 0.05$) between treatments is indicated by different letters (Boniferroni Test). Error bars indicate SEM (n = 3).

3.1.4 Lead

$CaCl_2$-extractable Pb concentrations showed more fluctuations for the treatments without S application compared to Zn or Cd. When S rate 1 (0.5 g kg^{-1}) was applied, $CaCl_2$-extractable Pb concentrations rose only in C and Lig1 (Figure 16). However, when S rate 2 (1.5 g Kg^{-1}) was added, Pb extractability increased in all treatments.

Time independent tests (between-subject) showed significant differences between treatments, S rate and their interactions ($p < 0.01$, Table 8). However, the interactions of time*treatment and time*treatment*S-rate (as within-subject factor) were not significant.

During immobilization, $CaCl_2$-extractable Pb concentrations of the C, BC and Lig treatments did not significantly differ to each other. In contrast, those of VC were significantly higher after 21 days of incubation ($p < 0.05$, Table 17). After S rates had been applied, the treatments could be divided into two groups: on the one hand, the C, both BC and the VC2 treatment with lower and on the other hand, both Lig and the VC1 treatment with significantly higher extractable Pb concentrations ($p < 0.05$, Table 17).

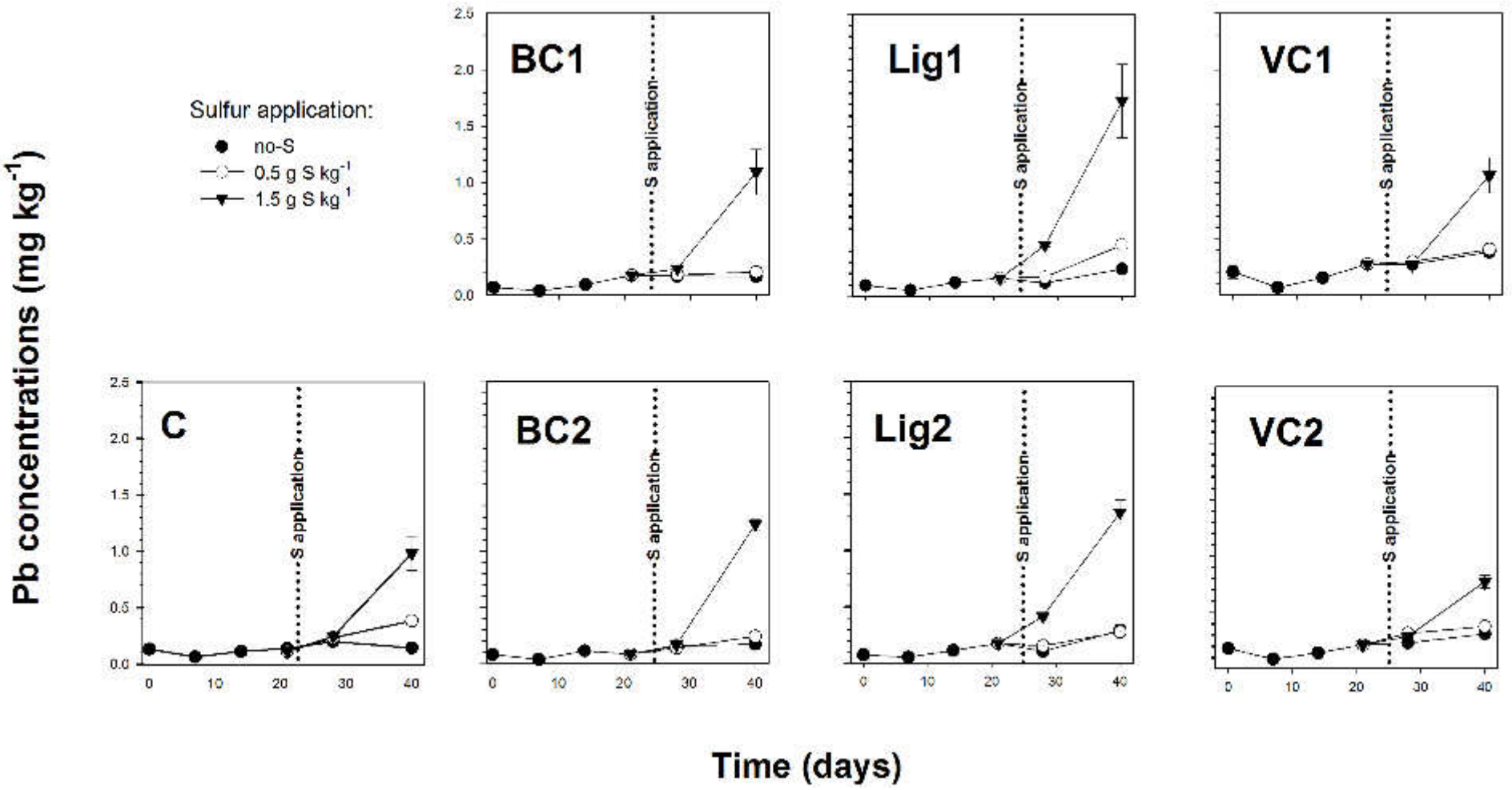

Figure 16: $CaCl_2$-extractable Pb concentrations (mg kg^{-1}) for different time steps (0, 7, 14, 21, 28 and 40 days), treatments (C = control, BC = biochar, Lig = lignite and VC = vermicompost) and S rates (no-S, 0.5 g S kg^{-1} and 1.5 g S kg^{-1}; added at day 22). Error bars present SEM (n = 3).

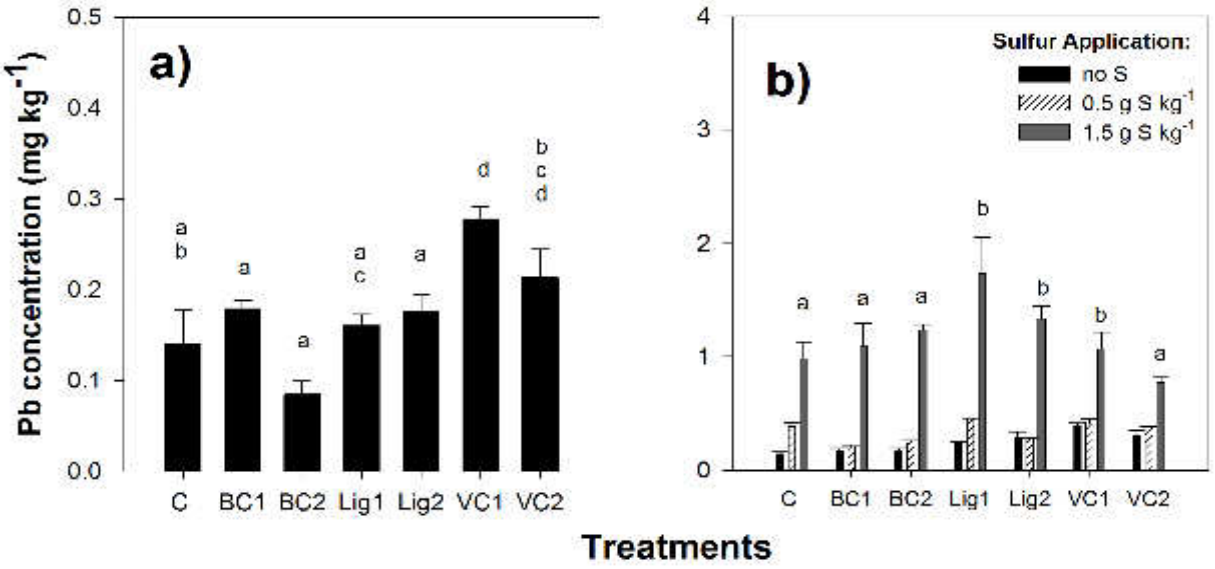

Figure 17: $CaCl_2$-extractable Pb concentrations (mg kg^{-1}) after a) 21 days before S was added and b) 40 days of incubation with three S rates. Significance ($p < 0.05$) between treatments is indicated by different letters (Boniferroni Test). Error bars indicate SEM (n = 3).

3.1.5 Correlation between pH and Metals (Zn, Cd, Pb)

Among all three exponential regressions, the $CaCl_2$-extractable concentrations of Zn showed the closest correlation with pH followed by Cd and with a clearly weaker correlation Pb (Figure 18). Especially in the samples without S application and S rate 1 (0.5 g kg^{-1}), the assumed exponential relation between pH and extractable Pb concentrations only explained a very small part of the whole variation (R^2 = 0.04 and R^2 = 0.10). Yet, all correlations were significant ($p < 0.05$).

The increase in $CaCl_2$-extractable metal concentrations per declining pH unit was much more pronounced in the S-amended samples compared to those without S.

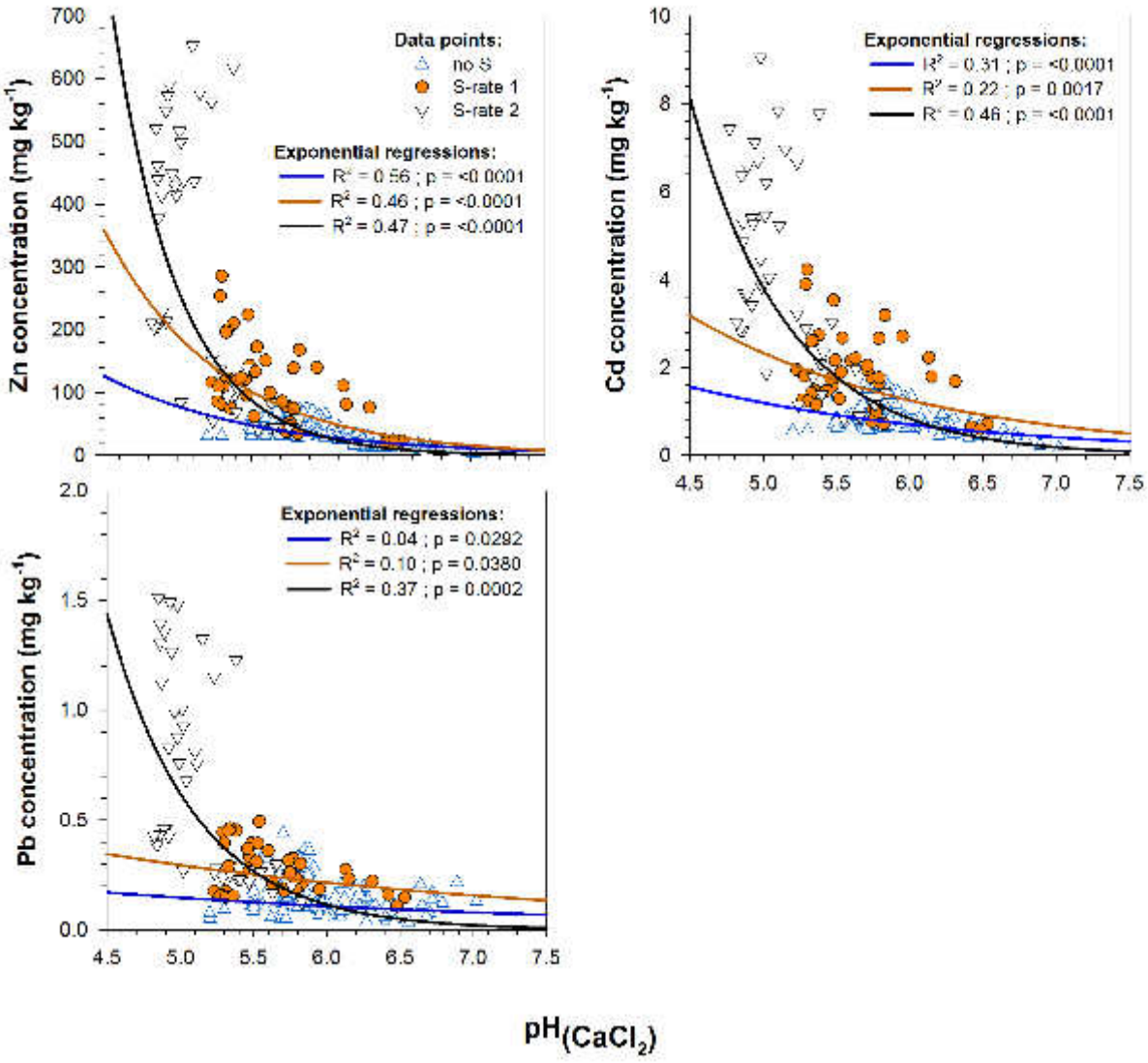

Figure 18: Exponential regressions of pH against $CaCl_2$-extractable concentrations of Cd, Zn and Pb (in mg kg^{-1}) calculated for each S rate (0.5 g kg^{-1}, 1.5 g kg^{-1}). R^2 and the significance level p are given in the legend of each plot (n = 126 for no-S, n = 42 for S rate 1 and 2).

3.2 Pot Experiment

3.2.1 Nutrient Availability in Pots

To assess plant nutrient availability, two different soil extraction methods were used: Olsen for the extraction of P (Figure 19) and $CaCl_2$ for the extraction of Fe, K, Mg, DOC, NO_3^-, NH_4^+ and total soluble N (Figure 20).

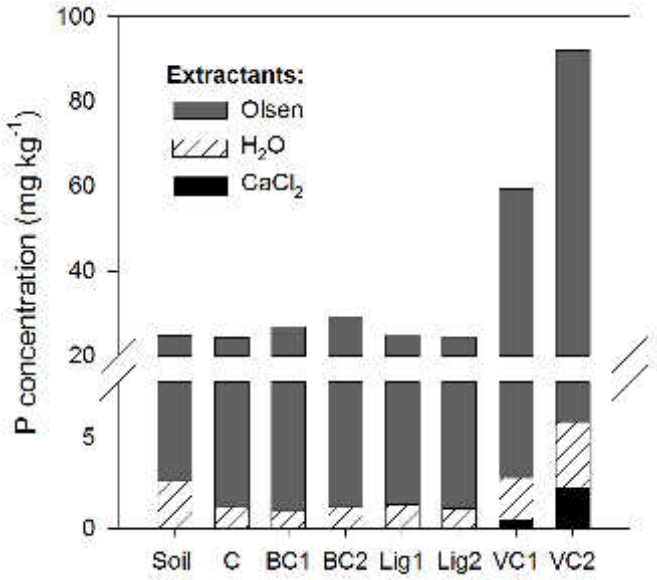

Figure 19: Different P extractions (mg kg^{-1}) according to Pierzynski (2000) three weeks after the sowing of maize. Given are means with n = 3. 'Soil' refers to air-dried Litavka soil.

$CaCl_2$-extractable P concentrations in VC amounted 0.47 ± 0.27 mg kg^{-1} (VC1) and 2.26 ± 0.02 mg kg^{-1} (VC2), respectively, while in other treatments no $CaCl_2$-extractable P could be measured. Similarly, VC displayed the highest water-extractable P concentrations followed by soil (i.e. unfertilized air-dried Litavka soil as reference), the C, Lig and BC treatments. When $NaHCO_3$ was used as extractant (Olsen), P extractability in the VC2 treatment was four times and in the VC1 treatment three times higher compared to all other treatments (Figure 19).

VC treatments showed the highest $CaCl_2$-extractable Fe concentrations (2.15 ± 0.25 mg kg^{-1}), whereas 25 % lower concentrations were measured in the C, both BC and the Lig2

treatments and approximately 50 % lower concentrations in the Lig1 treatment. The reference sample (i.e. 'soil') exhibited the lowest extractability of Fe (0.09 mg kg^{-1}).

Similar results could be obtained for the $CaCl_2$-extractable concentrations of K, Mg, DOC, NO_3^-, and total soluble N with the exception that the highest extractable DOC concentration was measured in the reference sample (Figure 20).

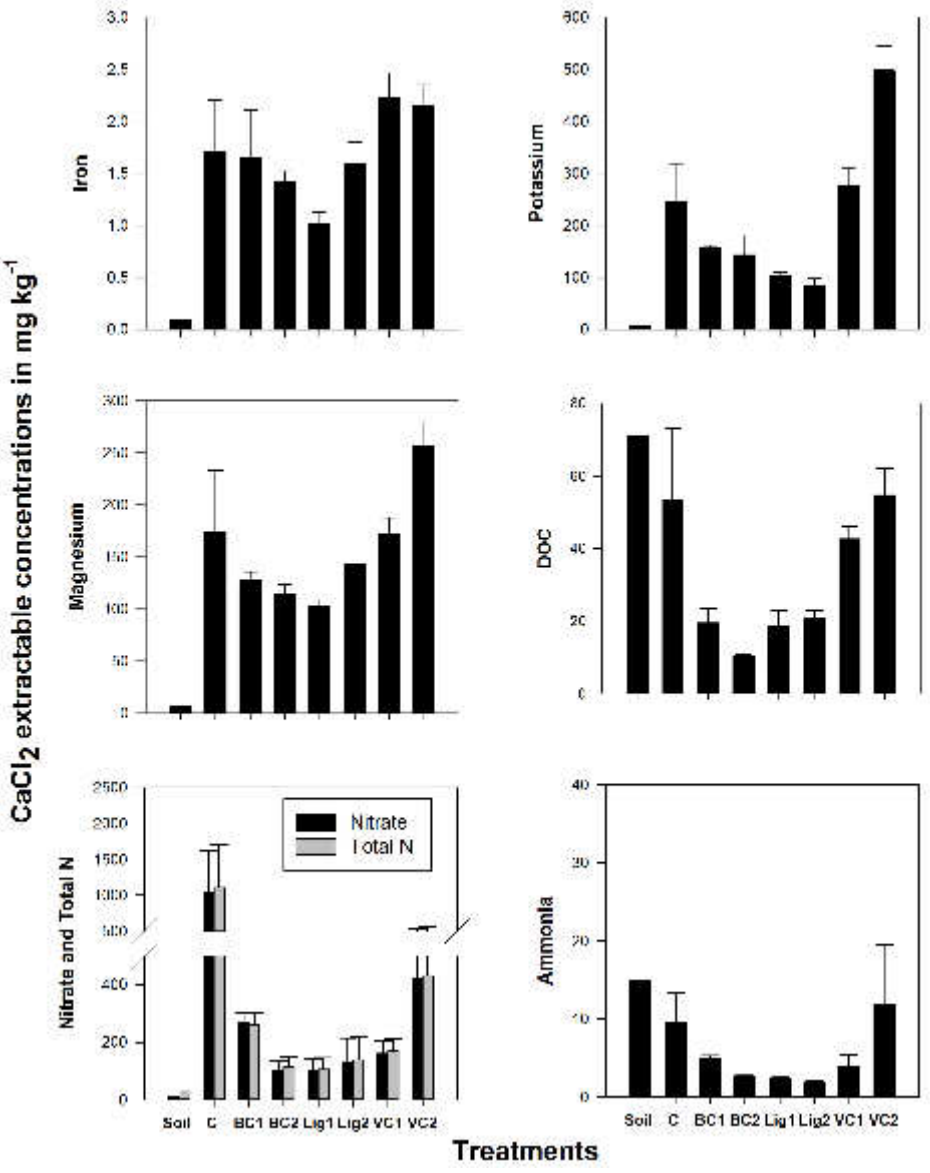

Figure 20: $CaCl_2$ extraction (mg kg^{-1}) of Fe, K, Mg, DOC and N species from pots three weeks after sowing. Error bars indicate SEM (n = 3). 'Soil' refers to air-dried Litavka soil.

