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Trace metal solubility and bio-availability changes in the rhizosphere of *Salix smithiana* in response to elemental sulfur amendments

Master thesis

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"What a good thing Adam had. When he said a good thing he knew nobody had said it before."

Mark Twain (1935), Notebook

Zusammenfassung

Phytoextraktion ist eine sanfte in-situ Technik um Spurenelemente (TMs) der Bodenmatrix zu entziehen und in die Pflanzenbiomasse zu verlagern. Die Extraktion erfolgt mithilfe von höheren Pflanzen und deren assoziierten Mikroorganismen in der Rhizosphäre.

Die vorliegende Arbeit befasst sich mit der Wirkung von elementarem Schwefel (S^0) als Bodenzusatz zu einem mäßig metallbelasteten Boden (ARNB-10) aus Arnoldstein (Österreich) und auf die Bioverfügbarkeit von Mn, Fe, Cu, Zn, Cd und Pb in der Rhizosphäre von *Salix smithiana*. In einem Rhizoboxexperiment untersuchten wir die durch S^0 Oxidation potentiell induzierte reduktive Auflösung von Mn Oxyhydroxiden und damit assoziierte Metalllösungsprozesse. Es wurden zwei S^0-Behandlungen (HS=0,51 g kg-1; S=1.02 g kg-1) getestet und Bodenwasserproben acht Mal in einem Zeitraum von 61 Tagen in der Rhizosphäre und im Bulk-Boden entnommen. Die Proben wurden auf pH-Wert, Anionen durch Ionen-Chromatographie (IC) und Metalle durch induktiv gekoppelte Plasma-Massenspektrometrie (ICP-MS) analysiert. Die Weiden wurden geerntet und nach einem Säureaufschluss auf ihre Metallkonzentrationen analysiert (ICP-MS). Am Experimentende wurden Bodenproben mit 0,05M $Ca(NO_3)_2$ extrahiert und die potentiell labilen Metallkonzentrationen bestimmt (ICP-MS).

Die Ergebnisse zeigen niedrigere pH-Werte in beiden S^0-Behandlungen, während die Mn, Zn, Cd und Pb Konzentrationen in den Bodenwasserproben stark erhöht waren. Die Metalllöslichkeiten in den Rhizosphären-Kompartimenten der S^0-Behandlungen waren signifikant ($p<0.05$) erhöht verglichen mit den Bulk-Böden-Kompartimenten. Unsere Daten deuten auf teilweise anaerobe Bedingungen hin, ausgelöst durch erhöhten O_2 Verbrauch bei der S^0 Oxidation und zusätzlich verstärkt durch die Wurzelatmung der Weide. In der Rhizosphäre beider S^0-Behandlungen führten diese Prozesse zu einer reduktiven Auflösung von Metallen verbunden mit Mn Oxyhydroxiden.

Abstract

The purpose of phytoextraction is to transfer trace metals (TMs) from polluted soils to plant shoot tissues, supported by the interaction of plants roots and microbial communities in the rhizosphere. Soil amendments and rhizosphere processes can increase TM bioavailability and flux in the soil.

Here, we investigate the effects of elemental sulfur (S^0) application on TM bioavailability in the rhizosphere of *Salix smithiana*, in a moderately Zn, Cd and Pb contaminated soil (ARNB-10) from Arnoldstein, Austria. Chemical and microbial sulfur oxidation acidifies the soil locally and decreases pH in the soil solution, which leads to enhanced metal solubilisation. Our focus was to investigate potential co-dissolution processes and other solubilisation mechanisms triggered by the S^0-amendments. A rhizobox experiment was conducted using a ARNB-10 and two amounts of S^0-amendments (HS=0.51 g kg^{-1}; S=1.02 g kg^{-1}). We sampled soil pore water eight times in the rhizosphere and bulk soil using Rhizon samplers over a period of 61 days. Samples were analyzed for pH, anions by Ion chromatography (IC), and TMs by Inductively coupled plasma mass spectrometry (ICP-MS). Willows were harvested, separated in roots, twigs and leaves, digested and analyzed (ICP-MS). For estimating potentially labile fractions in the soil, 0.05M $Ca(NO_3)_2$ extracts were measured for TMs using (ICP-MS).

Results show decreased pH in both S^0 treatments, whereas Mn, Zn, Cd and Pb solubility strongly increased. The S^0-amended rhizosphere compartments showed significantly ($p<0.05$) larger increase in metal solubility than the corresponding bulk soils. Our data indicate that partially anaerobic conditions triggered by S^0-oxidation further enhanced by O_2 depletion due to root respiration in the willow rhizosphere resulted in reductive co-dissolution of TMs associated with hydrous oxides of Mn. This process may be further explored for optimizing S-aided phytoextraction of Zn and Cd polluted soils.

Table of Content

List of Abbreviations

The following table describes the significance of various abbreviations and acronyms used throughout the thesis.

Abbreviation	Description
ARNB-10	Metal contaminated experimental soil from Arnoldstein, Austria
C, HS, S	Soil treatments: Control; Sulfur (HS=0.51g kg^{-1}, S=1.02g kg^{-1})
CEC / AEC	Cation exchange capacity / Anion exchange capacity
dwt	Oven dry weight (105℃)
Eh	Soil redox potential
IC	Ion chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
Milli-Q water	Millipore Elix3 water purification system, EMD Millipore Corporation, Billerica, MA, USA; EC <0.1 µS cm^{-1} Ultrapure water "Type1", trademark of Millipore Corporation
n	Sample size, number of replicates
PE / PP	Polyethylene / Polypropylene
RSD	Relative standard deviation
S^0	Elemental sulfur
SEM	Standard error of the mean
TEA(s)	Terminal electron acceptor(s)
TM(s)	Trace metal(s)
WHC	Water holding capacity (%)

1. Introduction

Soil contamination by inorganic contaminants is a global issue. Soils can have elevated levels of potential harmful metals from natural or anthropogenic origin e.g. industrial or agricultural production, mining activities, commercial activities, transport and services, military land use or recreational shooting. Toxic amounts of trace metals, often synonymously referred to as heavy metals (Duffus, 2002), pose a potential risk to the environment and human health.