3.2.2 Soil Solution and Leachate Sampling

3.2.2.1 pH, Metals (Zn, Cd, Fe, Pb, Mn) and Phosphorus

To assess changes over time, soil solution samples on three different dates and two locations were collected:

- before S application (= 8 weeks after sowing); sampling location: rhizosphere
- 19 days after S (= 12 weeks after sowing); sampling location: rhizosphere
- 27 days after S (= 13 weeks after sowing); sampling location: leachate

Soil solution pH (rhizosphere) increased in every treatment after S addition (Figure 21). This increase was significant in the C, BC1 and Lig1 treatments ($p < 0.05$, Table 10). Except for BC2, pH in the leachate sampling was even higher compared to the second solution sampling from the rhizosphere.

Soluble Cd and Zn concentrations (rhizosphere) decreased in C as well as BC2 and increased in both Lig and VC treatments. However, only the decrease of soluble Cd in the BC2 treatment was significant ($p < 0.05$, Table 10).

In both soil solution and leachate sampling, soluble Pb concentrations declined strongly (partly below detection limit). Due to high SEM, none of the changes in soluble Pb concentrations were significant (Table 10).

Soluble Mn concentrations decreased in every treatment except for VC2 (in the leachate sampling). Soluble Fe concentrations increased after S addition (except for Lig2 and VC1). Apart from a significant decrease of soluble Mn concentrations in the VC2 and a significant increase in the soluble Fe concentrations in the Lig1 treatment, no further significant changes of soluble Mn and Fe concentrations occurred (Table 10).

Soluble P concentrations obtained from the rhizosphere sampling increased after S addition in the C ($p < 0.05$), BC1 ($p < 0.05$), BC2, both Lig and VC1 treatments (Table 10).

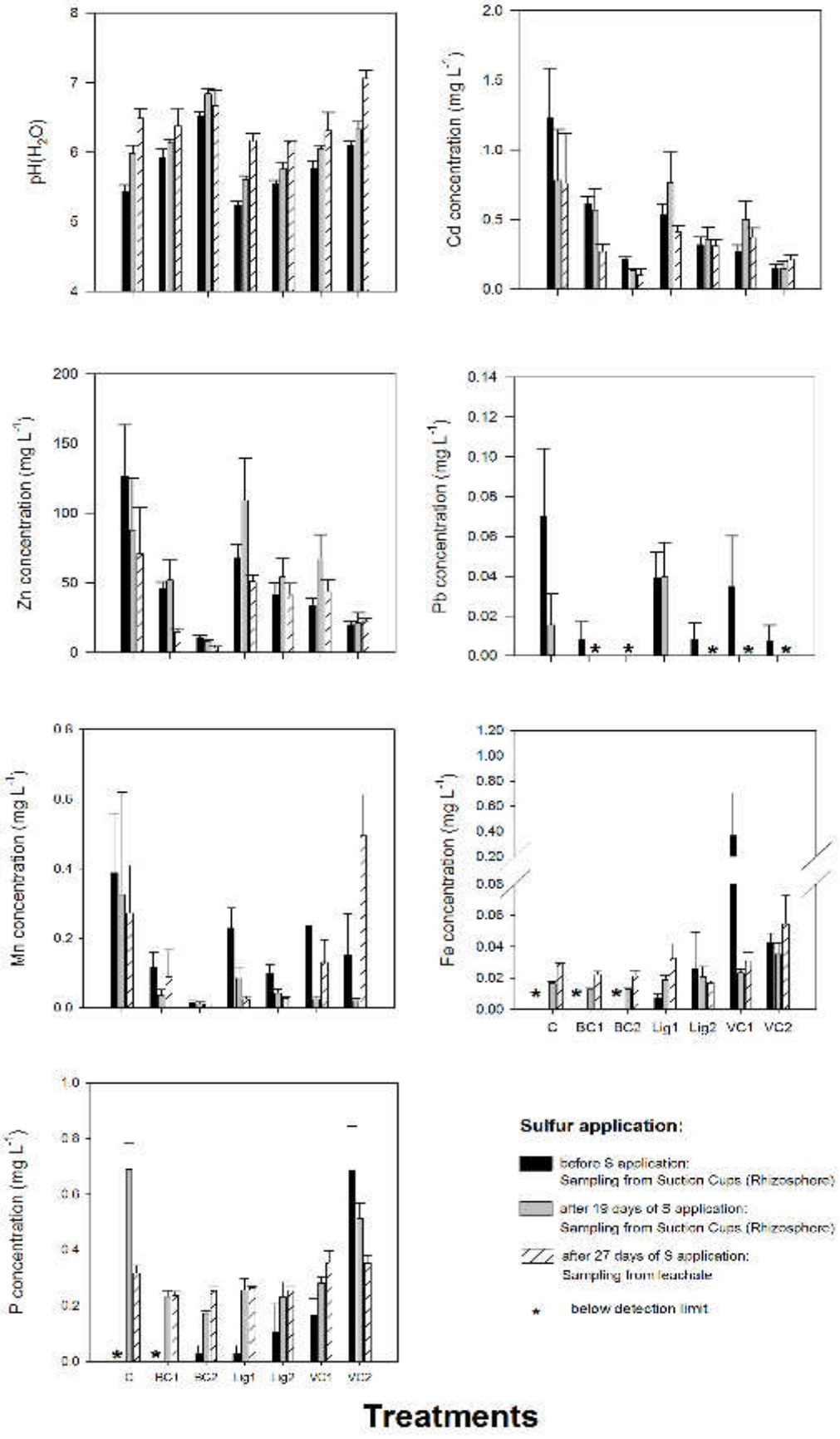

Figure 21: pH value, soluble metal (Cd, Zn, Pb, Mn, Fe) and P concentrations (mg L^{-1}) of three different solution samplings (from rhizosphere and leachate) before and after application of elemental S. Error bars indicate SEM (n = 3).

Table 10: Influence of sampling date (before and 19 days after S application) on pH, metal (Zn, Cd, Pb, Mn, Fe) and P concentrations in the soil solution within each treatment tested by ANOVA or Kruskal-Wallis tests (n = 4 before S and n = 3 after S application). Significant values are underlined (p < 0.05).

	Significance in treatment						
Parameter	C	BC1	BC2	Lig1	Lig2	VC1	VC2
pH	0.016[a]	0.216[a]	0.014[a]	0.004[a]	0.103[a]	0.067[a]	0.114[a]
Zn	0.503[a]	0.670[a]	0.241[a]	0.199[a]	0.429[a]	0.114[b]	0.864[a]
Cd	0.434[a]	0.773[a]	0.020[a]	0.301[a]	0.701[a]	0.117[a]	0.887[a]
Pb	0.252[a]	0.629[b]	1.000[b]	0.972[a]	0.629[b]	0.400[b]	0.629[b]
Mn	0.914[a]	0.201[b]	0.400[b]	0.111[a]	0.169[a]	0.091[a]	0.010[b]
Fe	0.057[a]	0.057[a]	0.057[a]	0.032[a]	0.400[b]	0.400[a]	0.480[a]
P	0.001[a]	0.001[a]	0.057[b]	0.057[b]	0.400[b]	0.170[a]	0.413[a]
[a]	ANOVA						
[b]	Kruskal Wallis						

3.2.2.2 Other Compounds in Leachate: Chloride, Sulfate, Citrate and Oxalate

Leachate concentrations of Cl^- were approximately five times higher in BC2 (173 ± 57.0 mg L^{-1}) and three times higher in BC1 (103 ± 9.36 mg L^{-1}) compared to the other treatments (Figure 22).

The C treatment showed the lowest SO_4^{2-} concentrations in the leachate, whereas those of BC, Lig and VC treatments were three to ten times higher. The treatments with a 90 g kg^{-1} addition rate (BC2, Lig2, VC2) displayed higher SO_4^{2-} concentrations than treatments with 45 g kg^{-1} addition rate (BC1, Lig1, VC1).

The treatments VC2 and Lig1 exhibited the lowest oxalate concentrations in the leachate followed by BC2, Lig2, BC1 and finally the control with an oxalate concentrations being 250 % higher than in Lig1 or VC2.

Citrate concentrations in the leachate were similar in C, both BC and Lig treatments (14.1 – 17.6 mg L^{-1}). In contrast, citrate concentrations in VC were one third (VC1) and two third (VC2) lower.

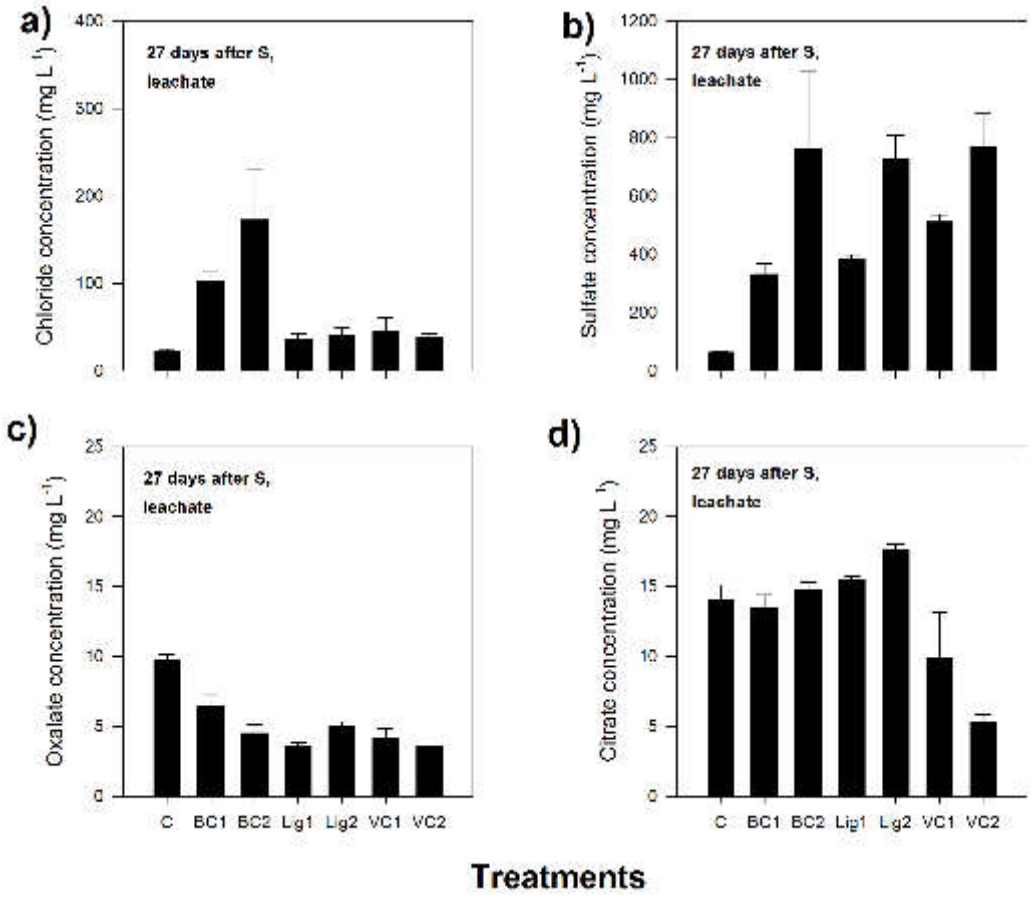

Figure 22: Soil leachate concentrations (mg L^{-1}) of a) Cl^-, b) SO_4^{2-}, c) oxalate and d) citrate measured 27 days after S application. Error bars indicate SEM (n = 3).

3.2.3 Soil Extraction with $CaCl_2$

3.2.3.1 pH and $CaCl_2$-extractable Concentration of Metals (Zn, Cd, Fe, Pb, Mn)

Before elemental S was applied, BC treatments exhibited the highest soil pH_{CaCl2} (BC1: 5.70 ± 0.03; BC2: 6.18 ± 0.15), while the pH in the other treatments ranged from 5.5 to 5.6 (Figure 23).

19 days after the application of S, only the following small changes in the soil pH_{CaCl2} could be measured: an increase in BC1, VC1 and VC2, a decrease in BC2 as well as Lig2 and no changes in the C and Lig1 treatments (Figure 23). In contrast, all treatments (except for C) exhibited a smaller soil pH 33 days after S application. However, only in Lig2 and VC2 these differences between the sampling dates (before and after S) were significant ($p < 0.05$, Table 11).

During the immobilization period (i.e. before S was added), BC reduced the $CaCl_2$-extractable concentrations of Cd by 70 %, Zn by 84 % and Pb by 56 % in comparison to the control (Figure 23). But also Lig and VC treatments notably reduced the $CaCl_2$-extractable concentrations of Zn and Cd. However, the extractable Pb concentration in both Lig and VC treatments were higher than those measured in the C treatment

After mobilization, $CaCl_2$-extractable concentrations of Zn, Cd and Pb showed the same trends over time: concentrations increased in both BC treatments, Lig1 ($p < 0.05$ for Zn), Lig2 ($p < 0.05$ for Zn, Cd, Pb) VC1 and VC2 ($p < 0.05$ for Zn, Cd), whereas the respective metal concentrations in the control did not change (Figure 23, Table 11).

$CaCl_2$-extractable Mn concentrations decreased in the control and increased in the Lig2 treatment over time ($p < 0.05$, Table 11). Fe extractability in the treatments did not significantly change during the pot experiment (Table 11).

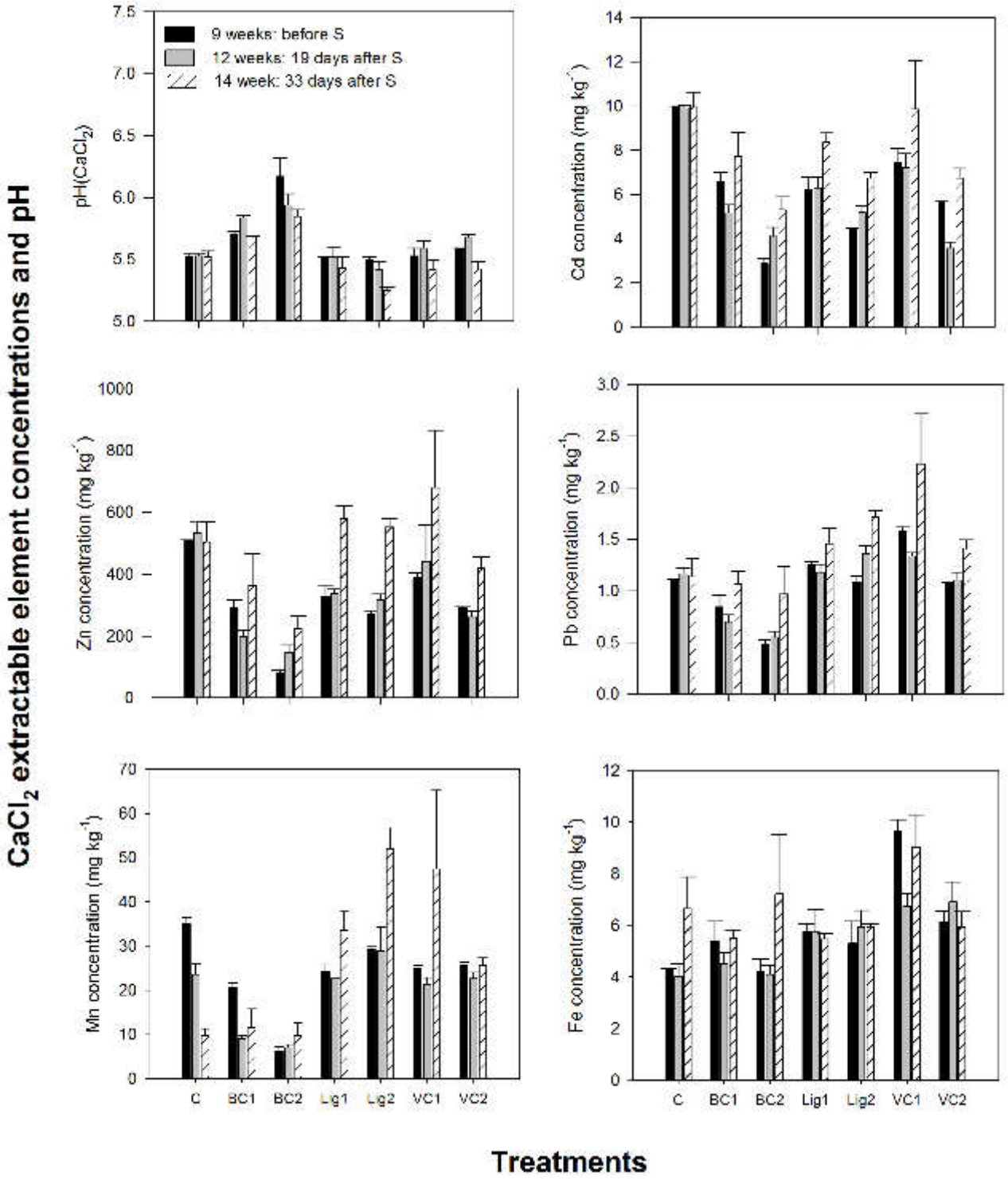

Figure 23: $CaCl_2$-extractable concentrations (mg kg^{-1}) of Zn, Cd, Pb, Mn, Fe and pH for three harvests before and after S addition. Error bars indicate SEM (n = 2 for 9 and 12 weeks | n = 3 for 14 weeks).

Table 11: Influence of harvest dates (before S application, 19 days and 33 days after S application) on pH, $CaCl_2$-extractable metal (Zn, Cd, Pb, Mn, Fe) and SO_4 concentrations within each treatment tested by ANOVA or Kruskal-Wallis tests (for 1st and 2nd harvest n = 2; for 3rd harvest n = 3). Significant values are underlined ($p < 0.05$).

	Significance in treatment						
Parameter	C	BC1	BC2	Lig1	Lig2	VC1	VC2
pH	0.983[a]	0.181[a]	0.133[a]	0.682[a]	<u>0.029</u>[a]	0.419[b]	<u>0.038</u>[b]
Zn	0.919[a]	0.452[a]	0.081[a]	<u>0.014</u>[a]	<u>0.002</u>[a]	0.438[b]	<u>0.041</u>[a]
Cd	0.997[a]	0.240[a]	0.062[a]	0.052[a]	<u>0.006</u>[a]	0.538[a]	<u>0.011</u>[a]
Pb	0.800[a]	0.181[a]	0.292[a]	0.524[b]	<u>0.006</u>[a]	0.326[a]	0.052[a]
Mn	<u>0.002</u>[a]	0.173[a]	0.574[a]	0.147[a]	<u>0.035</u>[a]	0.619[b]	0.381[b]
SO_4	0.713[a]	0.781[a]	0.212[a]	<u>0.014</u>[a]	<u>0.029</u>[b]	0.221[a]	0.361[a]
NO_3^-	0.524[a]	0.181[a]	<u>0.024</u>[b]	0.219[a]	0.419[a]	<u>0.021</u>[a]	0.657[a]
P	<u>0.048</u>[a]	<u>0.028</u>[b]	0.086[b]	0.554[a]	0.182[a]	0.619[b]	0.061[b]
[a]	ANOVA						
[b]	Kruskal Wallis						

3.2.3.2 Other $CaCl_2$-extractable compounds: Phosphorus, Sulfate, Nitrate and Oxalate

VC treatments showed the highest $CaCl_2$-extractable P concentrations among the treatments (Figure 24). Only for BC1 (45 % decrease), Lig2 (20 % increase) and VC2 (20 % increase) notable changes in the extractable P concentrations could be measured over time.

$CaCl_2$-extractable SO_4^{2-} concentrations increased in all treatments between the 1st sampling (before S) and 3rd sampling (33 days after S), however, only in Lig (both treatments) this increase was significant ($p < 0.05$, Table 11). While BC2, both Lig and VC treatments showed an increase in the $CaCl_2$-extractable SO_4^{2-} concentrations for the 2nd sampling (19 days after S) as well, the respective concentrations in C and BC1 did not change.

The $CaCl_2$-extractable concentrations of NO_3^- can be divided into two groups with contrasting behaviour: While NO_3^- extractability for C and both BC treatments started at a

high level (~750 – 800 mg kg^{-1}), a strong decrease after S application is visible. Both Lig and VC treatments just followed the opposite pattern: while initial NO_3^- concentrations have been low, the concentration could strongly increase after S addition.

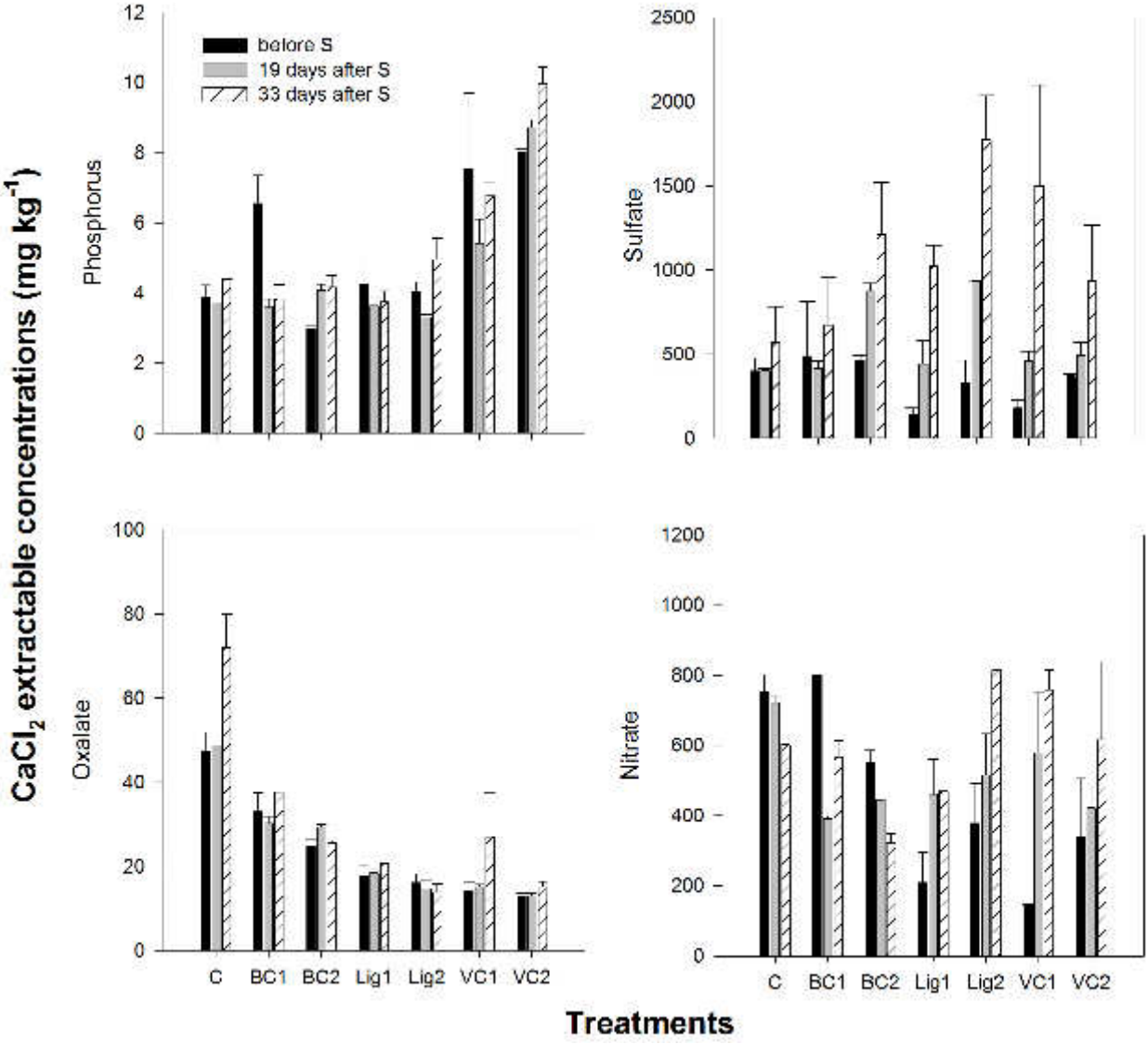

Figure 24: $CaCl_2$-extractable P, NO_3^-, SO_4^{2-} and oxalate concentrations (mg kg^{-1}) for three harvests before and after S addition. Error bars indicate SEM (n = 2 for 9 and 12 weeks; n = 3 for 14 weeks).

3.2.3.3 Regressions between $CaCl_2$-extractable Metal Concentrations and pH

To evaluate the correlation between pH and $CaCl_2$-extractable metal concentrations of Zn, Cd, Pb or Mn, an exponential regression model was used (Figure 25). For every sampling date (before S, 19 days after and 33 days after S applicatoin) a separate regression curve was calculated.

Except for the Cd concentrations 19 days after S application, a significant influence of pH on the respective $CaCl_2$-extractable metal concentrations could be found for every conducted regression (Figure 25).

Correlation coefficients were higher in the pot experiment compared to the incubation. However, the S application in the pots had only a small effect on the correlations between pH and extractable metal concentrations: with increasing S rates, the regression curves for Zn, Cd and Pb showed slightly higher concentrations at same pH values, while Mn concentrations showed a minimal decline (i.e. downward shift of the regression curve).

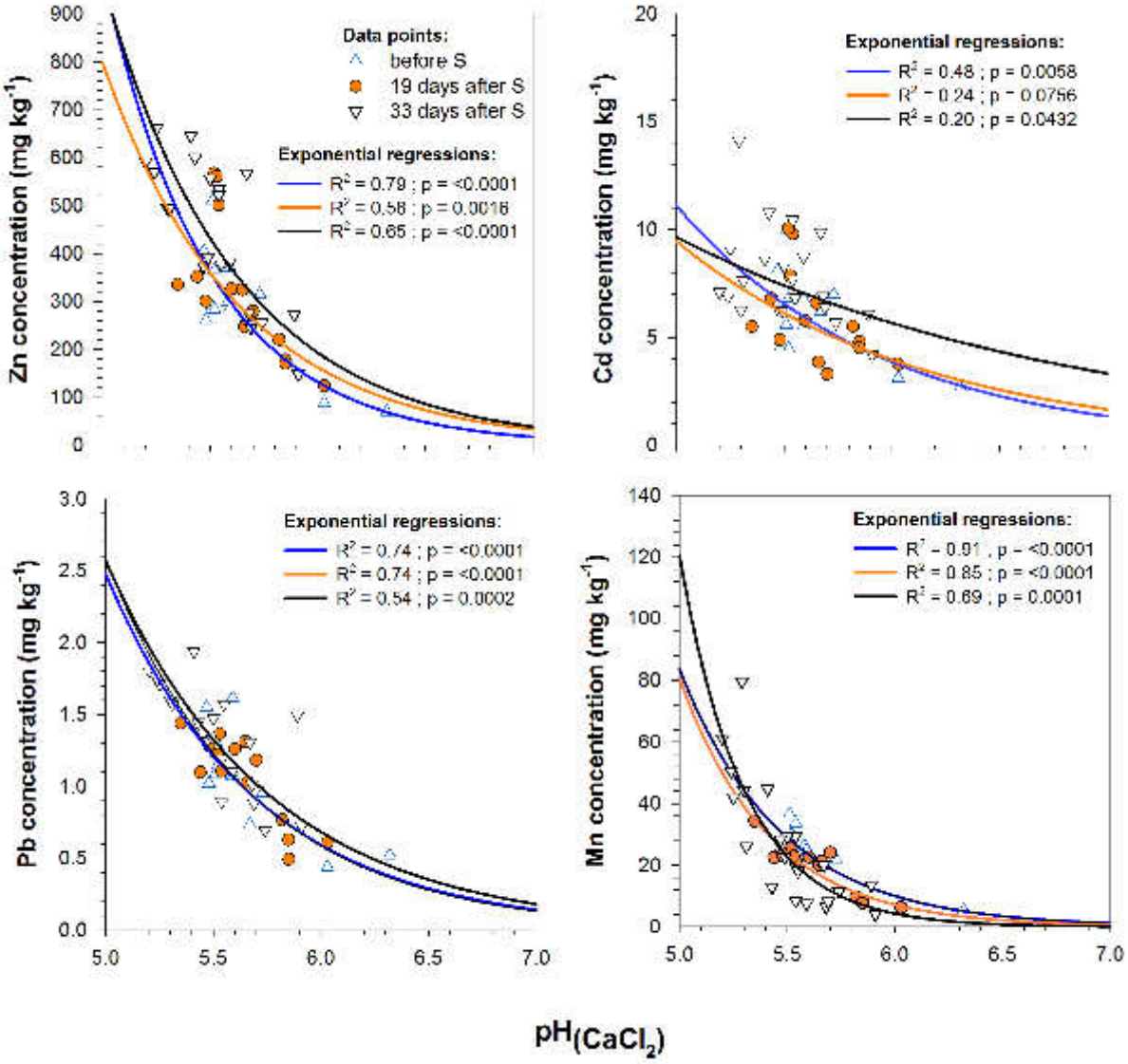

Figure 25: Exponential regressions of $CaCl_2$-extractable metal concentrations (Zn, Cd, Pb, Mn) against pH calculated separately for three sampling dates (before S application, 19 days and 33 days after S application). R^2 and the significance level p are given in the legend of each plot (n = 14 for before S and 33 days after S | n = 21 for 33 days after S).

3.2.4 Plant Responses during Pot Experiment

3.2.4.1 Growth of Maize and Plant Stress Symptoms

Treatments significantly influenced biomass production of roots and shoots after 14 weeks of growth ($p < 0.05$, Table 12) ranging from 1.53 to 28.3 g pot^{-1} for roots and 5.15 to 96.2. g pot^{-1} for shoots (Figure 26). The largest biomass production was found in VC2 and VC1 in both roots and shoots, whereas Lig2 reached only 30 %, Lig1 and BC2 20 % and BC1 and C only 7 % of the dry weight in VC2. While biomass dry weight in the C treatment did not change substantially over time, a decrease (roots and shoots) could be found in the BC1 treatment (during the 12th to the 14th week of growth). Both Lig and VC treatments steadily increased biomass dry weight during the whole experiment.

Table 12: Influence of treatments on biomass production of *Zea mays* (roots and shoots) after 14 weeks of growth tested by ANOVA or Kruskal-Wallis tests (n = 3). Significant values are underlined (p < 0.05).

			ANOVA		Kruskal Wallis	
Parameter	Levene	df	F	sig.	H	asym-sig.
Root	< 0.05	6.00	-	-	18.77	0.000
Shoot	0.40	6.00	102.96	0.000	-	-

After 8 weeks of growth, plant stress symptoms (i.e. toxicity and/or deficiency) could be found in all treatments (reddish and tapering leaves) except for VC (Figure 27a). After 11 weeks of growth, these symptoms fortified for the C, BC and Lig treatments and also in both VC treatments stress symptoms as tapering leaves could be found (Figure 27b). At the last harvest (13 weeks after sowing), the leaves of all plants were strongly affected by chlorosis and plants in the C and both BC treatments nearly died back (Figure 27c). Moreover, deficiency symptoms of K (BC1) and P (C, BC, Lig) could be identified (Figure 27 d,e,f).

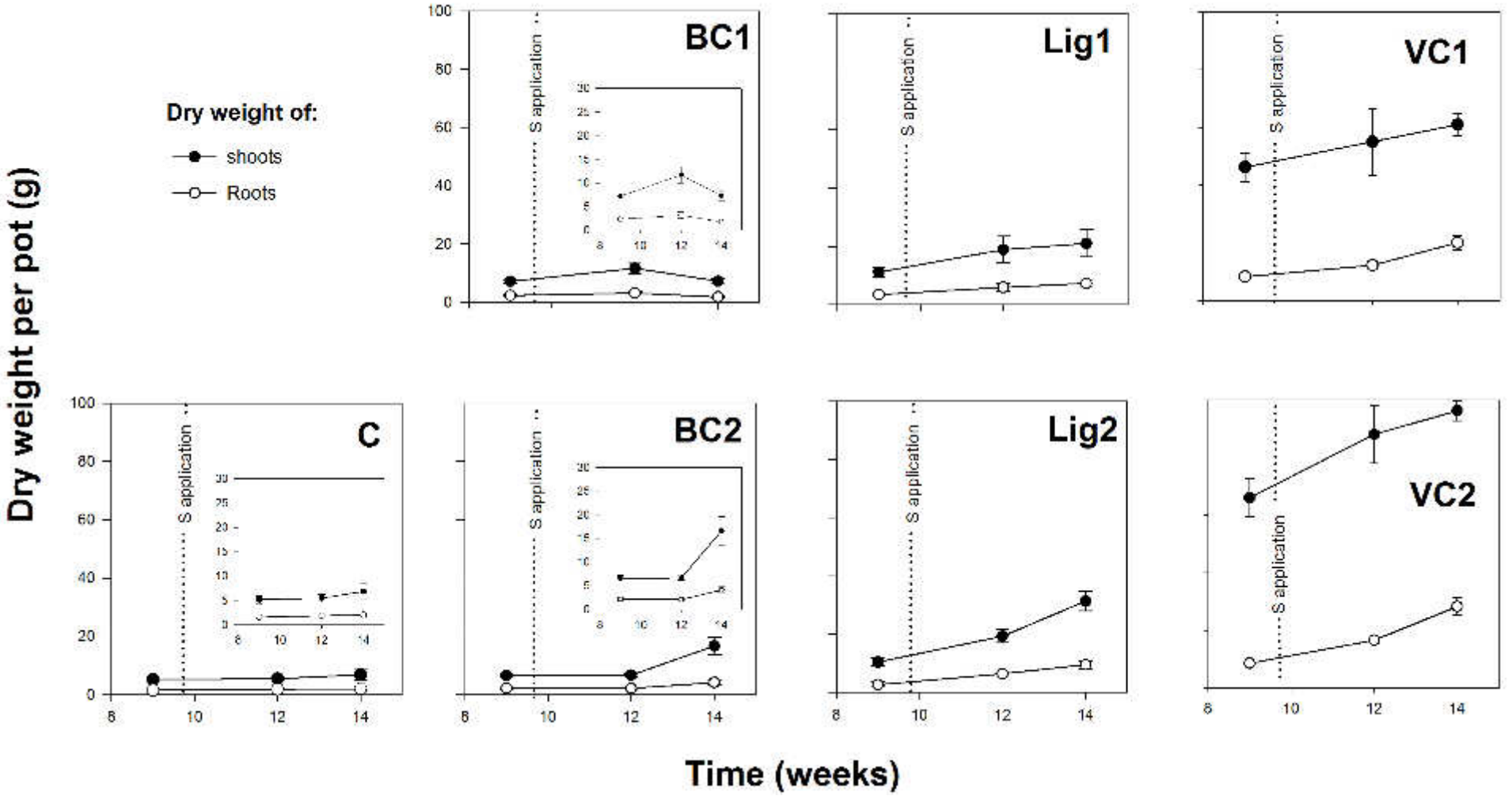

Figure 26: Dry weight (g) of maize plants for shoots and roots at different times for the treatments before and after S application of 0.5 g kg^{-1}. Error bars indicate SEM (n = 2 for first two harvests and n = 3 for last harvest)

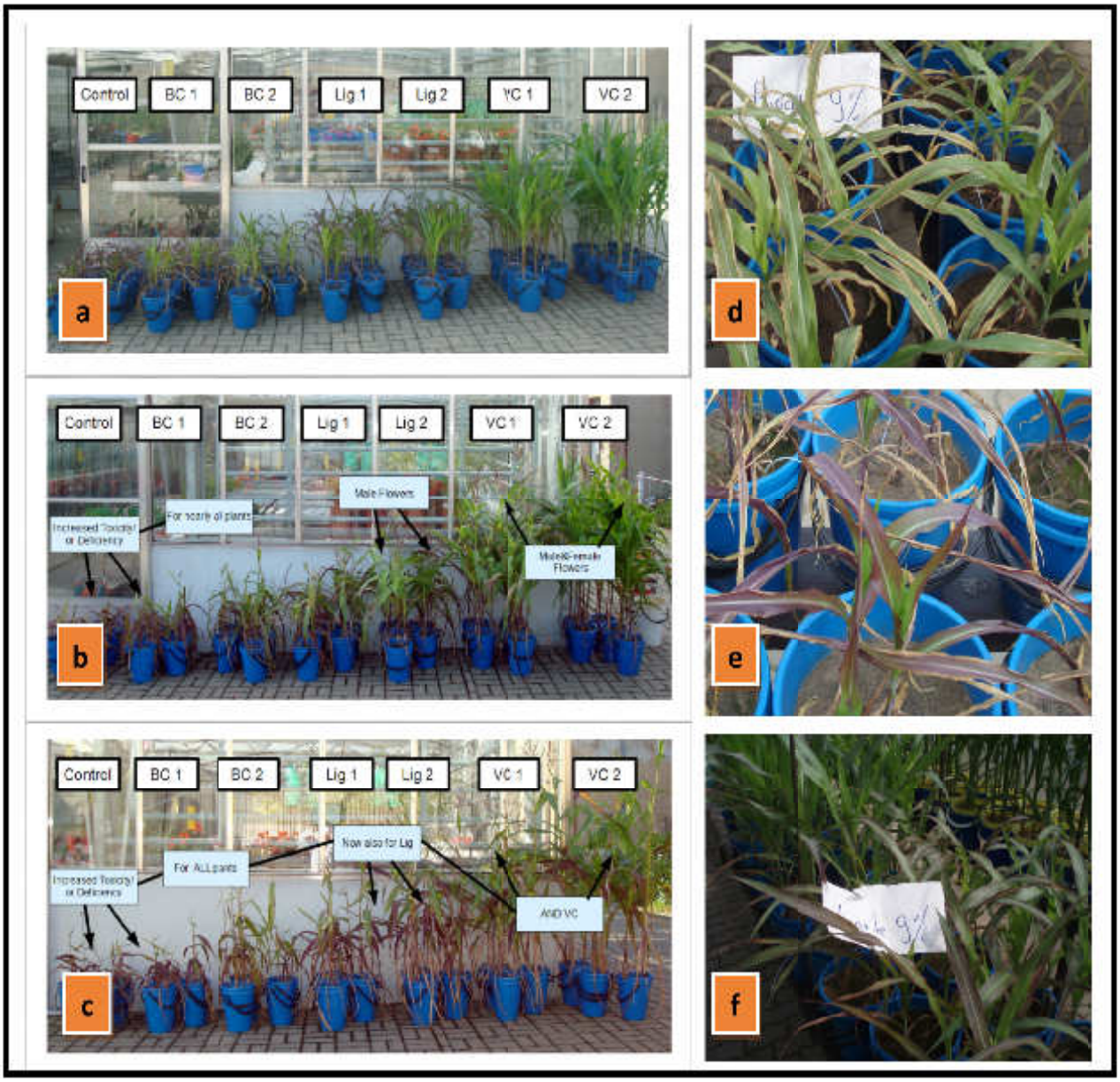

Figure 27: Photographic documentation of plant growth and stress symptoms. a) 8 weeks after sowing before S application; b) 11 weeks after sowing, 2 weeks after S application; c) 13 weeks after sowing and 4 weeks after S application; d) K deficiency symptoms for BC1 after 4 weeks; e) Toxicity or deficiency of BC2 and C after 4 weeks; f) P deficiency of Lig2, 6 weeks after sowing.