Trace metals in soils cannot be decomposed by microbial activity like most of the organic contaminants, they can only be relocated and chemically transformed. Common, technical remediation techniques are expensive and often rarely sustainable, such as excavating the soil material and dumping it on sealed surfaces. Therefore, less invasive in-situ techniques such as phytoremediation are taking advantage of plant - microbial interactions for either immobilizing or extracting TMs from the soil. These green in-situ technologies are emerging and show promising results in cleansing and restoring soil and environmental quality by the use of plants (Baker et al., 1994; Ernst, 2005; Wenzel, 2009). Extracting TMs from the soil is termed phytoextraction and aims to reduce the concentration of TMs to such a level that the soil can be used without danger for agriculture, horticulture, forestry or amenity. For phytoextraction it is favored to achieve high extraction rates of TMs from the soil, plant uptake and translocation into shoot biomass. It is regarded as non-destructive, in-situ option for permanently reducing TM concentrations from the soil (Ernst, 2005).

1.1. Enhanced phytoextraction

Early studies on phytoextraction aimed at "hyperaccumulating" plants which can take up large amounts of certain TMs but typically produce low biomass. Later studies used metal-tolerant, high-biomass producing plants, e.g. poplars or willows and/or soil amendments such as synthetic chelants to enhance bioavailable TM fractions in the soil and uptake in phytoextraction crops (Kayser et al., 2000; Schmidt, 2003). Both options resulted in advanced knowledge of the underlying processes but were critically assessed in terms of potential risks posed to the environment. Chelant-enhanced phytoextraction significantly increased extraction rates and accumulation by plants but is costly and endangers plant growth and ground water resources by enhanced and highly persistent, mobile amounts of TMs and leaching to groundwater (Wenzel et al., 2003; Nowack et al., 2006). Other studies explored the use of less persistent and/or slow-release amendments such as natural chelants and elemental sulfur (Kayser et al., 2000; Wenger et al., 2002; Wang et al., 2006a; Wang et al., 2006b).

In a recent study a novel, combined approach of immobilizing TMs and remobilizing them was proposed (Iqbal et al., 2012). Remobilization of TMs was achieved by the use of elemental sulfur amendments and enhanced phytoextraction substantially by showing locally enhanced bioavailable TMs in the rhizosphere while keeping the risk of metal leeching in the bulk soil low (Iqbal et al., 2012). The results looked promising but in-depth knowledge about the underlying rhizosphere processes is still limited. Questions emerged and need to be elucidated in detail to understand rhizosphere processes of sulfur transformation and related trace metal biogeochemistry.

1.2. Elemental sulfur

Sulfur (S^0) is a highly abundant element on earth and an essential nutrient for the growth of all organisms. It is a component in amino acids, proteins and in enzyme cofactors (-SH groups) (Kertesz et al., 2007). Since the 1980s numerous soils throughout the world are considered to be sulfur-deficient. Possible reasons include the increasing use of low-S-containing fertilizers e.g. triple superphosphate, reductions in the use of S^0 as fungicide and the effectiveness in SO_2-pollution abatement programs and content in fuel (Wainwright and Brady, 1984).

Naturally occurring sulfur in soils is mostly bound to polymeric organic molecules and therefore not plant-available. Organic sulfur is accounting for >95% of total sulfur in most humid and semi-humid regions. Inorganic sulfur exists in S^{-2}, S^0, S^{+2}, and S^{+6} oxidation states and can be utilized by plants (Tabatabai, 1996). In the soil-plant relationship, SO_4^{2-} is predominantly utilized by plants and primarily transported via the xylem to target tissues. Therefore, in soils, S^0 needs to be oxidized to become plant-available which is possible in two ways, (i) abiotic or (ii) biotic (Kertesz et al., 2007).

i) Abiotic sulfur oxidation is regarded to play a minor role, but it does occur in aerated soils. Hypothetically, this process may also involve intermediates until S^0 is oxidized to SO_4^{2-}, (Eq. 1). The oxidation is considered slow and steady and primarily catalyzed by microbes with adequate supply of air and moisture (Wainwright and Brady, 1984).

$$S^0 \rightarrow S_2O_3^{2-} \rightarrow S_4O_6^{2-} \rightarrow SO_3^{2-} \rightarrow SO_4^{2-} \qquad \text{Eq. (1)}$$

ii) Biotic sulfur oxidation can be achieved by a broad spectrum of soil microorganisms and fungi. Most abundant and well known S^0-oxidizers are microbes from the *Thiobacillus* genus and heterotrophic bacteria such as *Thiobacillus thiooxidans* and *T. ferrooxidans*. However putative microbial specialist species were not identified yet (Kayser et al., 2000;

Kertesz et al., 2007). Carbon-rich root exudates in the rhizosphere are generating important sites for microbial activity and can facilitate S^0 oxidation. In the rhizosphere soil, the amount of sulfate producers can be significantly higher compared to the bulk soil (Grayston and Germida, 1990).

To sustainably enhance the phytoextraction capacity, TM bioavailability needs to be improved in the rhizosphere, while possible leaching of metals needs to be reduced to a minimum in the bulk soil. Building on earlier work (Kayser et al., 2000; Wang et al., 2006a; Wang et al., 2006b), Iqbal et al. (2012) already showed that for a slow and steady enhancement of TM solubility, soils can be amended with S^0. Results from this study raised questions to which extent rhizosphere processes are involved. The authors proposed two fundamental bio-geochemical processes.

i) In aerated systems, S^0 oxidizes to sulfuric acid (H_2SO_4) and acidifies the soil locally. This changes soil redox state (Eh), soil reaction and lowers soil pH by proton release into soil pore water. Ions can get desorbed from soil particles and released into the bioavailable pool (Figure 1). The extent of the oxidation process is depending on the soil buffer capacity and the cation/anion exchange capacity (CEC, AEC), while soil pH is the governing factor (Schmidt, 2003; Marschner and Marschner, 2012). Trace metal speciation is thereby directly influenced by soil pH changes due to its impact on the net charge of metal complexes and their precipitation and dissolution reactions (Naidu et al., 2008).