3.2.4.2 Root Morphology Changes Before and After Sulfur Application

Before S application (9 weeks after sowing), the largest specific root length could be found in the maize plants of the Lig2 (4400 ± 534 cm g^{-1}) and BC2 (4160 ± 242 cm g^{-1}) treatment followed by BC1, C, Lig1 and with much lower values both VC treatments (Table 13). After elemental S was added to the pots, the specific root length of plants in the C, BC and Lig treatments declined strongly and in C and BC treatments also the total root length decreased. Only in VC treatments an increase of the specific root length could be observed after S application.

To obtain detailed information about the changes in root morphology before S application, total root length proportions were calculated for six diameter classes (Table 14): the development of thicker roots (diameter class > 1 mm) was more pronounced in the roots of both VC treatments, while a high root length proportion in the smaller diameter classes (0.0 – 0.2 mm and 0.2 – 0.4 mm) was predominantly found in the C, BC and Lig treatments (Table 14).

Considering the changes in total root length proportion of maize after S application, two distinguished trends can be identified (Figure 28): In the C, BC and Lig treatments a redistribution from the second (0.2 – 0.4 mm) to the first (0.0 – 0.2 mm) diameter class could be measured accompanied by a small increase in the diameter classes 0.6 to 8 mm and 8 to 10 mm. In contrast, maize roots in the VC treatments showed a 20 % increase of the total root length proportion in the first diameter class (0. – 0.2 mm) and a decrease in all other diameter classes (most pronounced in the diameter class > 1.0 mm).

Table 13: Dry weight (g), total and specific root length (cm g^{-1}) before and after the application of S. All data are calculated as means per root. SEM after ± is given with n = 4.

	Week 9: before S application			Week 12: 19 days after S application		
Treatment	**Root dry weight (g)**	**Total root length (cm)**	**Specific root length (cm g^{-1})***	**Root dry weight (g)**	**Total root length (cm)**	**Specific root length (cm g^{-1})***
C	0.52 ± 0.05	1830 ± 127	3580 ± 92.1	0.48 ± 0.04	1180 ± 161	2410 ± 126
BC1	0.70 ± 0.10	2590 ± 175	3940 ± 596	0.90 ± 0.16	2290 ± 470	2520 ± 170
BC2	0.68 ± 0.13	2780 ± 426	4160 ± 242	0.59 ± 0.19	1610 ± 414	2860 ± 224
Lig1	0.98 ± 0.19	2900 ± 145	3180 ± 394	1.19 ± 0.20	2920 ± 682	2420 ± 245
Lig2	0.79 ± 0.17	3230 ± 417	4400 ± 534	1.30 ± 0.20	3470 ± 453	2700 ± 118
VC1	2.37 ± 0.50	4020 ± 345	1970 ± 429	2.72 ± 0.33	7700 ± 622	2910 ± 231
VC2	2.48 ± 0.41	3790 ± 173	1660 ± 271	3.85 ± 1.85	8880 ± 2020	2300 ± 108

* specific root length was calculated as mean of root-length-dry-weight ratios for each root

Table 14: Proportion of total root length calculated for each treatment and diameter class. Values were measured after 9 weeks of growth and before S application.

	Proportion of total root length in diameter classes (mm)					
Treatment	**0.0 – 0.2**	**0.2 – 0.4**	**0.4 – 0.6**	**0.6 – 0.8**	**0.8 – 1.0**	**> 1.0**
C	0.35	0.31	0.10	0.07	0.07	0.11
BC1	0.39	0.31	0.09	0.04	0.04	0.12
BC2	0.40	0.31	0.11	0.04	0.03	0.11
Lig1	0.39	0.30	0.09	0.06	0.05	0.12
Lig2	0.48	0.24	0.08	0.05	0.05	0.10
VC1	0.35	0.25	0.12	0.06	0.04	0.17
VC2	0.29	0.24	0.14	0.08	0.05	0.20

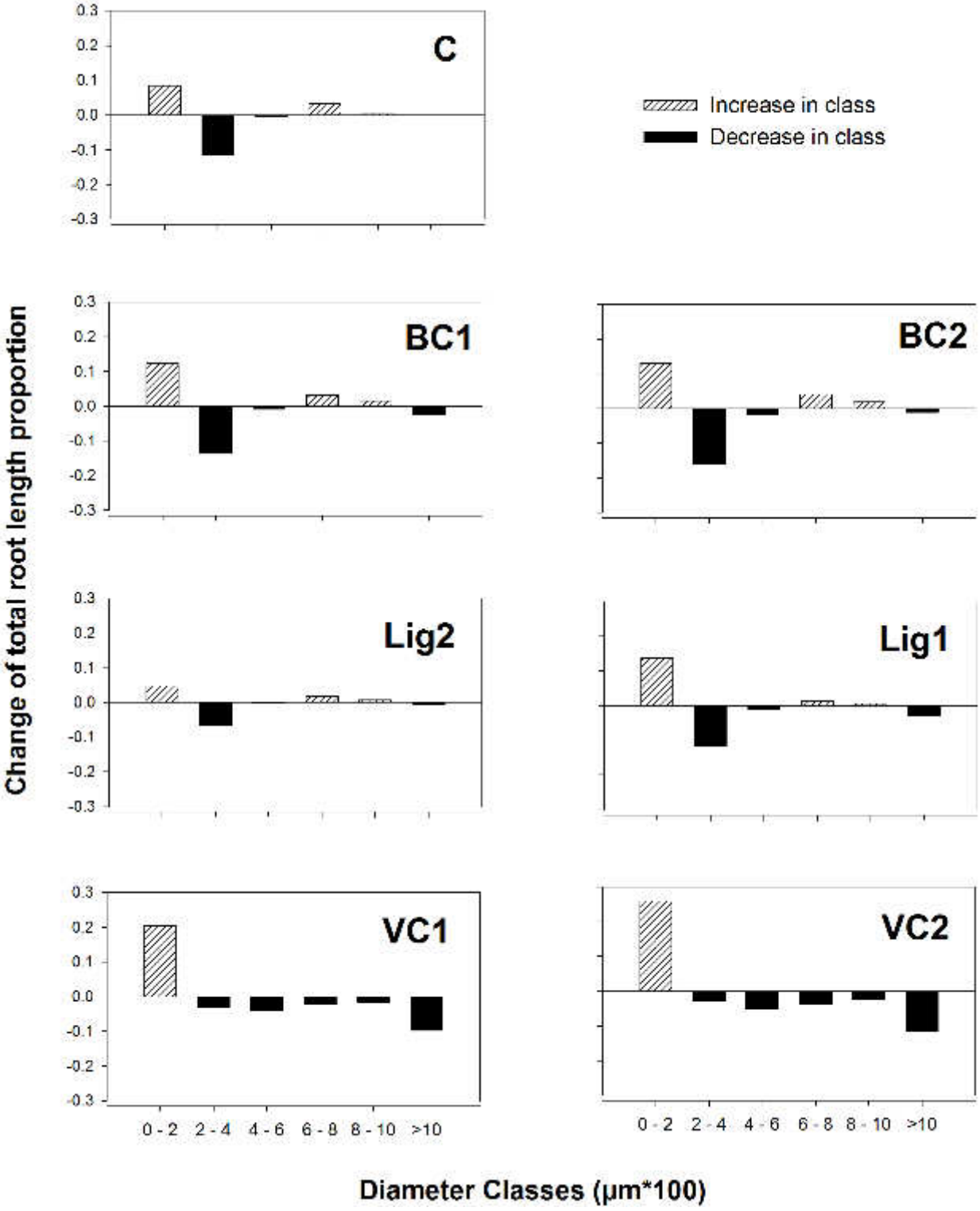

Figure 28: Changes of root length proportion (%) within each diameter class (mm) between 1st and 2nd harvest (i.e. after 9 and 12 weeks of growth).

3.2.4.3 Element Concentrations in Plant Tissue

3.2.4.3.1 Element Concentrations in Roots

Element concentration in maize roots varied considerably among treatments and harvests (Figure 29). In some samples (especially Pb, Mn and Fe) high SEM could be found.

Except for VC2, the Cd concentration in roots decreased in all treatments from the 1st (9 weeks of growth) to the 3rd harvest (14 weeks of growth). In contrast to other metalloid concentrations in maize shoots, the differences between the treatments were not significant for Cd after 14 weeks of growth ($p < 0.05$, Table 15).

The highest Zn concentrations in roots were measured in the C treatment followed by both VC, Lig and BC treatments. A significant increase of Zn concentrations in maize roots was only found in the VC2 treatment ($p < 0.05$, Table 16).

Mn concentrations in the roots of the C and BC1 treatment ranged from 100 to 480 mg kg^{-1} over the whole duration of the experiment. In contrast, Mn concentrations in the Lig1 and VC2 treatment increased by the factor 20 and 16 after 14 weeks of growth and reached 1670 and 3200 mg kg^{-1}, respectively. However, only in the VC2 treatment the increase of Mn concentrations in roots was significant ($p < 0.05$, Table 16).

Pb and Fe concentrations in the roots of maize showed an analogical development to Mn.

The S concentration in maize roots increased in Lig1 ($p < 0.05$, Table 16), Lig2 and VC2. After 14 weeks of growth, the highest S concentrations in roots were measured in Lig2 (6580 ± 624) and Lig1 (5744 ± 242) being approximately 30 % higher than in the other treatments.

P concentrations in roots were approximately 40 % higher in both VC compared to all other treatments. However, VC1 exhibited a decline from the 1st to the 3rd harvest, while VC2 showed steady increase in the P concentrations of maize roots.

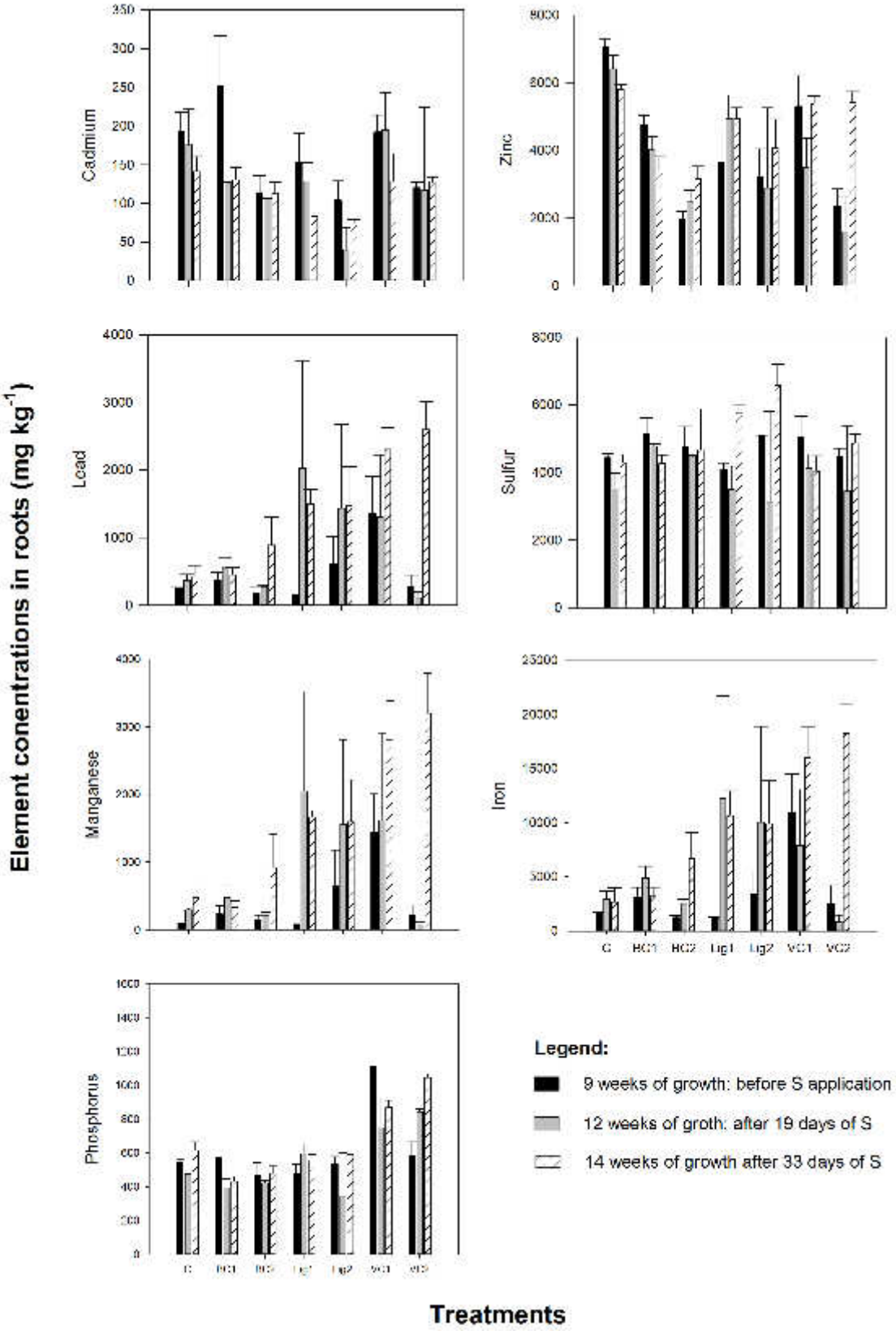

Figure 29: Metal (Cd, Zn, Pb, Mn, Fe), P and S concentrations (mg kg-1) in roots from three different dates before and after application of elemental S. Error bars indicate SEM (n = 2 for 9 and 12 weeks | n = 3 for 14 weeks).

Table 15: Influence of treatments on metal (Zn, Cd, Pb, Fe, Mn), P and S concentrations in roots of *Zea mays* after 14 weeks of growth (ANOVA, n = 3). Significant values are underlined (p < 0.05).

			ANOVA	
Parameter	Levene	df	F	sig.
Zn	0.59	6.00	5.97	0.003
Cd	0.15	6.00	2.12	0.115
Pb	0.34	6.00	6.02	0.003
Fe	0.53	6.00	5.63	0.004
Mn	0.31	6.00	6.19	0.002
P	0.88	6.00	38.13	0.000
S	0.14	6.00	3.53	0.024

Table 16: Influence of harvest dates (before S application, 19 days and 33 days after S application) on metal (Zn, Cd, Pb, Fe, Mn), P and S concentrations in the roots of *Zea mays* within each treatment tested by ANOVA or Kruskal-Wallis tests (1st and 2nd harvest n = 2; for 3rd harvest n = 3). Significant values are underlined (p < 0.05).

	Significance in treatment						
Parameter	C	BC1	BC2	Lig1	Lig2	VC1	VC2
Zn	0.105[b]	0.162[a]	0.160[a]	0.334[a]	0.807[a]	0.168[a]	0.019[a]
Cd	0.469[a]	0.098[a]	0.971[b]	0.048[a]	0.248[b]	0.438[b]	0.971[b]
Pb	0.688[a]	0.686[b]	0.350[a]	0.219[a]	0.743[b]	0.410[a]	0.048[b]
S	0.205[a]	0.267[b]	0.982[a]	0.048[a]	0.267[b]	0.387[a]	0.743[b]
Mn	0.481[a]	0.502[a]	0.383[a]	0.219[a]	0.619[b]	0.457[a]	0.014[a]
Fe	0.692[a]	0.458[a]	0.223[a]	0.339[a]	0.743[b]	0.371[a]	0.048[b]
P	0.657[b]	0.063[a]	0.734[a]	0.346[a]	0.657[b]	0.895[b]	0.004[a]
[a]	ANOVA						
[b]	Kruskal Wallis						

3.2.4.3.2 Element Concentrations in Shoots

Zn, Cd and to a less extent Pb, Mn and Fe concentrations in the shoots of maize shared a common pattern among treatments (Figure 30): The highest concentrations as well as a gradual increase of the respective metal concentration could be observed in the shoot tissue of the C and BC1 treatment. On the contrary, the other treatments (with some exceptions) exhibited a decline with proceeding sampling dates resulting in much lower shoot concentrations than for C or BC1. Metal concentrations (Zn, Cd, Pb, Mn and Fe) in shoots differed significantly between the treatments after 14 weeks of growth ($p < 0.05$, Table 17).

S concentrations in maize shoots increased in the C, BC and Lig treatments, however, only the increase in Lig1 and Lig2 was significant ($p < 0.05$, Table 18). Also the small decline of the S concentration in the shoots of the VC2 treatment proved to be significant ($p < 0.05$, Table 18). P concentrations in shoots decreased significantly in BC1, both Lig and VC treatments ($p < 0.05$, Table 18).