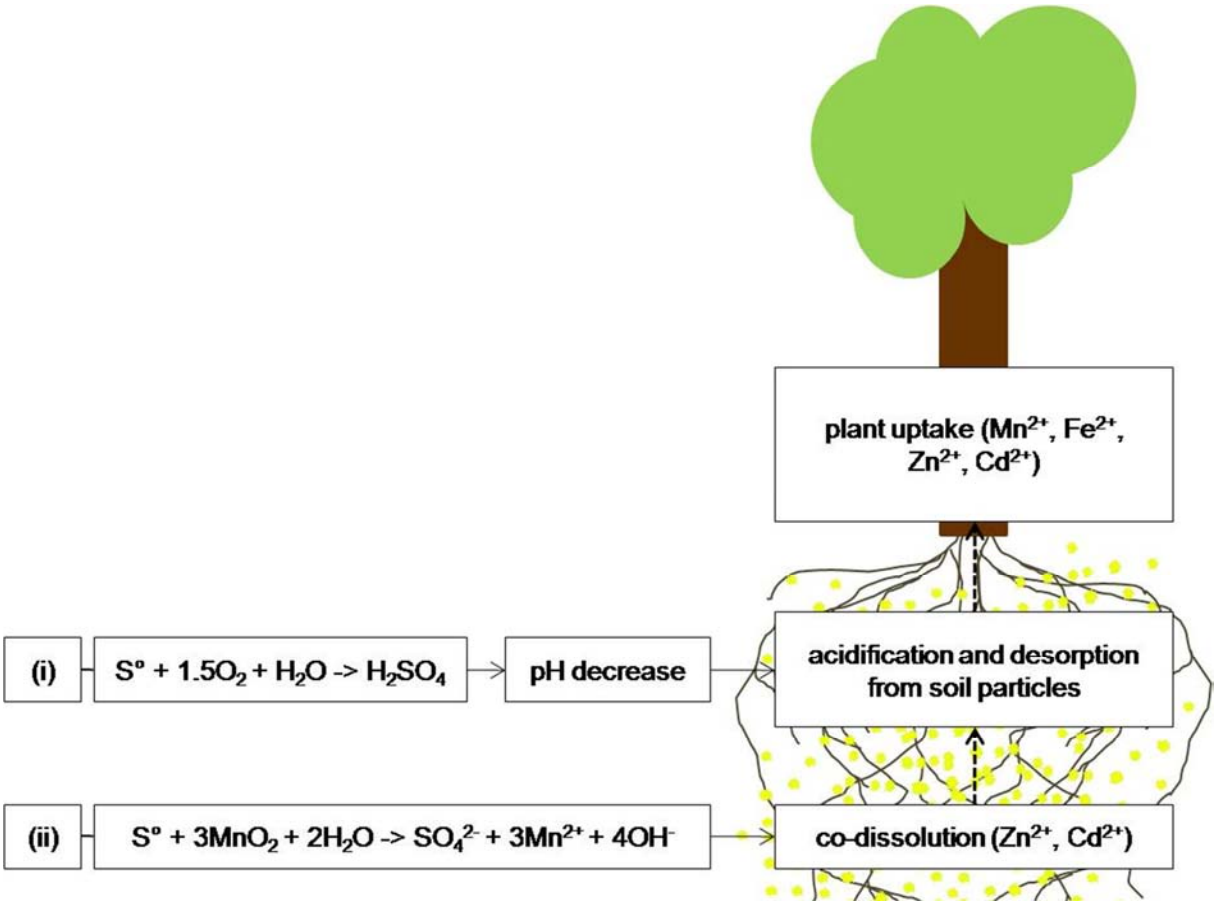


Figure 1: Elemental sulfur oxidation in aerated soils leads to (1) acidification of soil and desorption of metal cations from mineral surfaces, (2) co-dissolution of Mn (oxy)hydroxides and Zn^{2+}, Cd^{2+} under partly anaerobic conditions based on Iqbal et al. (2012).

ii) S^0 oxidizing bacteria may use (oxy)hydroxides of Mn^{4+} and Fe^{3+} as electron acceptors to oxidize S^0 under locally occurring anaerobic conditions in the rhizosphere. This process may result in reductive co-dissolution of Zn^{2+} and Cd^{2+} (Figure 1) (Iqbal et al., 2012). Such co-dissolution processes in the rhizosphere could be indicated by high concentrations of Mn and Fe in the soil pore water and associated high fractions of bioavailable Zn and Cd (Iqbal et al., 2012).

2. Hypothesis

The purpose of this study was to elucidate the fundamental processes involved in elemental sulfur (S^0) oxidation in the rhizosphere of *Salix smithiana* compared to the bulk soil and its effects on TM solubility, bioavailability and plant uptake using a rhizobox approach. As previous studies showed, elemental sulfur can be used as an inorganic amendment for enhancing phytoextraction of Zn and Cd (Kayser et al., 2000; Wang et al., 2006a; Wang et al., 2006b; Iqbal et al., 2012). Here, the focus was to further investigate the solubility and bioavailability of Mn, Fe, Cu, Zn Cd and Pb in the rhizosphere of *Salix smithiana* over time in response to two different S^0 application rates and to explore the processes involved.

Main objectives for this study included:

- to investigate the oxidation / reduction of two different amounts of S^0 (HS=0.51 g kg^{-1}; S=1.02 g kg^{-1}) in the rhizosphere of *S. smithiana* vs. bulk soil, its changes in soil reaction, TM solubility and bioavailability;
- to determine as to whether desorption, co-dissolution or precipitation processes, mediated by Mn and Fe (oxy)hydroxides magnify TM bioavailability in the rhizosphere and plant uptake by willows;
- to monitor the temporal variation of pH, dissolved TMs and anions in rhizosphere vs. bulk soil;
- to explore to which extent sulfur-enhanced phytoextraction using willows is based on locally increased TM fractions in the rhizosphere and less enhanced TM solubility (and consequently a lower risk of TM leaching and toxicity) in the bulk soil.