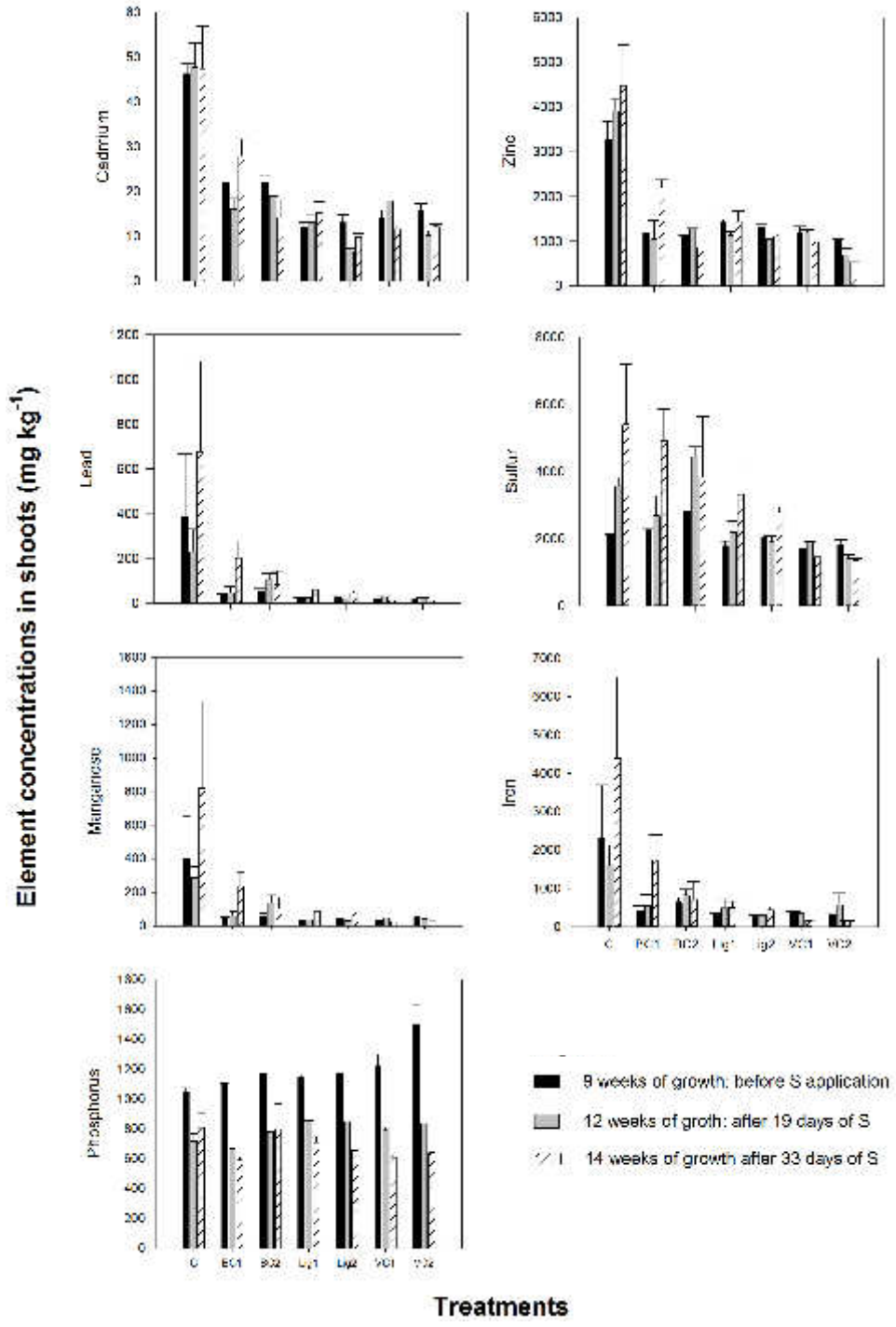

Figure 30: Metal (Cd, Zn, Pb, Mn, Fe), P and S concentrations in shoots (mg kg^{-1}) from three dates before and after S application. Error bars indicate SEM (n = 2 for 9 and 12 weeks | n = 3 for 14 weeks).

Table 17: Influence of treatments on metal (Zn, Cd, Pb, Fe, Mn), P and S concentrations in shoots of *Zea mays* after 14 weeks of growth tested by ANOVA or Kruskal-Wallis tests (n = 3). Significant values are underlined (p < 0.05).

			ANOVA		Kruskal Wallis	
Parameter	Levene	df	F	sig.	H	asym-sig.
Zn	< 0.05	6.00	-	-	16.96	0.009
Cd	0.23	6.00	9.74	0.000	-	-
Pb	< 0.05	6.00	-	-	15.97	0.014
Fe	< 0.05	6.00	-	-	15.97	0.014
Mn	< .05	6.00	-	-	15.72	0.015
P	0.46	6.00	72.23	0.000	-	-
S	< 0.05	6.00	-	-	14.89	0.021

Table 18: Influence of harvest dates (before S application, 19 days and 33 days after S application) on metal (Zn, Cd, Pb, Fe, Mn), P and S concentrations in the shoots of *Zea mays* within each treatment tested by ANOVA or Kruskal-Wallis tests (1st and 2nd harvest n = 2; for 3rd harvest n = 3). Significant values are underlined (p < 0.05).

	Significance in treatment						
Parameter	C	BC1	BC2	Lig1	Lig2	VC1	VC2
Zn	0.565^{a}	0.067^{b}	0.627^{a}	0.562^{b}	0.044^{a}	0.067^{b}	0.029^{b}
Cd	0.994^{a}	0.105^{b}	0.347^{a}	0.617^{a}	0.029^{b}	0.105^{b}	0.035^{a}
Pb	0.668^{a}	0.199^{a}	0.794^{a}	0.233^{a}	0.067^{b}	0.016^{a}	0.442^{a}
S	0.351^{a}	0.130^{a}	0.780^{a}	0.242^{a}	0.029a	0.050^{a}	0.033^{a}
Mn	0.658^{a}	0.153^{a}	0.694^{a}	0.196^{a}	0.042^{a}	0.002^{a}	0.174^{a}
Fe	0.549^{a}	0.245^{a}	0.949^{a}	0.971^{b}	0.302^{a}	0.067^{b}	0.067^{b}
P	0.132^{a}	0.029^{b}	0.214^{a}	0.002^{a}	0.001^{a}	0.029^{b}	0.029^{b}
^{a}	ANOVA						
^{a}	Kruskal Wallis						

3.2.4.4 Total Content of Metals (Zn, Cd, Pb) in the *S*hoots of *Zea Mays*

Total metal (Zn, Cd, Pb) contents in maize shoots were calculated by multiplying the shoot dry weight with the respective metal concentration in the shoot tissue. Subsequently, means for each treatment and harvest were calculated. Statistical tests (i.e. ANOVA or Kruskal-Wallis) showed that the treatments significantly influenced Zn and Cd but also S and P contents in the shoots of maize 14 weeks after sowing ($p < 0.05$ Table 19).

The highest total Zn contents in shoots were measured in the VC1 and VC2 treatment reaching almost 60 mg pot^{-1} after 14 weeks of growth (Figure 31). While the total Zn content in the shoots of the VC1 treatment increased from the 1st to the 3rd harvest, those of VC2 declined. In contrast, total Zn contents in shoots of all other treatments increased over time ($p < 0.05$ for Lig2, Table 20) but ranged only from 12.9 mg pot^{-1} (BC2) to 35.3 mg pot^{-1} (Lig1) after 14 weeks of growth. Total contents of Cd in maize shoots displayed a similar development, however, not only the increase in Lig2 but also in the BC2 treatment was significant ($p < 0.05$, Table 20).

After 14 weeks of growth, the highest total content of Pb was found in the shoots of the C treatment (3.23 ± 0.99 mg pot^{-1}) exceeding the Pb content of the other treatments by more than 100 % (Figure 31). Although total Pb contents in shoots of maize increased strongly (3 to 9 times) in both BC and Lig treatments over time, only the increase in Lig1 was significant ($p < 0.05$, Table 20).

Table 19: Influence of treatments on total metal content (mg pot^{-1}) in the shoots of *Zea mays* after 14 weeks of growth tested by ANOVA or Kruskal-Wallis tests (n = 3). Significant values are underlined (p < 0.05).

			ANOVA		Kruskal Wallis	
Parameter	Levene	df	F	sig.	H	asym-sig.
Zn	0.27	6.00	27.94	0.000	-	-
Cd	0.06	6.00	54.91	0.000	-	-
Pb	0.34	6.00	2.17	0.109	-	-
P	0.46	6.00	72.23	0.000	-	-
S	< 0.05	6.00	-	-	16.10	0.013

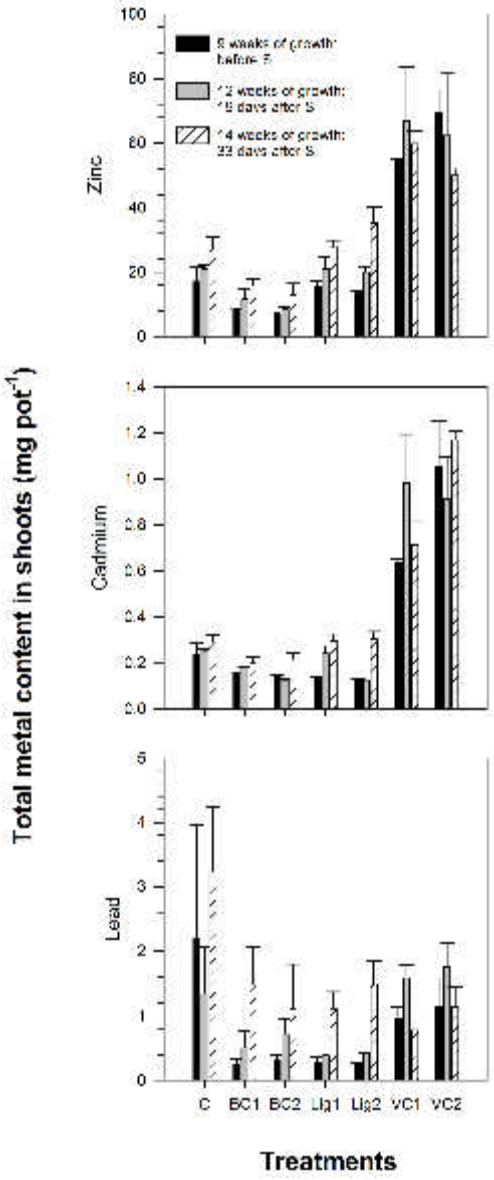

Figure 31: Total metal (mg pot^{-1}) content in the shoots of *Zea mays* for different harvests and treatments. Error bars indicate SEM (n = 2 for 9 and 12 weeks | n = 3 for 14 weeks).

Table 20: Influence of harvest dates (before S application, 19 days and 33 days after) on total metal contents (mg pot^{-1}) in the shoots of *Zea mays* within each treatment tested by ANOVA or Kruskal-Wallis tests (1st and 2nd n = 2; for 3rd harvest n = 3). Significant values are underlined (p < 0.05).

	Significance in treatment						
Parameter	C	BC1	BC2	Lig1	Lig2	VC1	VC2
Zn	0.209^{a}	0.155^{a}	0.619^{a}	0.052^{a}	<u>0.029</u>^{b}	0.971^{b}	0.524^{b}
Cd	0.599^{a}	0.467^{a}	<u>0.029</u>^{b}	0.105^{b}	<u>0.029</u>^{b}	0.324^{b}	0.362^{b}
Pb	0.657^{a}	0.251^{a}	0.637^{a}	<u>0.029</u>^{b}	0.065^{b}	0.089^{a}	0.474^{a}

^{a} ANOVA

^{b} Kruskal Wallis

3.2.5 Sulfur Balance

Except for VC2, total S contents in shoots clearly exceeded those in the roots (Table 21). However, the ratio of the total S content between roots and shoots increased with increasing biomass production (i.e. wide ratio in C and narrow ratio in VC2).

The total S content in soil was comprised of (i) the background content in Litavka soil (Table 3), (ii) the addition of elemental S (with a rate of 0.5 g S kg^{-1} soil) and (iii) the total S content in the additives. In the latter, huge differences could be found: while BC1 increased the total S content in soil by 0.29 g pot^{-1}, Lig1 (with the same application rate) increased the total S content in soil by 5.12 g pot^{-1}. The treatments Lig2, VC1 and BC2 showed the highest $CaCl_2$-extractable S-SO_4 contents ranging from 1.62 to 2.37 g pot^{-1}, followed by Lig1, VC2, BC1 and C.

Table 21: S balance with different S components calculated per pot and for the 3rd harvest.

		Total content (g pot^{-1}) in treatment						
Balance Component		**C**	**BC1**	**BC2**	**Lig1**	**Lig2**	**VC1**	**VC2**
Plant uptake	Roots	0.008	0.008	0.021	0.042	0.064	0.078	0.139
	Shoots	0.031	0.036	0.056	0.063	0.088	0.090	0.130
	Total	0.039	0.044	0.077	0.105	0.152	0.168	0.269
Total S in soil	Soil Litavka	---------- 1.49 ----------						
	S application	---------- 2.00 ----------						
	S in additive	0.00	0.29	0.58	5.12	10.33	2.01	4.03
	Total	3.49	3.78	4.07	8.61	13.82	5.50	7.52
$CaCl_2$ extr. S	S-SO_4	0.77	0.90	1.62	1.36	2.37	2.00	1.25
	Total extractable	0.43	0.98	2.31	1.74	2.54	2.36	1.17

4. Discussion

4.1 Effect of Additives on the Immobilization of Metals (Cd, Zn, Pb)

The different effects of the used additives on soil pH_{CaCl_2} (Figure 10 and 23) can be attributed to differences in the treatments' chemical composition: Lig has a high humic acid content of about 45 % (MIBRAG, 2013) and also VC contains humic acids (Aguiar et al., 2010; Arancon et al., 2006) lowering soil pH. In contrast, BC exhibits numerous functional groups as for instance hydroxyl (-OH), aliphatic or ester groups depending on the conditions of pyrolysis (e.g. temperature and oxygen concentration) (Chun et al., 2004; Zheng et al., 2010). Similar to our findings, Beesley et al. (2011, p. 3275) reported a “liming effect” of biochars, especially when applied to (slightly) acidic soils.

At day 40 of incubation, both BC treatments showed the lowest increase in proton activity between the samples without S and 1.5 g S kg^{-1} (Table 9) but their $CaCl_2$-extractable concentrations increased the most (Table 9, Figure 12 and Figure 14). In contrast to this, both Lig treatments exhibited the largest increase in proton activity (between the samples with S and 1.5 g S kg^{-1}) but a lower increase in the extractable Zn and Cd concentrations compared to BC. Therefore, we suggest that the increase in $CaCl_2$-extractable metal concentrations after S addition does not solely depend on the change in proton activity (due to the oxidation of S to sulfuric acid) but also on the immobilization mechanism which can be predominantly found in the used soil amendment. Immobilization of Cd and Zn in BC-amended treatments may be owed to the higher pH and the corresponding adsorption of Zn and Cd to the large specific surface of BC being reversed when proton activity increases. In contrast, complexation of Cd and Zn by organic matter could be the predominant immobilization mechanism for Lig and VC thus being more resistant to an increased proton activity (Pusz, 2007; Sklodowski et al., 2006).

Various researches showed that several compost and carbon amendments (as coal for instance) are able to efficiently decrease extractable Pb concentrations in soil (Fellet et al., 2011; Janoš et al., 2010; Pusz, 2007). However, compared to the control, we measured higher $CaCl_2$-extractable Pb concentrations in VC and Lig treatments in both incubation and pot experiment (Figure 16 and Figure 23). Since total Pb concentrations in Litavka soil

reach 3300 mg kg^{-1} (Table 3), those of the amendments cannot be the reason for the increase in Pb extractability: in 20 g of Litavka soil (i.e. the weight of soil in one incubation bottle) approximately 66 mg of Pb were contained, while only 0.0036 mg Pb were additionally added to the sample by the application of lignite (calculated for the application rate 90 g kg^{-1}) (MIBRAG, 2013).

Beesley et al. (2010) found that the application of greenwaste compost and biochar decreased the water-extractable Zn and Cd concentrations due to their liming effect (i.e. pH increase). In contrast, Cu, As and Pb water-extractable concentrations increased by the application of the respective amendments. The authors suggest that the amount of water soluble Zn and Cd are predominantly controlled by pH while for Cu, As and Pb the increase in DOC due to compost and biochar application may be the governing factor. Clemente and Bernal (2006) focussed on the immobilization effect of humic acids (isolated from compost and peat) on the different fractions of Zn, Pb, Cu, Fe and Cd. Similar to our findings, the $CaCl_2$-extractable Pb concentrations increased significantly during the incubation. We suggest that the elevated $CaCl_2$-extractable Pb concentrations in the BC, Lig and VC treatments can be attributed to the high organic matter content of the additives. This may have increased DOM as well and led to the increase in Pb extractability by the two following processes (Jordan et al., 1997): (i) DOM complexes Pb thus inhibiting its adsorption to soil; (ii) DOM (and complexes of DOM with metals) compete with Pb for non-specific adsorption sides.

Vondráčková et al (2013) investigated the immobilization potential of quick lime and dolomite when applied to Litavka soil (same sampling location) with rates of 15, 30 and 60 (g kg^{-1}) using a similar experimental design (incubation). While the $CaCl_2$-extractable (0.01 M) concentrations of Zn, Pb and Cd in the dolomite-amended treatments were comparable to our measured concentrations, those in the lime-amended treatments were three times lower for Zn, 15 times lower for Cd but 20 higher for Pb. This illustrates that BC, VC and Lig can be an efficient alternative for the frequently used lime and dolomite immobilization agents.

4.2 Remobilization of Metals due to S Oxidation

One of the main targets of this thesis was to evaluate whether the application of elemental S after a preceding immobilization period leads to a steady decline of soil pH and mobilization of metals (foremost Zn, Cd and Pb). Depending on the experiment (incubation or pot), different answers have to be given.

In the **incubation experiment**, $CaCl_2$-extractable concentrations of Zn, Cd and Pb did not only significantly increase when elemental S was added but were also significantly affected by interactions of treatments and S rate (Table 8). The application of elemental S manipulated the correlation between pH_{CaCl_2} and $CaCl_2$-extractable Zn, Cd and Pb concentrations: S-amended samples did not only show increasing $CaCl_2$-extractable metal concentrations with decreasing pH but also a steeper slope of the exponential regression curve could be identified (Figure 18). Precedent results of Iqbal et al. (2012) and Höfer (2013) showed a similar influence of the addition of elemental S to soil on metal concentrations, however, these measurements were made in the soil solution. The increase in soluble metal concentrations was attributed to the reduction and dissolution of Mn oxides (Eq. 2) under locally-occurring anaerobic conditions in the rhizosphere of *Salix smithiana* leading to the co-dissolution of Cd, Zn and other metals. In our incubation experiment, no plants were involved. Therefore, no rhizosphere effect could have influenced microbial populations. However, the incubation bottles were only aerated every three days. Between those intervals microbes may have consumed oxygen (due to respiration and oxidation of S) and used Mn or Fe oxides as electron acceptors. By comparing the C treatments (without S application) to the S-amended treatments, Iqbal et al. (2012) measured an increase in soluble metal concentrations of 10 to 20 times (for Zn) and 10 to 40 times (for Cd). In contrast, we found an increase in $CaCl_2$-extractable Zn concentrations of 8 to 41 times and a respective increase for Cd of 6 to 14 times.

The evaluation of the intended S oxidation and metal mobilization is more difficult in the **pot experiment** since inconsistencies between the results of the soil solution sampling and $CaCl_2$ extraction occurred (Figure 21, Figure 23 and Figure 24). Naturally, the element concentrations measured in the soil solution and $CaCl_2$ soil extraction differ from each other concerning the order of magnitude, however, a consistent trend in both methods should exist. The $CaCl_2$ soil extraction method (using low molar concentrations of $CaCl_2$) is

based on the dissociation of $CaCl_2$ in demineralized water leading to the exchange of Ca^{2+} ions from the solution with protons (or other cations) attached to soil particles. Therefore, a slightly lower pH and a faster equilibration are associated with this method. Nonetheless, these methodological differences cannot explain the different results of the soil solution sampling and the $CaCl_2$ extraction. Both soil solution sampling and $CaCl_2$ extractions were taken approximately at the same date (+/- 1 day) but while the soil extraction samples were refrigerated and analysed within few hours, soil solution samples were cooled and stored for 12 h before the analysis, which could have influenced the measurements.

Despite the described differences, only few changes in pH and metal concentrations proved to be significant in both soil solution and $CaCl_2$ extraction over time (Table 10 and Table 11). For this reason, we conclude that the S oxidation and mobilization of metals (Zn, Cd, Pb, Mn and Fe) in the C, BC and VC treatments was limited in the pot experiment. This finding is supported by the results of the exponential regressions of $CaCl_2$-extractable metal concentrations (Zn, Cd, Pb, Mn) and pH (Figure 25): in contrast to our incubation experiment and the findings of Iqbal et al. (2012) and Höfer (2013), the addition of S had no strong effect on the correlation between pH and metals. In fact, the regression curve of the $CaCl_2$-extractable Mn concentrations and pH shifted downwards after S addition indicating that at same pH values even lower Mn concentrations were measured.

However, an exception can be seen in the Lig2 treatment since Zn and Cd concentrations in the soil solution sampling did not change over time (Figure 21, Table 10) but $CaCl_2$-extractable concentrations of Zn, Pb, Cd, Mn, sulfate and pH increased significantly with proceeding sampling dates (Figure 23, Figure 24 and Table 11). Even the total metal content of Zn and Cd in maize shoots rose significantly (Table 20). Similar results could be found for the Lig1 treatment in the pot experiment, however, only $CaCl_2$-extractable sulfate and Zn concentrations rose significantly over time (Table 11).

According to Ruamsap & Akaracharanya (2002) and Hagedorn (2010), the S pool in brown coals can be classified into two fraction: (i) inorganic S consisting mainly of sulfate and pyritic S and (ii) organic S bound to carbon (C-S) or esters (C-O-S). While inorganic S can be easily accessed by microbes (as for instance S oxidizing or reducing bacteria), the availability of organic S compounds strongly depends on the integrity of the coal and the “release of S from ester sulfates through extracellular enzymatic hydrolysis” (Churka Blum

et al., 2013, p. 157). Therefore, the milling of Lig (and also of other amendments) before mixing with soil could have increased the microbial availability of the organic S fraction since its integrity (i.e. structure) was destroyed (Ruamsap and Akaracharanya, 2002). When incubating four different plant residue amendments and a corn starch biochar with an Oxisol for 90 days, Churka Blum et al. (2013) proved that microbial S transformation in soil was strongly influenced by the starting material (i.e. amendments and soil): not the total S content in the amendments determined S mineralization (since the additives were normalized to the same S content), but their composition of different S forms. Hence, we suggest that easy accessible S forms in Lig (and to a less extent in the other amendments) functioned as driver for S oxidation thus stimulating S oxidizing bacteria probably even before S (in a rate of 0.5 g kg^{-1}) was applied to soil. This could explain the increase in $CaCl_2$-extractable sulfate concentrations in the Lig, BC2 and VC treatments, while in the control no notable change was measured after S addition (Figure 24). Moreover, the high sulfate concentrations in the leachate samples of BC, Lig and VC treatments support our findings being 5 to 12 times higher than in the control (Figure 22).

The oxidation of inorganic pyrite in brown coal is performed by *Thiobacillus ferroxidans* and frequently used in industrial desulfurisation (bio-leaching) of coal (Ruamsap and Akaracharanya, 2002): in acidic, aerobic environments (i.e. pH 2 - 4) ferrous iron is first oxidized to ferric iron and subsequently used as electron acceptor for the oxidation of pyrite to elemental S and further on to sulfate:

$$4FeSO_4 + O_2 + 2H_2SO_4 \rightarrow 2Fe_2(SO_4)_3 + 2H_2O \qquad \text{(Eq. 3)}$$

$$Fe_2(SO_4)_3 + FeS_2 \rightarrow 3\,FeSO_4 + 2S^0 \qquad \text{(Eq. 4)}$$

Theoretically, S-oxidation can also occur under anaerobic conditions in which *Thiobaccili spp.* use ferric iron as electron acceptor (Pronk et al., 1992; Sugio et al., 1985):

$$S + 6Fe^{3+} + 4H_2O \rightarrow H_2SO_4 + 6Fe^{2+} + 6H^+ \qquad \text{(Eq. 5)}$$

Similar to the examples above, different S bacteria may have used lignite-supplied S groups and oxidized them to elemental S, thiosulfate and sulfate . In general, S oxidation

occurs within a wide pH range of 1.9 to 8.5 and is performed by numerous species (Hagedorn, 2010) depending on the environmental conditions (Table 22). Heterotrophic and mixotrophic S oxidizing bacteria may have benefited from the increased availability of organic carbon contained in BC, VC and Lig improving their growth conditions (Graff and Stubner, 2003). However, the influence of organic matter input on S oxidizing bacteria is discussed controversially in literature: Wainwright et al. (1986) studied the influence of wheat straw and sugar beet pulp on S oxidation: after an initial stimulation of S oxidizing bacteria and an increase of LiCl-extractable sulfate concentrations, a decline below the sulfate concentrations of the control was observed at the end of the incubation period (after 7 weeks). In contrast, Karimi et al. (2012) found significant interactions between cattle manure and S application to soil due to the stimulation of S oxidizing bacteria.

For future researches, the testing of further brown coals and their impacts on the combined phytoremediation approach could be promising, since our results suggest that lignite may have beneficial impacts on both the immobilization period (leading to higher biomass production) and the subsequent mobilization (by facilitating S oxidation).

Table 22: Examples for mixotrophic, autotrophic and heterotrophic colourless S bacteria (Hagedorn, 2010; Maheshwari, 2011)

Classification	Species	pH	Motility	Temperature
Mixotrophic	*Thiobacillus novellus*	6 - 8	-	25 - 30
	Thiobacillus versutus	6 - 8	+	30 - 35
	Thiobacillus acidophilus	2 - 4	+	25 - 30
Autotrophic	*Thiobacillus thioparus*	6 - 8	+	25 - 30
	Thiobacillus ferroxidans	2 - 4	+	30 - 35
	Thiomicrospira denitrificans	7	-	20 - 25
Heterotrophic	*Thiomonas perometabolis*	2 - 9	+	30 - 35
	Beggiatoa spp.	6 - 8	+	varying

The deviant outcomes of the incubation and pot experiment raise the question which differences in the experimental designs might have influenced microbial processes in the rhizosphere of maize plants. Among several factors (Table 23), the differences concerning the application of S may have played a major role: while S and soil could be mixed

homogeneously in our incubation as well as in previous experiments of Höfer (2013) and Iqbal et al. (2012), the subsequent S application in the pot experiment was restricted to the area above the root zone of maize (~8 cm depth). This may have diminished the contact surface for S oxidizing bacteria substantially. Since elemental S is highly water insoluble, some S oxidizing bacteria as *T. thiooxidans* use a hydrophobic wetting agent (consisting of phospholipid and fatty acids) to initiate adhesion to the S surface (Beebe and Umbreit, 1971; Schaeffer and Umbreit, 1963). When S is present in too high amounts, heterogeneously distributed in soil or insufficient wetting agents are present, S oxidation is inhibited (Cook, 1964).

In further experiments, the application method for elemental S should be improved to guarantee both a homogeneous and subsequent application.

Table 23: Differences between incubation and pot experiment and their potential impacts.

		Comparison		
	Experiment parameter	**Incubation**	**Pot experiment**	**Potential impacts on**
Abiotic	Temperature (°C)	25 (constant)	Depending on weather	Microbial activity
	Water content (%)	60 (of mWHC[a])	Varying	Eh[b] and S oxidation
	O_2 supply	Frequent aeration	Diffusion	
Design	Mixing of S	Homogeneous	Heterogeneous	Accessibility for microbes
	Watering	None	Daily	Eh
	Fertilization	None	Beginning	
	S rates (g kg^{-1})	No-S, 0.5, 1.5, 2.5	0.5	
Biotic	Experimental plant	None	*Zea mays*	O_2 concentration, microbial activity, element uptake

[a] maximum water holding capacity [b] Redox potential

While aeration was supplied manually in the incubation experiment, oxygen supply in the pots depended on diffusion through the soil pores. Respiration of maize roots and microbes in the rhizosphere, the influence of the amendments on soil physical properties

(i.e. hydraulic conductivity) and daily watering may have caused oxygen depletion and thus limited S oxidation (Zhou et al., 2002).

In contrast to Iqbal et al. (2012), the addition rates of VC, Lig and BC were almost twice as high (Table 24). Since we observed frequent water logging and increased soil compaction in the pots of the BC treatments, we suppose that instead of local anaerobic patches, whole areas with oxygen depletion could have occurred. While Höfer (2013) supplied water to the rhizoboxes with glass fibre twigs and Iqbal et at. (2012) watered every two or three days, we supplied water on a daily basis reinforcing the formation of anaerobic conditions.

Table 24: Differences between Höfer (2013), Iqbal et al. (2012) and our experiment concerning plants, experimental design and amendments

		Comparison		
	Experiment parameter	**Höfer (2013)**	**Iqbal et al. (2012)**	**Our experiment**
Plant	Species	---- *Salix* x *smithiana Willd*. ----		*Zea mays*
	Plants per pot	1	1	3
	Growth period (days)	61	160	98
Design	Environment	--------- Greenhouse ---------		Outside
	Array	Rhizobox	Pot	Pot
	Amount of soil (kg)	0.6	2.5	4.0
	Rhizosphere compartment	Yes	-	-
	Water supply	Wicks	2 – 3 days	Daily
Immobilization	Amendment	-	RM[a] and GS[b]	BC, Lig, VC
	Rate (g kg^{-1})	-	50 (25 for each)	45 and 90
	Material	-	Inorganic	Organic
Mobilization	Rate (g kg^{-1})	0.51 and 1.02	1.02 and 1.82	0.5
	S addition time	Beginning	Beginning	After 60 days
	Mixing of S	Homogeneous	Homogeneous	Heterogeneous

[a] Red mud

[b] Gravel sludge

Although the experimental soils used by Höfer (2013), Iqbal et al. (2013) and our experiments have comparable textures (Litavka soil and PR2: sandy loam; ARNB: loam), the soil type differs substantially: while ARNB (used by Höfer and Iqbal) is an Eutric Cambisol, Litavka soil can be classified as Gleyic Fluvisol (eutric) according to the IUSS Working Group WRB (2006). Litavka soil has undergone several flood events in the past (Boruvka and Vacha, 2006; Mikanova, 2006; Vanek et al., 2008) and the microflora may have adapted to the periodic change of aerobic and anaerobic conidtions. Hence, different redox pathways may occur compared to the soils used by Höfer (2013) and Iqbal et al. (2012).

If the redox potential was sufficiently lowered by oxygen depletion in the maize rhizosphere, even occassional microbial reduction of sulfate could have occured. According to Husson (2012, p. 398) sulfate reduction is found in “waterlogged and submerged fields“ and requires a pH about 6 and Eh of -100 mV. The input of organic matter (in form of the amendments) may have further decreased the redox potential in the pots (Figure 32), since organic matter is considered as most reduced compound in soil representing an important source of electrons (Chesworth, 2004; Husson, 2012; Kijjanapanich et al., 2014). By reducing sulfate, heterotrophic sulfate reducing bacteria may have oxidized organic compounds according to the following generic equation leading to a increase in pH (Chesworth, 2004; Sawyer et al., 2003):

$$SO_4^{2-} + organic\,matter \rightarrow HS^- + CO_2 + H_2O \qquad \text{(Eq. 6)}$$

Hence, the precipitation of metals with sulfides could have limited the metalloid concentrations in the soil solution sampling. Weber et al. (2009) found a strong decrease of Cd and Pb concentration due to precipitation with sulfide (using a sequential extraction procedure). Similar to Litavka, a Gleyic Fluvisol (texture: silty loam) was used and sulfate concentrations were monitored during an artifically created flood regime. Furthermore, a pH increase from 5.7 to 6.7 and the dissolution of Fe(III) and Mn(IV,III) oxides were observed. The typical sequence of electron acceptors under anaerobic conditions commences with oxygen (until its complete depletion or the occurrence of anaerobic patches) and continues with nitrate, Mn(IV) and Fe(III) as electron acceptors. Not until then sulfate is consumed as electron acceptor (Brümmer, 1974; Nguyen, 2009). This stays in contrast to our measurements, since apart from a few exceptions in the Lig and VC2

treatments, a significant increase in the Fe or Mn concentrations could be neither found in the soil solution sampling nor in the $CaCl_2$ extraction (Table 10 and Table 11).

In future researches, redox potential changes in the maize rhizosphere should be regularly monitored to test the possibility of microbial sulfate reduction when using Litavka soil and organic matter containing amendments.

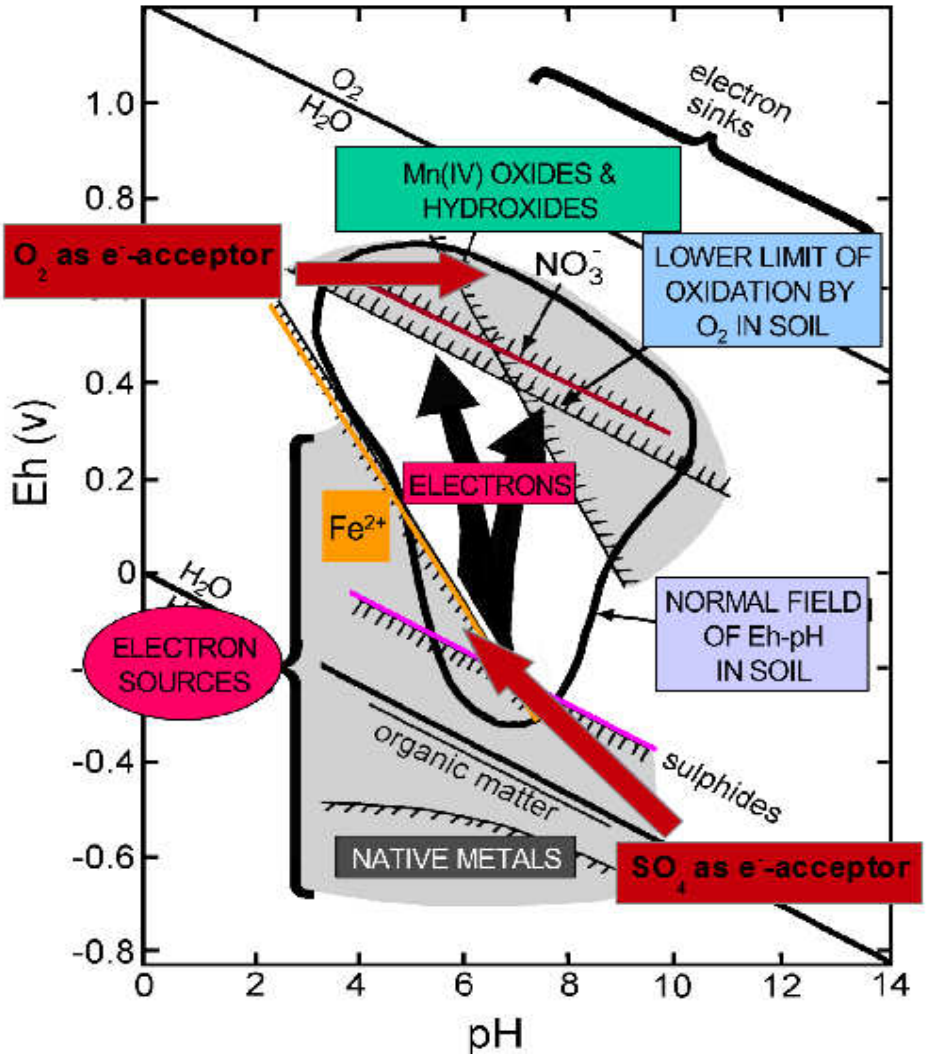

Figure 32: Pourbaix diagrammes for various electron donors and acceptors in soil (Chesworth 2004, p.40)

4.3 Nutrient Availability, Plant Responses and Phytoremediation Efficiency

Three weeks after sowing, BC and Lig treatments showed the lowest $CaCl_2$-extractable concentrations of Mg, K, Fe and DOC (Figure 20), which can be attributed to the adsorption of cations (and DOC) to the large specific surface of BC and Lig (Beesley et al., 2011; Zheng et al., 2010). Pusz (2007) showed that the CEC of two experimental soils increased with increasing addition rates of brown coal. Besides adsorption, also the complexation of metals at the surface of BC and Lig may have decreased the extractability of the respective elements (Beesley et al., 2011). Despite the lowest Zn, Cd and Pb concentrations in both soil solution and $CaCl_2$ extraction (Figure 21 and 23), BC treatments showed a substantially lower biomass production than Lig and VC (Figure 26). Therefore, we conclude that the application of VC and Lig led to a steady release of nutrients, while in BC sorption mechanisms decreased the availability of nutrients during the whole experimental period and thus limited plant growth.

$CaCl_2$-extractable nitrate concentrations decreased in the C and BC treatments after S was applied to soil (Figure 24). Similar to our results, Höfer (2013) found a significant decrease of soluble nitrate concentrations in S-amended treatments. The decline was attributed to locally occurring anaerobic conditions (i.e. inhibiting nitrification), plant uptake and microbial denitrification. Hagedorn et al. (2010) reported, that some facultative *Thiobacillii* are capable of denitrification under anaerobic conditions. The respective microbes use nitrate as terminal electron acceptor and reduce this compound subsequently to NO_2^- and after further intermediates to N_2 (Maheshwari, 2011). In contrast, $CaCl_2$-extractable nitrate concentrations in Lig and VC treatments increased over time, although plant uptake was likely to be higher than in the C and BC treatment (due to the higher biomass production). The application of VC and Lig may have influenced the mineralization of N leading to a sustained release from organic compounds. This is in line with findings from Murugan & Swarnam (2013) who demonstrated the slow but steady mineralization of N in vermicompost-amended soil.

According to the results of the P extraction using Olsen's method (Figure 19), no indication for a low P availability for plants could be found: while 10 mg kg^{-1} Olsen-extractable P can be considered as optimum for plants (Pierzynski, 2000), the lowest measured extractable P concentration amounted 21.9 mg kg^{-1}. This stays in contrast to the reddish color of

leaves in the maize plants of the C, BC and Lig treatments (Figure 26) indicating a P deficiency (Nagajyoti et al., 2010). Since Litavka soil has high total concentrations of Zn and Pb, precipitation could have limited the availability of P (Nagajyoti et al., 2010; Schachtman et al., 1998). Therefore, we calculated the ionic products of $Zn_3(PO_4)_2$ and $Pb_3(PO_4)_2$ using the measured Zn, Pb and P concentrations from the soil solution sampling (Figure 21) and compared them with the solubility products obtained from ChemBuddy (2010). Since we only measured the <u>total</u> P concentrations in the soil solution and leachate, no information about the speciation of soluble P are available: besides inorganic dissolved P (i.e. orthophosphate), also dissolved organic P species (e.g. P esters) could be present in considerable amounts (McDowell and Koopmans, 2006; Ron Vaz et al., 1993). Chapman et al. (1997) investigated the speciation of P in the leachate and soil solution of a temperate grassland soil which had a similar texture (sandy loam) but a higher organic carbon content (45 g kg^{-1}) as Litavka soil (36 g kg^{-1}). While the inorganic orthophosphate concentrations in the leachate represented 71 % of the total dissolved P concentration, those in the soil solution represented only 54 %. According to these findings, we assumed that total dissolved P in our measurements is comprised of 50 % inorganic orthophosphate in the soil solution and 70 % in the leachate. For the calculation, the following equations and solubility products (K_{sp}) were used:

$$Zn_3(PO_4)_2 \rightarrow 3\mathrm{Zn}^{2+} + 2\mathrm{PO}_4^{3-} \qquad \text{(Eq. 7)}$$

$$K_{sp} = 10^{-32} mol^5 L^{-5} = [Zn^{2+}]^3 [PO_4^{3-}]^2 \qquad \text{(Eq. 8)}$$

$$Pb_3(PO_4)_2 \rightarrow 3\mathrm{Pb}^{2+} + 2\mathrm{PO}_4^{3-} \qquad \text{(Eq. 9)}$$

$$K_{sp} = 10^{-43.5} mol^5 L^{-5} = [Pb^{2+}]^3 [PO_4^{3-}]^2 \qquad \text{(Eq. 10)}$$

The ionic product of the Zn and phosphate concentrations in soil solution and leachate exceeded the solubility product by several orders of magnitude in all treatments (Figure 33). Hence, precipitation of Zn phosphate may have strongly limited the availability of P. In contrast, precipitation of Pb phosphate may have only been present in the soil solution of the C, Lig and VC treatments.