3. Material and Methods

The rhizobox experiment and sample analysis was carried out at the Institute of Soil Research, University of Natural Resources and Life Sciences Vienna, UFT Campus Tulln. Ion chromatography (IC) was conducted, in the laboratories of the same institute in Vienna, Austria.

3.1. Experimental soil

The experimental, Eutric Cambisol (IUSS Working Group WRB, 2006) was collected close to a former metal smelter in Arnoldstein, Carinthia, Austria in the year 2010. The A horizon (0-20 cm) of the experimental soil (ARNB-10) shows moderately elevated concentrations of Zn, Cd and Pb and was used in the experiment. Increased soil metal concentrations were caused by more than hundred years of deposition from the surrounding smelter activities which date back to 1495 and ended in 1992 (Friesl et al., 2006). In Table 1, selected soil properties are compiled from previous investigations.

Table 1: Selected soil properties of ARNB-10 (Iqbal et al., 2012).

Soil characteristics	Unit	Experimental soil (ARNB-10)	
Sand	g kg^{-1}	486	
Silt	g kg^{-1}	359	
Clay	g kg^{-1}	155	
WHC	g kg^{-1}	470	
pH (H_2O)	-	5.6	
EC	µS cm^{-1}	27.5	
$CaCO_3$	g kg^{-1}	0	
Organic carbon	g kg^{-1}	25.5	
CEC	cmolc kg^{-1}	1.6	
Total N[a]	g kg^{-1}	3.3	
Trace metals		Total (aqua regia)	NH_4NO_3-extractable
Mn	mg kg^{-1}	1010	-
Fe	mg kg^{-1}	3.5	-
Cu	mg kg^{-1}	54.3	0.06 ± 0.01
Zn	mg kg^{-1}	463	43.6 ± 5.1
Cd	mg kg^{-1}	4.7	0.7± 0.1
Pb	mg kg^{-1}	753	3.3 ± 0.4

[a]Data from (Friesl-Hanl, 2012).

3.2. Experimental plant

As experimental plant we used a willow clone, known for its suitability to translocate and accumulate large amounts of TMs into the shoot biomass. *Salix* x *smithiana Willd*. (*S. caprea L.* x *S. viminalis L.*, clone BOKU 03 CZ-001) was originally obtained from Silva Tarouca Research Center for Landscape and Decorative Horticulture, Průhonice, CZ. In previous studies *Salix smithiana* was shown to grow on metal polluted soils, and to efficiently phytoextract Zn and Cd. The clone is able to take up >400 mg kg^{-1} Cd and >2000 mg kg^{-1} Zn in leaves dry weight (dwt) from contaminated soil (Dos Santos Utmazian and Wenzel, 2007; Dos Santos Utmazian et al., 2007; Wieshammer et al., 2007; Puschenreiter et al., 2013).

Prior to the start of the experiment, fresh willow cuttings (length approx. 20 cm, stem diameter approx. 1 cm) were pre-grown in a commercially available potting mixture for 39 days. This ensured a high root biomass at start and sufficient plant vitality. For the rhizobox experiment only vital and homogenously grown willows were selected from a pool of approx. 40 pre-grown specimen.

3.3. Rhizobox experiment

3.3.1. Experimental design

The used rhizobox design, based on Fitz et al. (2003) (Figure 2), allowed to distinguish between three compartments, (1) mixed soil-root compartment for *S. smithana* (120 x60 x40 mm), (2) rhizosphere compartment (120 x60 x2 mm) and (3) bulk soil compartment (120 x60 x30 mm). For separating rhizosphere soil from bulk soil and plant roots, two 30-µm PE nettings (03-30/18, SEFAR, Switzerland) were installed to confine compartment 2.

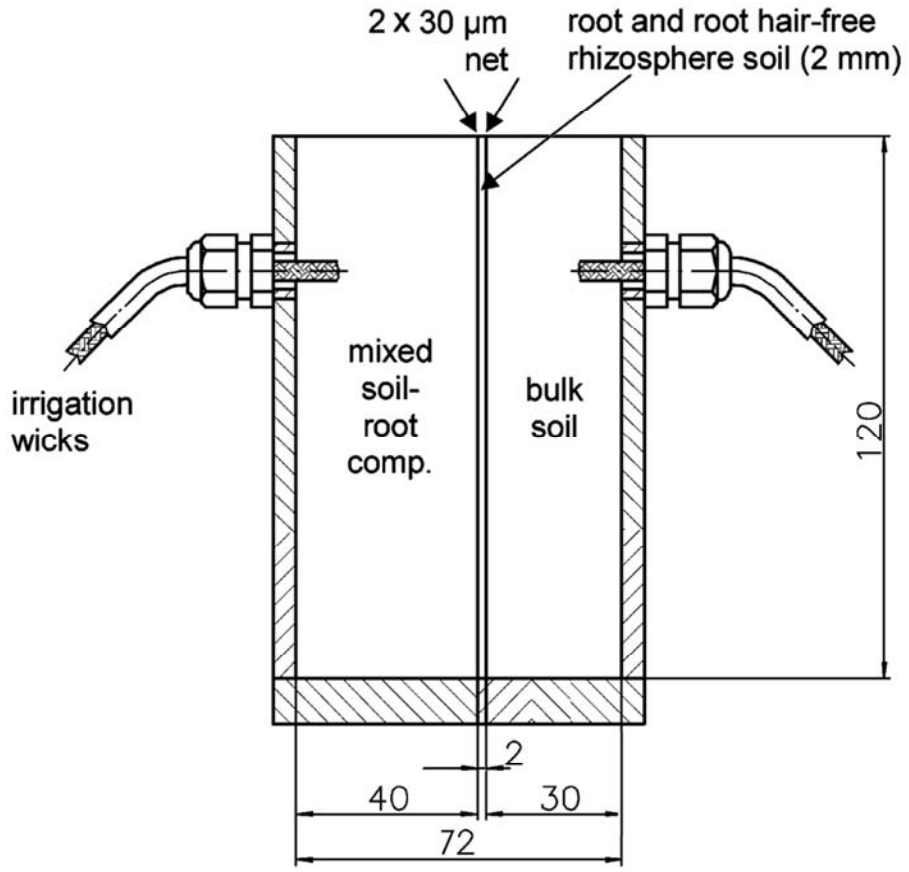


Figure 2: Rhizobox design sketch, without Rhizon samplers and plant, modified after Fitz et al. (2003), dimensions in mm, depth 60mm.

In the root and bulk soil compartment, 50-mm acid washed (0.01M HNO_3, rinsed two times with Milli-Q water) Rhizon samplers (Rhizosphere research products, Wagenigen, The Netherlands) were installed in central position 20 mm above the rhizobox bottom to repeatedly collect soil pore water during the 61 days of the experiment duration.

Three soil treatments (Control, HS, S) with four replicates each were prepared. Elemental sulfur (Sulfur AnalaR NORMAPUR, VWR) was manually crushed with a plastic spatula to gain homogenous powder, sieved (<200 µm) and weighted. The soil was air dried, sieved (<2 mm) and homogenized. All treatments were separately prepared and manually mixed end-over-end in clean, sealed plastic bags for approximately ten minutes. The treatments were then incubated in air-dry conditions at room temperature (21℃) for 24 h in the dark to equilibrate before filling the rhizoboxes.

The amount of sulfur for the treatments was chosen to reach a certain target pH according to a preliminary incubation experiment (Iqbal et al., 2012). Table 2 shows the applied S^0 amounts.

Table 2: Elemental sulfur soil amendment (Control, HS, S) to reach a certain target pH when added to the experimental soil (ARNB-10).

Name	Units	S^0 per kg soil dwt	Target pH
Control	g kg^{-1}	-	5.5
HS	g kg^{-1}	0.51	4.5
S	g kg^{-1}	1.02	4.0

For filling, the treatments were pre-moistened with Milli-Q water using a spray bottle. Soil was filled by careful compaction using a plastic tamper (approx. 600 g per rhizobox to obtain a soil bulk density of 1.4 g cm^{-3}). During filling, pre-grown willows cuttings were carefully transferred into the rhizoboxes after washing off the remaining growth substrate from the roots with tap water. *Salix smithiana* was cut back to one single twig to reduce leaf transpiration and ensure uniform growth conditions at experiment start. Soil was added cautiously to the roots to guarantee intimate contact of roots and soil. During this step, Rhizon samplers (Rhizosphere research products, Wagenigen, The Netherlands) were placed close to the stem in the root zone. For irrigation, two PE-coated glass fiber wicks (TRIPP Kristallo Rundschnur, 4 mm, IDT, Germany) were installed in the bulk soil and plant root compartment (Figure 3).

Figure 3: Photographs of the rhizobox assembly: (1) bulk soil and 2-mm rhizosphere compartment; (4) willow planting and installation of Rhizon sampler; (2) detailed view of final rhizobox - bulk soil, rhizosphere and root compartment including Rhizon samplers; (3) rhizobox at start of the experiment including watering system; (5) replicates for all treatments before setting up the experiment in the greenhouse.

At start of the experiment, the rhizoboxes were saturated to 80% WHC. To prevent light irradiation, causing algae growth, and loss of potential leaching water, the rhizoboxes were covered in aluminum foil and placed into clean plastic bags.

3.3.2. Monitoring and soil pore water sampling

The rhizobox experiment was carried out in an automated greenhouse using day-light lamps (Master HPI-T Plus 400W, IP65, Philips) (16 h per day) at 60% rel. humidity, from 23rd April until 23rd June, 2012 (61 days). During growth, water content was kept constant and pots were rotated several times. The measured climatic data during the experiment is summarized in Table 3. Temperature and humidity were recorded every hour continuously during the whole experiment by an automated system.

Table 3: Greenhouse climatic monitoring data, 23rd April to 23rd June, 2012 (mean ± SEM).

	Temperature (C°)	**Relative humidity** (%)	**Light intensity** (µmol m^{-2} s^{-1} PAR)
Mean	24.1	51.1	300
SEM ±	0.05	11.6	-
Min	14.9	12.8	-
Max	36.2	76.2	-

Soil pore water was sampled by applying vacuum, using acid-washed equipment (soaked in 5% HNO_3, rinsed two times with Milli-Q water). The Rhizon samplers were connected with 10 mL luer lock syringes (Rhizosphere research products, Wagenigen, The Netherlands) to sample approx. 4 mL of soil pore water per compartment (Figure 4). After collection, the samples were directly transferred in 5-mL sample vials (PP Tuben, Semadeni, Switzerland), measured for pH (ORION 3 Star, Thermo Scientific) and frozen at -20℃ for further analysis of anions (IC) and trace metals (ICP-MS).

Figure 4: Willows with attached pore water sampling syringes in the greenhouse (4). Control (1), HS treatment (2), S treatment (3). Pictures are taken on May, 30th, 2012.

Sampling was repeated eight times, at days 4, 14, 18, 22, 26, 37, 47, 59 after start of the experiment. Dropped leaves were collected using plastic tweezers and stored in separate paper bags for each replicate. Weed was manually removed from the pots and collected. Sampling intervals were chosen to monitor sulfate concentrations, and related changes in TM solubility.

3.3.3. Harvest

3.3.3.1. Plant

On day 25, one replicate of the S treatment (n=3) and on day 37, one replicate from the control treatment (n=3) had to be removed from the experimental plot due to death of the plants. We could not identify any specific reasons, such as visible signs of plant toxicity. At that time, the plants apparently suffered from temporarily high temperatures in the greenhouse.