The Olsen method is based on the extraction of P with 0.5 M $NaHCO_3$ and the adjustment of the pH to 8.5. However, at such high pH values, Zn and Pb are strongly sorbed to the soil matrix, which was demonstrated by the regressions between pH and the $CaCl_2$-extractable concentrations of Zn or Pb (Figure 25). Hence, the use of Olsen as P extraction method for highly contaminated Litavka soil may overestimate the amount of plant available P, since the formation of metal phosphates may be disabled due to the high pH involved in this method.

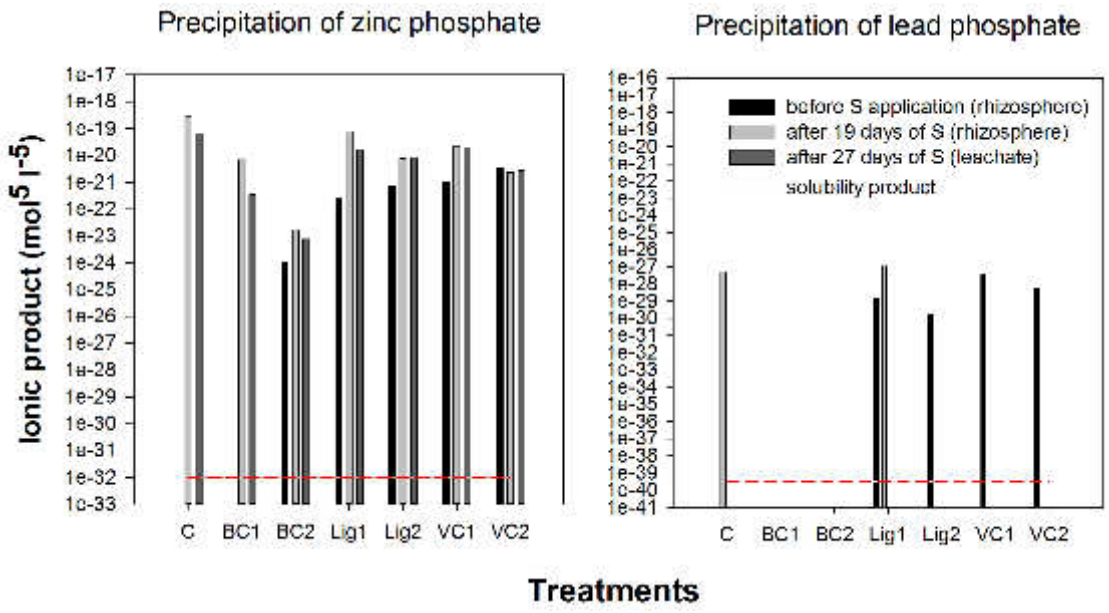

Figure 33: Solubility and ionic products of zinc phosphate and lead phosphate calculated for different sampling times of the soil solution and leachate sampling.

In general, distinguishing between toxicity symptoms and deficiencies was rather difficult during the whole experiment. Certainly, a combination of both occurred: while P and K deficiencies might have reduced plant growth, the high $CaCl_2$-extractable concentrations of Zn, Cd and Pb induced plant stress and a poor performance for BC and C. After 13 weeks of growth, maize plants of all treatments showed plant stress symptoms (Figure 27): besides the Zn-induced P deficiency (see above), we found tapering leaves and a reduction in the leaf area as a consequence of Zn toxicity (Reichmann, 2002; White, 2012). These findings can be supported by comparing the measured extreme concentrations of metals (Zn, Cd, Pb, Fe, Mn), S and P in the shoot tissue of maize with the sufficiency ranges recommended in literature (Table 25). Zn, Cd and Pb concentrations

in the maize shoots of all treatments lay within the toxicity range, however, those in the control were always the highest. Mn and Fe concentrations in shoots lay within the sufficiency range for both VC treatments but within the toxicity range for the control. P concentrations in shoots indicate a deficiency for the plants of all treatments.

Table 25: Sufficiency and toxicity levels of metals (Zn, Cd, Pb, Fe, Mn), S and P compared to measured extreme values in the shoots of maize in all harvests.

Element	Sufficiency level (mg kg^{-1})	Toxicity level (mg kg^{-1})	Occurring extreme concentrations in shoots	
			Min (mg kg^{-1})	Max (mg kg^{-1})
Zn[1]	15 - 30	100 - 700	520 (VC2)	4480 (C)
Cd[1]	-	5 - 10	6.50 (Lig2)	47.7 (C)
Pb[1]	-	10 - 20	11.8 (VC2)	679 (C)
Fe[1]	50 - 250	>500	140 (VC2)	4410 (C)
Mn[1]	10 - 20	200 - 5300	24.1 (VC1)	821 (C)
S[2]	1500 - 5000	-	1350 (VC2)	5420 (C)
P[2]	3000 - 5000	-	591 (BC1)	1500 (VC2)

[1] data from White et al. (2012) [2] data from A&L Great Lakes Laboratories (2009)

As shown by Carlvalhais et al. (2011), Fe-deficient maize plants exudate an increased amount of citrate. In accordance to these findings, the citrate concentrations in the leachate were clearly increased for the C, BC and Lig treatments compared to VC (Figure 22). The total concentration of Fe in Litavka soil is very high, however, plant uptake may be restricted by an antagonism with Zn (occuring at high concentrations). This phenomenon was already described by Reichmann (2002) and also other experiments of our institute using willows showed a high Fe deficiency (unpublished yet).

Oxalate is a strong chelating agent and usually exudated in response to Al toxicity. Ma and Miyasaka (1998) proved the ameliorative effect of oxalate exudation for *Taro*: the addition of oxalate increased the relative root length of *Taro* when grown in Al polluted soil. Albeit, exudation of oxalate in response to Zn is less explored, Li (2012) proved an increased exudation of oxalic acid by Chinese cabbage when grown in a Zn polluted soil. Similar to these results, maize plants in our pot experiment may have exudated oxalate in response to very high Zn concentrations in the soil solution (Figure 22).

The increase of the total root length in Lig and VC treatments after S application can be attributed to less plant stress and toxicity symptoms in those treatments compared to C or BC1 and BC2 (Table 13). Moreover, an increase in the total root length proportion in the diameter classes 0.6 – 0.8 mm and 0.8 – 1.0 mm was measured in the roots of the C, BC and Lig treatments (Figure 28): this thickening of roots is commonly reported in literature as toxicity response symptom (Arduini et al., 1995; Reichmann, 2002). Probst et al. (2009) proposed that an enzymatic induced thickening of root cell walls may restrict the absorption of metals in the roots of *V. faba L*. Lux et al. (2011) reviewed that Cd polluted soils induce changes in the root morphology leading to a decrease in root length up to 50 %. Similar findings were made by Sen et al. (2013) observing reduced root length of Indian mustard in a Cd and Pb contaminated soil, which is in line with our results.

Due to high concentrations (both total and $CaCl_2$-extractable) in Litavka soil, large amounts of metals (Zn, Cd, Pb, Mn and Fe) could be transported to and taken up by the roots of *Zea mays* (Figure 29). Since maize belongs to the group of strategy II plants (Ignatova et al., 2000), the exudation of phytosiderophores is used for the chelation of Fe^{3+}. Subsequently, those complexes can be taken up as a whole by special transporters, so called yellow-stripe 1 proteins (Curie et al., 2008). Similarly, other metals such as Zn, Ni, Cd, Cu and Mn can be chelated by phytosiderophores and then taken up at higher concentrations (von Wirén et al., 1996; White, 2012). Another important transporter group are the so-called Zn-regulated transporters and iron-regulated transporter-like proteins (ZIP). Purposely, ZIPs take up Fe, Mn, Ni, Cu or Zn, but also Al, Cd, Hg, Pb (Llamas et al., 2008). When plants experience stress in form of toxicity or deficiency, membrane selectivity may deteriorate leading to an uncontrolled uptake of metals. Moreover, the break down of the Caspian Stripe under high toxicity stress (Pourrut et al., 2011) may have caused an excessive uptake of Pb thus explaining the high Pb concentrations in the shoots of the C treatment (Figure 30).

In general, metal (Zn, Cd, Pb, Mn, Fe) concentrations in the shoots of maize were the highest in the treatments with low biomass (i.e. C and BC) and the lowest in the treatments with the highest biomass production (Figure 30). Hence, we conclude that biomass production caused a dilution effect for the respective metal concentrations in the shoots of maize.

Phytoremediation efficiency could be substantially increased in the VC and to a less extent in the Lig treatment, since the total Zn and Cd content in the shoots of maize were higher than in the C treatment (Figure 31). However, different conclusions concerning the success of S aided mobilization have to be drawn: while the total content of Zn and Cd in the maize shoots of the VC treatments were not significantly affected by the application of S, those of the Lig2 treatment increased significantly (Table 20) but were more than 50 % lower than in the VC treatments. Therefore, we conclude that rather the effective immobilization and provision of nutrients in the VC treatments than the oxidation of elemental S increased the efficiency of phytoremediation. Moreover, a subsequent mobilization of metals might be not necessary in the highly contaminated Litavka soil, since the soluble Zn and Cd concentrations during our immobilization period (Figure 21) were as high as the respective concentrations measured by Höfer (2013) after a successful mobilization with S. Alternatively, a high metal accumulating plant could be used in further experiments being able to take up larger amounts of Zn, Cd and Pb without suffering from toxicity.

5. Conclusion

Based on the previous work of Iqbal et al. (2012), we combined two phytoremediation strategies by applying VC, BC and Lig as immobilization and elemental S as mobilization agent in an incubation (without plant) and pot experiment (with *Zea mays*). In contrast to the previous experiment, (i) elemental S was not added together with the immobilization agents but after a period of several weeks, (ii) *Zea mays* instead of *S. smithiana,* (iii) organic instead of inorganic immobilization additives and (iv) a highly contaminated Gleyic Fluvisol as experimental soil were used.

During the immobilization period, the application of VC, BC and Lig significantly ($p < 0.05$) reduced the $CaCl_2$-extractable Zn and Cd concentrations in both experiments, while those of Pb increased in comparison to the control.

When S was applied in the incubation experiment, pH_{CaCl2} declined significantly ($p < 0.05$) and the $CaCl_2$-extractable concentrations of Zn increased by 8 to 41 times and those of Cd by 6 to 14 times in comparison to samples without S application. In accordance with the findings of Iqbal et al. (2012) and Höfer (2012), the increase in $CaCl_2$-extractable metal concentrations per decreasing pH unit was much more pronounced after 0.5 g S kg^{-1} and 1.5 g S kg^{-1} were added to soil.

During the pot experiment, metal concentrations (Zn, Cd, Pb, Mn and Fe) in the soil solution and $CaCl_2$ extraction of the C, BC and VC treatments were not significantly affected by S addition. Similarly, the total content of Zn, Cd and Pb in the shoots of maize did not significantly increase in the respective treatments. While S and soil could be mixed homogeneously in the incubation, the subsequent S application in the pot experiment was restricted to the area above the root zone of maize thus limiting the contact surface for S oxidizing bacteria. Moreover, the soil type (Gleyic Fluvisol), high addition rates of the additives (i.e. changing hydraulic conductivity and causing water logging), oxygen depletion in the pots (due to root respiration and frequent watering) and the use of organic immobilization agents (i.e. introducing reduced organic compounds to soil as electron donors) might have influenced the redox chemistry in soil by lowering the redox potential and thus limited S oxidation.

An exception can be seen in the Lig2 treatment since $CaCl_2$-extractable concentrations of Zn, Pb, Cd, Mn and sulfate increased and pH decreased significantly ($p < 0.05$) after S application. We suppose that easy accessible S forms in Lig functioned as driver for microbial S oxidation thus stimulating S oxidizing bacteria probably even before S was applied to soil.

VC and to a less extent Lig treatments were associated with a steady nutrient supply for plants and an increased P availability compared to the other treatments. Consequently, the highest biomass yields and lowest toxicity as well as deficiency symptoms could be observed in the VC and Lig treatments, while BC produced even less biomass than the control and severe toxicity symptoms were found during the whole experiment. These findings could be confirmed by the observation of root morphological changes before and after S addition: only plants in both VC treatments were able to increase the specific root length, while the remaining treatments showed a strong decrease.

The highest total Zn and Cd contents in the shoots of maize were measured in the VC treatment thus increasing PR efficiency by 100 % for Zn and 400 % for Cd in comparison to the control.

Further research is needed to investigate the influence of organic amendments on S oxidation taking into account the specific microbiology and redox reactions in Litavka soil. For future experiments with the same soil, we suggest the use of a high metal accumulating plants being able to take up large amounts of pollutants without suffering from toxicity stress. Moreover, synergistic effects between the application of lignite and the oxidation of S should be evaluated and the subsequent application of S should be improved to guarantee a homogeneous distribution in soil.

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Annex A: Incubation Experiment Data

The following tables contain the most important data of the incubation experiment. All values are given as means (number of replicates is given in the description) plus standard error of the mean (SEM).

Table A1: Mean Cd concentrations (mg kg^{-1}) in the incubation experiment given for different time steps, treatments and S rates (n = 3).

Time	S-Rate	$CaCl_2$-extractable Cadmium concentrations in mg kg^{-1}													
		C		BC1		BC2		Lig1		Lig2		VC1		VC2	
		mean	+/- SEM	mean	+/- SEM	mean	+/- SEM	mean	+/- SEM	mean	+/- SEM	mean	+/- SEM	mean	+/- SEM
0	No S	1.36	0.01	0.73	0.04	0.45	0.03	0.83	0.01	0.56	0.00	0.77	0.08	0.65	0.01
7		1.31	0.03	0.68	0.03	0.42	0.02	0.82	0.01	0.58	0.01	0.79	0.01	0.57	0.01
14		1.44	0.03	0.84	0.06	0.42	0.02	0.84	0.00	0.61	0.01	0.87	0.01	0.63	0.00
21		1.60	0.01	0.63	0.04	0.34	0.05	0.94	0.04	0.64	0.03	0.90	0.01	0.66	0.02
28		1.65	0.04	0.81	0.02	0.25	0.05	0.89	0.03	0.71	0.01	1.16	0.11	0.70	0.01
40		1.59	0.06	0.82	0.11	0.48	0.06	1.06	0.04	0.85	0.04	1.07	0.02	0.79	0.01
28	0.5 g kg^{-1}	1.88	0.17	1.92	0.09	0.65	0.24	1.86	0.04	1.21	0.03	1.15	0.07	0.82	0.07
40		3.88	0.20	2.86	0.17	1.90	0.16	2.65	0.04	1.45	0.02	2.14	0.32	1.93	0.12
28	1.5 g kg^{-1}	2.09	0.14	3.04	0.09	1.52	0.24	3.59	0.09	2.91	0.06	1.43	0.18	0.87	0.02
40		6.18	0.55	7.87	0.68	7.13	0.33	6.36	0.62	4.61	0.48	5.81	0.38	4.11	0.18

Table A2: Mean Zn concentrations (mg kg^{-1}) in the incubation experiment given for different time steps, treatments and S rates (n = 3).

Time	S-Rate	$CaCl_2$-extractable Zinc concentrations in mg kg^{-1}											
		C		BC1		BC2		Lig1		Lig2		VC1		VC2	
		mean	+/- SEM	mean	+/- SEM	mean	+/- SEM	mean	+/- SEM	mean	+/- SEM	mean	+/- SEM	mean	+/- SEM
0	No S	61.28	3.43	28.90	1.76	15.07	1.24	41.78	0.34	31.06	0.42	37.65	2.26	31.56	0.32
7		63.09	1.55	23.56	1.06	12.52	0.76	39.36	0.33	30.96	0.57	34.32	0.54	25.72	0.38
14		71.59	2.27	29.88	2.84	12.10	0.66	40.58	0.13	33.27	0.40	38.02	0.40	29.03	0.29
21		67.15	3.62	20.36	1.59	8.04	1.48	46.13	1.89	35.48	1.83	40.15	0.55	30.07	0.73
28		71.78	2.01	28.84	0.84	6.32	1.72	46.93	1.23	41.48	0.43	51.49	1.40	32.23	0.35
40		76.14	2.96	33.09	6.32	14.28	2.38	65.08	4.22	57.33	2.47	55.33	1.41	42.49	0.59
28	0.5 g kg^{-1}	84.21	9.05	82.01	3.65	20.70	11.96	112.02	2.14	80.58	2.91	53.54	4.19	39.77	3.37
40	0.5 g kg^{-1}	254.46	17.68	148.92	9.56	89.49	10.72	201.90	4.22	121.80	1.04	136.89	22.72	135.17	8.67
28	0.5 g kg^{-1}	95.18	5.88	142.92	5.37	62.09	11.96	224.11	5.32	206.99	2.65	72.11	11.04	43.66	1.52
40	0.5 g kg^{-1}	508.56	44.35	609.90	83.93	585.85	16.55	578.40	100.74	471.15	56.09	492.74	40.73	427.20	6.92

Table A3: Mean Pb concentrations (mg kg^{-1}) in the incubation experiment given for different time steps, treatments and S rates (n = 3).

Time	S-Rate	$CaCl_2$-extractable Lead concentrations in mg kg^{-1}													
		C		BC1		BC2		Lig1		Lig2		VC1		VC2	
		mean	+/- SEM	mean	+/- SEM	mean	+/- SEM	mean	+/- SEM	mean	+/- SEM	mean	+/- SEM	mean	+/- SEM
0	No S	0.13	0.01	0.07	0.01	0.08	0.01	0.10	0.01	0.07	0.01	0.21	0.06	0.18	0.01
7		0.06	0.00	0.04	0.00	0.04	0.00	0.05	0.00	0.05	0.00	0.07	0.00	0.09	0.00
14		0.11	0.01	0.10	0.01	0.11	0.00	0.12	0.01	0.11	0.01	0.16	0.00	0.14	0.01
21		0.14	0.04	0.18	0.01	0.08	0.01	0.16	0.01	0.18	0.02	0.28	0.01	0.21	0.03
28		0.20	0.01	0.17	0.02	0.16	0.03	0.12	0.00	0.11	0.00	0.28	0.01	0.23	0.02
40		0.14	0.02	0.17	0.02	0.17	0.02	0.24	0.00	0.29	0.05	0.36	0.03	0.31	0.04
28	0.5 g kg^{-1}	0.23	0.01	0.18	0.00	0.14	0.02	0.17	0.01	0.15	0.00	0.30	0.02	0.31	0.01
40		0.39	0.04	0.21	0.01	0.24	0.02	0.46	0.00	0.28	0.01	0.41	0.05	0.37	0.01
28	0.5 g kg^{-1}	0.25	0.01	0.24	0.01	0.17	0.02	0.45	0.01	0.42	0.01	0.27	0.01	0.29	0.01
40		0.98	0.15	1.09	0.20	1.24	0.05	1.73	0.33	1.34	0.11	1.06	0.16	0.77	0.06

Table A4: Mean pH values in the incubation experiment given for different time steps, treatments and S rates (n = 3).