At harvest, willows were cut directly above the soil surface using a pruning shear and separated into roots, shoots and leaves. Shoots and leaves were separated using ceramic scissors, and washed with Milli-Q water in a stainless steel sieve (<2 mm) for three minutes, and put into paper bags for oven drying at 65℃ for 72 h (UFE 600, Memmert GmbH, Germany). Prior to root separation, the plant compartments were air dried because of the high water content during the experiment. Soil particles were gently sieved away using a stainless steel sieve (<2 mm). Larger root particles were manually separated using plastic tweezers. The roots covered in remaining soil were stored overnight for further air drying and then washed in a plastic sieve (<2 mm) with Milli-Q water for three minutes. The remaining part of the stem (below ground) was removed and not included into the root or twigs fraction. To remove metals from the apparent free space of the root tissues, the roots were washed in 250-mL acid-washed beakers with 0.05M $CaCl_2$ (Calcium Chloride Dehydrate, MERCK, Austria) in an ultrasonic bath (RK510, Brandelin Sonorex, Germany) for ten minutes. Afterwards the roots were sonicated again for 10 minutes in Milli-Q water, put on tissue paper and into paper bags for oven drying at 65℃ for 72 h (UFE 600, Memmert GmbH, Germany).

Plant samples were finely milled using a clean stainless steel grinder (IKA A11 electric coffee grinder, IKA®-Werke GmbH & CO. KG, Germany). Subsamples of 0.2 g were digested in a mixture of 69% HNO_3 (EMPARTA®, ACS, Merck) and 30% H_2O_2 (TraceSELECT® Ultra, Fluka, Sigma Aldrich) (5:1) plus one drop of Iso-Octanole in a closed microwave digestion system (Anton Paar Multiwave 3000, Perkin Elmer). For quality management, two blanks and two certified reference material samples (Oriental Tobacco Leaves, CTA-OTL-1) were included in each microwave run. In a preliminary experiment, microwave settings (Table 4) were tested to ensure full digestion of the willow material (data not shown). The digests were then filtered using 45-µm filter paper (150 mm diameter, Munktell 14/N), collected and filled up with Milli-Q water to 40 ml total volume in 50-mL sample vials.

Table 4: Microwave digestion settings for willow plant material.

	Digestion (1300 Watt)	Cleaning (1300 Watt)
Ramp time	20	20
Holding time	35	20
Cooling time	15	15

The equipment used for harvesting was washed with Milli-Q water after each replicate to avoid cross contamination between control and the treatments while the following sequence was retained: control (C), half sulfur treatment (HS), sulfur treatment (S).

3.3.3.2. Soil

At harvest, mean soil moisture across all treatments and compartments was 75% ± 6 % WHC. For measuring pH and EC, 10 g of soil (<2 mm) on a dry weight basis, were measured in Milli-Q water at a soil:solution ratio of 1:2.5 (w/v) after 2 h of equilibration according to standardized procedures (ÖNORM, 2006).

For determining potentially labile TM fractions, bulk, rhizosphere and plant compartment soils were extracted using $Ca(NO_3)_2$ according to McLaren (2007) as modified by Iqbal et al. (2012), with a soil:solution ratio of 1:5 (m/v). Five gram of soil dwt (<2 mm) and 25 mL of 0.05M $Ca(NO_3)_2$ (Calcium nitrate tetahydrate puriss pa Reag., ACS, Sigma Aldrich) were added to 50 ml acid-washed centrifuge vials (Cs500, Centrifuge Tube, VWR), closed and put into an end-over-end shaker for 2 h at 20 revelations per minute. The extracts were then centrifuged at room temperature for 5 min at 613 x g relative centrifugal force (Multifuge X3R, Fiberlite F14-6x250 LE rotor, Thermo Scientific). After centrifugation, the solution was filtered through 45-µm filter paper (150 mm diameter, Munktell 14/N), collected in sample vials and acidified using 0.3 ml 65% HNO_3 (EMSURE, ISO, Merck). For quality control, four soil reference materials and four blanks were added.

3.3.4. Soil and plant analysis

Soil pore water samples were analyzed for anions (NO_3^-, SO_4^{2-}, Cl^-) in three batches to keep time between freezing and thawing as short as possible. Two different dilutions per sample were measured to cover IC detection limits and to ensure quality control. All samples were measured on IC (DX-500, Dionex).

For trace metal analysis (Mn, Fe, Cu, Zn, Cd and Pb), soil pore water samples, plant digestions and $Ca(NO_3)_2$ extractions were diluted using 1% double sub-boiled HNO_3 and

measured on ICP-MS (Elan 9000 DRCe, Perkin Elmer) using ^{115}In as internal standard. Quality control and blanks were measured after every 10th sample and at the end of each batch. Obtained values were blank-corrected and compared with a second measurement using a different sample dilution. ICP-MS calibrations were assumed as simply linear and measured before and after the batch followed by a homemade quality management standard.

3.4. Statistical evaluation

For the statistical evaluation, multivariate general linear models (GLMs) with repeated measurements were used. GLMs were calculated for element concentrations in the soil solution. For within-subject effects depending on repeated measures (time; time*compartment; time*treatment; time*compartment*treatment) the degrees-of-freedom-corrected Greenhouse-Geisser test was used for the interpretation because prior tests on sphericity failed and the sample size was small. For time-independent, between-subject effects (compartment; treatment; compartment*treatment) a Bonferroni correction was used. Bonferroni is the most conservative correction and therefore appropriate if the equality of error variances is not significant for all measures (Rasch et al., 2006). For evaluating a potential influence of pH decrease on biomass production an ANOVA was calculated after testing the data for normal distribution (Bühl, 2008; Kirkpatrick and Feeney, 2011). Model building and statistical analysis was carried out using SPSS Statistics Version: 20, IBM, with α=0.05.

Graphical illustrations were plotted using Systat Software, SigmaPlot Version 11. To calculate the x-fold increase in the trace metal concentrations in the soil solution of the treatments Eq.(2), and in plant trace metal concentrations Eq.(3) was used.

$$\text{x-fold\_soil} = \frac{\text{mean conc. (treatment, compartment, time)}}{\text{mean conc. in control (compartment, time)}} \qquad \text{Eq.(2)}$$

$$\text{x-fold\_plant} = \frac{\text{mean conc. (treatment, plant tissue)}}{\text{mean conc. in control (plant tissue)}} \qquad \text{Eq.(3)}$$

4. Results and Discussion

4.1. Soil pore water

4.1.1. Soil acidification and sulfate release

Soil solution pH was substantially lowered in the sulfur treatments compared to the control. In both, treatments and compartments, the soil was acidified due to subsequent S^0 oxidation and reached the targeted pH-values after approx. 25 days. At the last sampling time, mean pH values were: 5.7 ± 0.1 (rhizo control), 5.8 ± 0.2 (bulk control), 4.4 ± 0.1 (rhizo HS), 4.8 ± 0.3 (bulk HS), 3.8 ± 0.1 (rhizo S) and 4.2 ± 0.1 (bulk S).