Time	S-Rate	pH_{CaCl2} in mg/kg											
		C		BC1		BC2		Lig1		Lig2		VC1		VC2	
		mean	+/- SEM	mean	+/- SEM	mean	+/- SEM	mean	+/- SEM	mean	+/- SEM	mean	+/- SEM	mean	+/- SEM
0	No S	5.50	0.00	6.03	0.03	6.30	0.06	5.67	0.07	5.23	0.03	5.50	0.00	5.60	0.06
7		5.98	0.02	6.43	0.02	6.43	0.09	5.69	0.00	5.68	0.03	6.05	0.02	6.17	0.05
14		5.89	0.02	6.26	0.02	6.40	0.04	5.82	0.02	5.94	0.01	5.94	0.02	6.06	0.03
21		5.77	0.01	6.21	0.01	6.68	0.01	5.77	0.02	5.73	0.00	5.81	0.01	5.84	0.02
28		5.83	0.01	6.23	0.01	6.90	0.07	5.72	0.02	5.54	0.03	5.88	0.02	5.86	0.01
40		5.73	0.02	6.21	0.06	6.69	0.03	5.66	0.02	5.61	0.02	5.79	0.04	5.67	0.01
28	0.5 g kg^{-1}	5.72	0.05	5.74	0.03	6.48	0.06	5.27	0.02	5.31	0.03	5.68	0.08	5.78	0.02
40	0.5 g kg^{-1}	5.36	0.06	5.86	0.05	6.20	0.06	5.35	0.02	5.37	0.03	5.50	0.02	5.53	0.04
28	0.5 g kg^{-1}	5.27	0.13	5.33	0.07	5.74	0.06	4.90	0.01	4.84	0.01	5.33	0.00	5.62	0.04
40	0.5 g kg^{-1}	5.02	0.05	5.01	0.04	5.25	0.07	4.83	0.03	4.89	0.02	4.94	0.00	5.00	0.02

Annex B: Pot Experiment Data

The following tables contain the most important data of the pot experiment. All values are given as means (number of replicates is given in the description) plus standard error of the mean (SEM).

Table B1: Mean root concentrations and SEM (mg pot^{-1}) for *Zea mays* according to different treatments and harvests (n = 3 for 14 weeks and n = 2 for 9 and 12 weeks).

Age	Treatment	P (mg kg^{-1}) mean +/- SEM		Fe (mg kg^{-1}) mean +/- SEM		Mn (mg kg^{-1}) mean +/- SEM		Cd (mg kg^{-1}) mean +/- SEM		Pb (mg kg^{-1}) mean +/- SEM		Zn (mg kg^{-1}) mean +/- SEM		S (mg kg^{-1}) mean +/- SEM	
9 weeks	Control	546.32	14.88	1585.49	108.00	98.60	3.27	192.67	24.96	247.74	10.63	7067.15	243.36	4453.32	108.65
	BC1	573.39	21.37	3126.69	922.46	235.40	115.48	252.24	64.65	368.21	119.11	4768.58	270.93	5148.99	462.98
	BC2	467.00	75.31	1240.51	152.69	141.17	74.71	113.89	22.15	189.53	68.91	1976.13	216.43	4759.31	599.19
	Lig1	481.57	51.83	1153.79	102.87	82.24	21.58	153.92	37.16	158.56	29.04	3673.04	906.67	4098.70	188.53
	Lig2	536.83	40.51	3425.01	2149.65	657.27	517.93	103.89	24.75	604.69	413.05	3219.29	831.15	5093.93	8.73
	VC1	1113.12	350.12	10976.91	3517.65	1440.80	571.20	192.11	22.53	1367.33	533.51	5309.33	908.65	5058.34	611.66
	VC2	586.42	79.93	2455.47	1770.77	212.12	159.24	120.60	7.05	267.32	181.23	2377.44	478.01	4489.80	224.21
12 weeks	Control	476.72	137.59	2974.66	719.50	300.28	18.34	176.30	45.41	364.64	88.21	6421.56	395.73	3526.91	469.29
	BC1	394.07	51.86	4865.35	1121.33	479.47	178.10	125.94	1.24	570.00	141.05	4028.29	371.56	4780.08	67.05
	BC2	421.91	15.78	2537.72	352.14	215.97	48.21	106.20	0.34	279.44	20.54	2476.20	344.00	4480.78	20.25
	Lig1	594.82	59.28	12249.55	9425.65	2052.63	1466.39	128.76	23.85	2033.77	1585.52	4946.11	687.51	3502.30	687.34
	Lig2	343.70	256.70	10041.17	8853.87	1556.23	1257.81	39.65	29.40	1429.79	1245.41	2888.49	2365.75	3132.82	2678.51
	VC1	750.14	182.43	7915.76	5163.25	1618.07	1290.34	195.02	47.82	1303.68	922.69	3485.42	872.03	4131.33	409.48
	VC2	841.75	18.86	877.41	596.62	80.39	36.39	116.83	107.52	109.33	81.89	1601.98	1054.21	3459.87	1928.77
14 weeks	Control	615.21	49.72	2686.88	1285.50	476.24	254.33	141.78	18.25	424.06	166.46	5801.73	149.04	4291.50	249.60
	BC1	430.09	31.88	3192.57	860.30	330.42	98.02	130.68	16.17	445.80	111.29	3374.34	438.93	4270.15	251.93
	BC2	477.65	44.06	6663.87	2454.92	917.77	502.49	113.10	14.91	888.49	421.23	3154.38	369.33	4682.45	1203.09
	Lig1	557.69	33.84	10652.35	2291.15	1668.57	89.12	83.50	2.86	1496.24	216.35	4944.75	310.52	5743.83	241.57
	Lig2	589.22	17.73	9904.94	3940.37	1603.26	612.65	73.74	4.76	1479.36	566.92	4077.29	837.69	6581.73	623.61
	VC1	870.62	41.48	15984.81	2911.75	2809.78	570.63	128.16	36.15	2304.71	316.55	5393.64	228.70	4047.61	442.65
	VC2	1045.68	21.49	18291.87	2621.04	3200.80	593.17	127.94	6.42	2597.60	405.78	5407.38	330.82	4874.65	257.23

Table B2: Mean shoot concentrations and SEM (mg pot^{-1}) for *Zea mays* according to different treatments and harvests (n = 3 for 14 weeks and n = 2 for 9 and 12 weeks).

Age	Treatment	P (mg kg^{-1}) mean	+/- SEM	Fe (mg kg^{-1}) mean	+/- SEM	Mn (mg kg^{-1}) mean	+/- SEM	Cd (mg kg^{-1}) mean	+/- SEM	Pb (mg kg^{-1}) mean	+/- SEM	Zn (mg kg^{-1}) mean	+/- SEM	S (mg kg^{-1}) mean	+/- SEM
9 weeks	Control	1046.28	31.54	2316.56	1377.46	405.04	249.35	46.40	2.07	384.66	285.41	3254.95	428.39	2124.14	11.21
	BC1	1094.94	9.57	432.18	121.91	41.70	10.68	21.98	2.22	33.05	8.86	1171.33	20.06	2269.16	14.59
	BC2	1175.63	34.79	637.77	132.97	56.15	16.45	22.01	1.65	50.49	15.53	1099.49	29.45	2840.15	268.71
	Lig1	1151.16	13.58	337.59	24.32	32.66	4.43	12.02	1.18	23.50	4.50	1421.81	73.16	1787.54	151.48
	Lig2	1171.49	6.01	267.55	54.47	36.97	5.52	13.03	1.67	24.74	5.51	1297.65	74.12	2022.07	96.81
	VC1	1220.85	78.12	380.62	20.56	35.18	1.66	13.98	1.89	20.23	2.02	1201.60	126.90	1691.91	24.11
	VC2	1502.87	124.84	323.80	14.87	50.18	7.32	15.82	1.42	16.74	5.18	1055.84	0.79	1841.37	124.75
12 weeks	Control	718.06	49.10	1599.87	529.40	285.88	64.71	47.74	5.49	231.59	101.28	3907.33	284.36	3576.22	264.28
	BC1	666.48	8.38	555.10	285.01	56.03	29.42	16.07	2.28	47.65	29.59	1051.96	416.40	2689.85	584.66
	BC2	781.02	2.51	829.78	143.19	136.85	47.00	18.87	0.16	105.71	28.89	1279.16	34.53	4441.22	302.25
	Lig1	854.93	2.55	490.85	237.76	34.69	1.42	13.19	1.48	21.37	3.94	1133.55	74.24	2189.56	337.48
	Lig2	848.24	40.23	279.63	28.32	29.52	1.93	6.53	0.65	21.15	1.09	1033.59	20.66	1907.75	167.74
	VC1	791.25	19.04	373.08	1.81	46.47	0.66	17.82	0.02	29.48	2.75	1205.91	46.35	1887.34	35.73
	VC2	835.87	24.74	576.20	295.42	40.74	3.26	10.25	0.94	20.81	6.64	692.36	144.59	1424.87	106.22
14 weeks	Control	808.52	99.83	4406.39	2102.52	820.83	515.19	47.41	9.62	676.88	403.28	4478.62	907.28	5417.16	1796.12
	BC1	590.86	20.39	1747.02	640.71	239.07	79.59	27.92	3.78	203.46	76.43	2193.32	174.59	4931.49	931.94
	BC2	797.36	173.16	730.74	470.52	101.36	68.05	14.11	4.22	83.95	62.33	867.50	363.89	3844.97	1805.73
	Lig1	708.68	43.49	507.89	158.11	88.63	26.04	15.14	2.53	61.44	19.87	1437.40	240.79	3314.39	683.52
	Lig2	653.39	21.26	439.32	91.29	69.81	9.48	9.77	0.84	46.82	7.64	1118.26	35.67	2780.13	165.06
	VC1	609.09	15.74	157.37	17.74	24.14	1.76	11.59	0.86	12.63	2.09	982.71	3.34	1461.66	100.13
	VC2	641.52	21.52	140.38	21.83	33.54	4.26	12.17	0.53	11.88	2.96	520.34	12.55	1354.24	50.05

Table B3: Mean total content of Zn, Cd and Pb for *Zea mays* (mg pot^{-1}) according to different treatments and harvests (n = 3 for 14 weeks and n = 2 for 9 and 12 weeks).

Age	Treatment	Zn (mg pot^{-1}) mean +/- SEM		Cd (mg pot^{-1}) mean +/- SEM		Pb(mg pot^{-1}) mean +/- SEM	
9 weeks	Control	17.08	4.65	0.24	0.05	2.20	1.76
	BC1	8.42	0.44	0.16	0.00	0.24	0.08
	BC2	7.24	0.47	0.14	0.00	0.32	0.07
	Lig1	16.73	1.67	0.13	0.01	0.27	0.09
	Lig2	13.40	0.92	0.13	0.00	0.25	0.03
	VC1	54.93	0.20	0.64	0.02	0.95	0.20
	VC2	69.68	6.81	1.05	0.20	1.14	0.45
12 weeks	Control	21.08	1.38	0.26	0.01	1.34	0.73
	BC1	11.60	3.08	0.18	0.00	0.51	0.27
	BC2	8.52	0.81	0.13	0.01	0.72	0.24
	Lig1	21.10	3.71	0.24	0.03	0.39	0.02
	Lig2	19.90	1.86	0.12	0.00	0.41	0.03
	VC1	66.98	16.54	0.98	0.21	1.59	0.19
	VC2	62.35	19.68	0.91	0.19	1.76	0.37
14 weeks	Control	27.48	3.37	0.29	0.03	3.24	0.99
	BC1	15.89	2.07	0.20	0.03	1.49	0.58
	BC2	12.90	3.53	0.21	0.03	1.11	0.69
	Lig1	27.96	1.92	0.30	0.03	1.11	0.26
	Lig2	35.32	4.88	0.30	0.03	1.49	0.37
	VC1	59.95	3.70	0.71	0.10	0.79	0.18
	VC2	50.06	2.22	1.17	0.04	1.14	0.30

Table B4: Root and shoots dry weights (g) of *Zea mays* shown for different harvests and treatments (n = 3 for 14 weeks and n = 2 for 9 and 12 weeks).

Age	Treatment	Root (g) mean +/- SEM		Shoot (g) mean +/- SEM	
9 weeks	Control	1,53	0,06	5,15	0,75
	BC1	2,35	0,26	7,20	0,50
	BC2	2,11	0,37	6,60	0,60
	Lig1	3,46	0,45	11,15	1,75
	Lig2	2,64	0,88	10,40	1,30
	VC1	8,38	0,68	46,25	5,05
	VC2	8,57	0,74	66,00	6,50
12 weeks	Control	1,82	0,30	5,45	0,75
	BC1	3,18	0,69	11,70	1,70
	BC2	2,09	0,15	6,65	0,45
	Lig1	5,93	1,34	18,91	4,51
	Lig2	6,40	0,79	19,30	2,20
	VC1	12,33	0,29	55,10	11,60
	VC2	16,55	1,11	87,95	10,05
14 weeks	Control	2,00	0,42	6,83	1,74
	BC1	1,80	0,20	7,30	1,01
	BC2	4,07	0,70	16,63	2,97
	Lig1	7,33	1,31	21,03	4,71
	Lig2	9,50	1,42	31,37	3,42
	VC1	20,17	2,45	61,03	3,95
	VC2	28,27	3,05	96,20	3,41

Table B5: $CaCl_2$-extractable concentrations (mg kg^{-1}) of metals, sulfate and P according to different treatments and harvests (n = 3 for 14 weeks and n = 2 for 9 and 12 weeks). First part of the data set.

Age	Treatment	P (mg kg^{-1}) mean	+/- SEM	Fe (mg kg^{-1}) mean	+/- SEM	Mn (mg kg^{-1}) mean	+/- SEM	Cd (mg kg^{-1}) mean	+/- SEM	Pb (mg kg^{-1}) mean	+/- SEM	Zn (mg kg^{-1}) mean	+/- SEM	SO_4 (mg kg^{-1}) mean	+/- SEM
9 weeks	Control	3.88	0.34	4.29	0.03	35.09	1.34	9.96	0.03	1.10	0.01	507.46	3.84	133.95	25.69
	BC1	6.56	0.80	5.38	0.77	20.71	0.93	6.60	0.39	0.85	0.11	291.36	23.82	163.27	108.56
	BC2	2.99	0.09	4.22	0.48	6.30	0.87	2.88	0.24	0.48	0.04	79.24	9.15	155.43	9.85
	Lig1	4.27	0.57	5.74	0.32	24.39	1.43	6.20	0.60	1.26	0.03	327.59	37.84	48.24	12.35
	Lig2	4.05	0.26	5.29	0.86	29.29	0.74	4.49	0.00	1.08	0.06	272.13	11.13	110.46	44.90
	VC1	7.55	2.15	9.63	0.47	24.93	0.74	7.45	0.65	1.58	0.03	388.04	16.92	60.42	14.72
	VC2	8.02	0.09	6.11	0.42	25.68	0.68	5.64	0.09	1.07	0.00	292.26	6.79	123.96	3.05
12 weeks	Control	3.74	0.47	4.03	0.47	23.55	2.50	9.93	0.12	1.16	0.06	534.49	32.95	135.08	4.02
	BC1	3.62	0.22	4.51	0.42	8.97	0.73	5.16	0.36	0.70	0.07	199.68	21.09	138.52	14.91
	BC2	4.07	0.18	4.09	0.36	6.98	0.75	4.14	0.37	0.55	0.06	147.49	23.45	292.36	15.32
	Lig1	3.64	0.24	5.72	0.87	22.63	0.08	6.29	0.51	1.18	0.08	339.17	12.79	147.50	45.35
	Lig2	3.32	0.07	5.95	0.63	28.79	5.55	5.20	0.31	1.36	0.08	318.15	17.67	311.74	2.45
	VC1	5.42	0.69	6.75	0.47	21.36	1.45	7.21	0.63	1.34	0.03	442.35	117.92	152.94	20.21
	VC2	8.73	0.21	6.90	0.79	22.66	1.39	3.58	0.28	1.11	0.08	263.48	16.26	164.39	26.22
14 weeks	Control	4.38	0.02	6.64	1.25	9.66	1.65	9.98	0.64	1.15	0.16	505.05	64.48	191.77	69.70
	BC1	3.85	0.39	5.49	0.34	11.58	4.25	7.73	1.08	1.07	0.12	362.75	102.85	224.95	93.35
	BC2	4.18	0.34	7.20	2.34	9.72	2.88	5.31	0.57	0.97	0.26	225.40	38.96	405.42	102.87
	Lig1	3.75	0.31	5.46	0.23	33.59	4.20	8.36	0.41	1.46	0.15	579.91	41.92	341.24	42.19
	Lig2	4.95	0.62	5.91	0.14	51.97	4.93	6.75	0.26	1.72	0.06	552.54	28.91	591.67	88.42
	VC1	6.78	0.36	9.04	1.25	47.52	17.78	9.86	2.19	2.23	0.49	680.22	185.45	500.23	200.86
	VC2	9.95	0.49	5.92	0.60	25.69	1.67	6.73	0.45	1.41	0.08	419.92	37.21	312.38	111.19

Table B6: $CaCl_2$-extractable concentrations (mg kg^{-1}) for nitrate, oxalate and pHaccording to different treatments and harvests (n = 3 for 14 weeks and n = 2 for 9 and 12 weeks). Second part of the data set.

Age	Treatment	NO_3 (mg kg^{-1}) mean	+/- SEM	Oxalate (mg kg^{-1}) mean	+/- SEM	pH mean	+/- SEM
9 weeks	Control	754.90	46.70	47.50	4.40	5.53	0.02
	BC1	802.10	249.40	33.00	4.50	5.70	0.03
	BC2	549.90	36.60	24.95	1.65	6.18	0.15
	Lig1	210.00	85.40	17.90	2.20	5.52	0.00
	Lig2	378.15	114.05	16.25	2.05	5.50	0.02
	VC1	144.80	2.30	14.15	2.05	5.53	0.06
	VC2	340.00	166.30	12.60	0.80	5.59	0.00
12 weeks	Control	722.00	17.50	48.60	4.50	5.53	0.01
	BC1	390.85	8.55	30.35	1.55	5.84	0.01
	BC2	445.65	48.35	29.30	0.70	5.94	0.09
	Lig1	461.70	98.50	18.45	0.85	5.52	0.08
	Lig2	515.95	118.15	14.70	2.10	5.42	0.07
	VC1	579.65	170.65	15.20	0.50	5.59	0.06
	VC2	422.60	61.30	13.05	0.65	5.68	0.02
14 weeks	Control	602.27	88.64	72.00	7.93	5.52	0.05
	BC1	567.13	46.30	37.67	1.81	5.68	0.01
	BC2	321.03	27.48	25.53	0.77	5.85	0.05
	Lig1	469.30	67.77	20.60	0.15	5.43	0.09
	Lig2	814.77	194.47	13.90	1.97	5.25	0.03
	VC1	758.10	57.85	27.03	10.43	5.42	0.08
	VC2	617.90	221.63	15.13	1.25	5.42	0.06

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