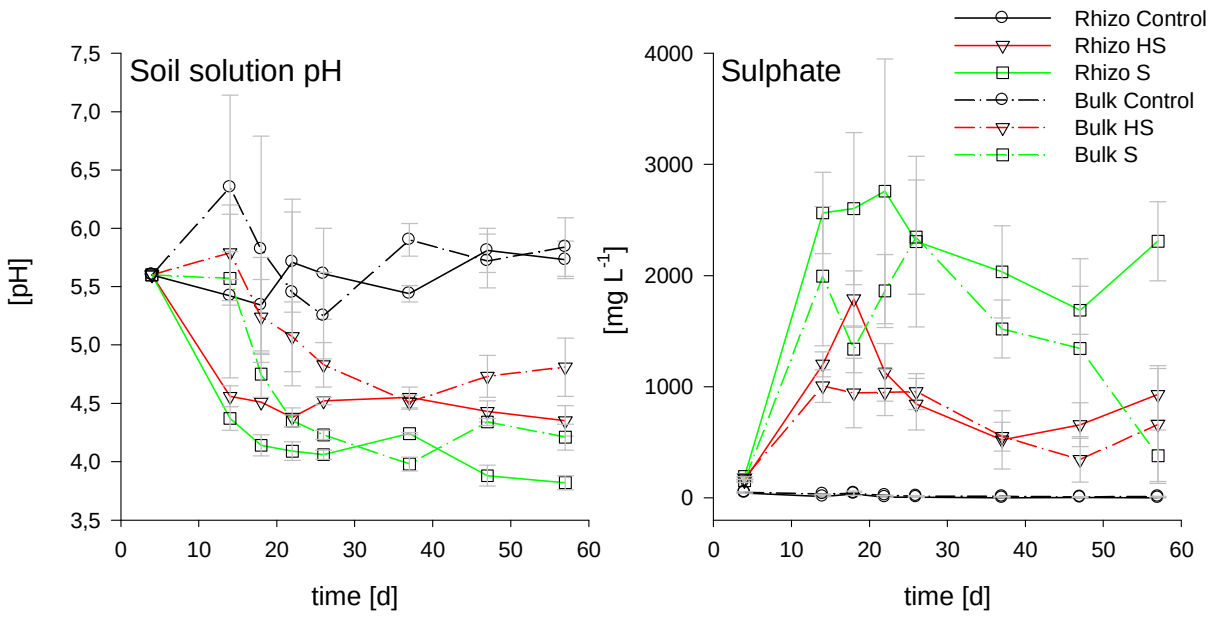


Figure 5: Soil pore water pH and sulfate in root and bulk soil compartments (mean ± SEM) for the Controls (n=3), HS (n=4) and S treatments (n=3).

The repeated measurements showed that time-dependent and time-independent tests for differences in soil solution pH were significant. We observed significant rhizosphere effects on pH in both sulfur treatments at all sampling times (within-subject effects) (Table 5), i.e. significantly different pH between the root and bulk soil compartments. Six out of seven time points showed lower pH values in the rhizosphere compared to bulk soil (Figure 5).

Time-dependent and time-independent tests for sulfate concentrations showed significant differences between the treatments, but not between the compartments. Therefore, differences in S^0 oxidation cannot be associated with plant root interaction instantaneously. However, the decrease in pH is to some extent related to the oxidation of S^0 to SO_4^{2-} (Figure

5). The amount of sulfur added, resulted in three significant different treatments for both pH and SO_4^{2-} (Table 5).

Table 5: Statistical results of GLMs for pH (H^+) and SO_4^{2-}.

	H+		SO4-	
	F	p	F	p
Test of Within-Subject Effects[a,b,d] Greehnouse-Geisser				
time	30.4	.000	9.7	.000
time*compartment	10.1	.000	1.7	**.173**
time*treatment	13.4	.000	3.8	.002
time*compartment*treatment	6.0	.000	.8	**.560**
Tests of Between-Subject Effects[b,c] Bonferroni				
intercept	203.6	.000	115.3	.000
compartment	16.6	.001	3.5	**.082**
treatment	57.3	.000	35.6	.000
compartment*treatment	6.0	.013	1.6	**.235**
Post-hoc test for homogenous subsets[c] (Tukey's-b)				
Control	4.9E-06		18.25	
HS-treatment	2.4E-05		800.55	
S-treatment	6.2E-05		1711.15	

[a] Mauchly's Test on Spericity failed (p<0.05) (data not shown) therefore Greenhouse-Geisser test was used
[b] Bold values are not significant (p>0.05)
[c] Between-Subject factors (Compartment; rhizo n=10, bulk n=10) (Treatment; Control n=6, HS n=8, S n=6)
[d] Within-Subject factors (Measurements 1-8)

The observed rhizosphere effect on pH can be explained by the fact that plants are known to exude protons or hydroxyl ions to compensate excess uptake of cations and anions (Marschner and Marschner, 2012). Since S^0 in soils is primarily microbially oxidized, different microbial communities may have contributed to an increased S^0 oxidation in the rhizosphere and/or were possibly present at a higher population density (Grayston and Germida, 1990; Marschner and Marschner, 2012).

4.1.2. Trace metals in soil solution

Trace metal concentrations of Mn, Zn, Cd and Pb increased significantly in both sulfur treatments, whereas Cu and Fe showed different patterns. The increase in water-soluble TMs by desorption can be explained predominantly due to the sulfur oxidation-triggered soil acidification (Figure 5). However, the TM concentrations increased up to more than one order of magnitude in the root compartments (after 25 days) and can therefore hardly be explained only by acidification effects only. As Iqbal et al. (2012) proposed, biotic S^0 oxidation in partly anaerobic conditions in the rhizosphere with resulting reductive co-dissolution of Zn^{2+} and Cd^{2+} may better explain the unusually high mobilization of TMs in the S^0-amended

rhizosphere compartments. In the S^0-treatments we also observed time-dependent differences in Mn, Zn, Cd and Pb concentrations in the soil solution between rhizosphere and bulk soil (Figure 6). Our results are clearly confirming the rhizosphere effect in the magnification of TM solubility as proposed by Iqbal et al. (2012). Bulk soil concentrations were generally lower and corroborate to the hypothesis of Iqbal et al. (2012), that sulfur oxidizing microorganisms use Mn (oxy)hydroxides and probably to a lower extent Fe compounds as TEA under partly anaerobic conditions in the rhizosphere of *S. smithiana*. Root activities and respiration of rhizosphere-associated microbial communities may have additionally lowered soil redox potential and oxygen availability compared to the bulk soil. Because Mn^{2+} is soluble and predominantly found under reducing conditions, substantially increased Mn concentrations in soil solution are strongly indicating reducing conditions and a low redox potential.

Table 6: Mean x-fold increase in trace metal concentration in the soil pore water for both S^0-treatments (HS, S) compared to the control (C), over time. Grey shaded values are indicating significantly higher increase in the rhizosphere relative to bulk soil. Ratios between rhizosphere and bulk soils were calculated for day 57 to compare relative differences of bioavailable fractions in the soil pore water.

Treatment	t [d]	Mn		Fe		Cu		Zn		Cd		Pb	
		rhizo	bulk	rhizo	bulk	rhizo	bulk	rhizo	bulk	rhizo	bulk	rhizo	bulk
HS / C	4	6	5	2.4	2.1	0.9	0.7	8	2	5	2	13	4
	14	65	26	6	1	0.8	1.3	7	2	6	2	13	5
	18	37	4	3	0	1.1	1.5	5	4	5	4	11	4
	22	229	85	8	3	1.1	0.7	16	3	15	2	31	13
	26	238	74	5	6	2	6	7	3	8	3	17	9
	37	1001	10	9	6	0	1	21	7	36	7	12	8
	47	680	90	13	7	7	8	26	11	53	13	13	9
	57	572	164	19	10	5.6	5.6	26	12	56	16	14	9
		3.5		2.0		1.0		2.2		3.5		1.7	
S / C	4	17	8	7	2	1.1	0.6	10	3	17	3	14	5
	14	126	42	7	2	1.6	1.1	16	4	18	3	19	7
	18	44	3	4	0	1.6	1.3	8	4	7	4	15	4
	22	369	166	9	4	2.1	1.0	32	7	38	4	51	24
	26	673	203	5	7	2.8	2.9	19	11	29	14	30	20
	37	5022	30	9	8	1.4	1.9	91	19	260	24	28	17
	47	2869	240	26	9	7	8	97	18	316	26	25	12
	57	2938	192	33	6	6.2	7.4	103	11	349	12	30	10
		15.3		5.4		0.8		9.1		28.6		3.1	

4.1.2.1. Manganese

Soluble Mn showed the strongest increase in the S^0-treated soils compared to the other measured TMs (Figure 6). The highest Mn concentrations were obtained in the rhizosphere compartment of the S treatment (245.1 ± 15.6 mg L^{-1}, last sampling). Thus, Mn in the rhizosphere increased 3.5-fold in HS- and 15.3-fold in the S treatment relatively to the bulk soil (Table 6). Time-dependent and time-independent tests for differences were significant for both, treatments and compartments (Table 7). The repeated sampling shows, that until the treatments reached the targeted pH (day 25), Mn concentrations in bulk soil and in the rhizosphere increased to a similar extent. Subsequently, Mn concentrations in the bulk soil decreased but continued to increase in the rhizosphere.

Manganese is considered to be a "redox-sensitive element". Its solubility is strongly influenced by pH and redox potential (Eh) in the soil. S^0 oxidation to sulfate may reduce Eh in the soil, primarily due to the oxygen demand for the reaction. The resulting oxygen depletion may result in reduction of Mn (oxy)hydroxides, thus enhancing Mn solubility. Therefore, high Mn concentrations are likely to indicate local reducing and anoxic conditions in the soil. Mn^{2+} is predominantly found under reducing conditions, while Mn^{3+} and Mn^{4+} is found in oxidized environments (Harrison, 2007). We found significantly higher concentrations in the rhizosphere of the S^0-treatments (Table 7), which is indicating that root activities and rhizosphere-associated microorganisms further contributed to the reducing conditions compared to bulk soil.

4.1.2.2. Iron

For Iron, the observed trend was less distinct than for Mn (Figure 6). Time-dependent tests for differences were only significant for the treatments. Time-independent test were significant for compartments and treatments, while post-hoc results showed only two subgroups i.e. C and HS/S (Table 7). Therefore, S^0-amendments had a significant effect on Fe solubility. Fe concentrations in the rhizosphere increased 2-fold in the HS- and 5.4-fold in the S treatment (last sampling) (Table 7).

The amount of sulfur added, affected Fe solubility to a lesser extent than Mn (Table 6). Most likely the conditions in the rhizosphere provided an oxic to anoxic soil environment were electrons in the in the soil solution were plentiful to support NO_3^- and Mn^{4+} reduction and to a lesser extent Fe^{3+} reduction (Sposito, 1994). Microbes likely preferred Mn (oxy)hydroxides as TEA over Fe due to their different redox-potential and the apparent electron activity in solution (McBride, 1994). Additionally, microorganisms may benefit from Mn as TEA, since

coating of Mn can protect them from predation, viral attack and trace metal toxicity (Tebo et al., 2005).

4.1.2.3. Copper

Copper concentrations show different results compared to Mn, Zn, Cd and Pb (Figure 6). Until day 36, soluble copper concentrations were generally low in all treatments (<0.1mg L^{-1}). Thereafter, in the last two sampling points, Cu concentrations strongly increased in both sulfur treatments to a similar extent in rhizosphere and bulk soil relatively to the control (Table 6). Time-dependent and time-independent tests showed significant differences only for the sulfur treatments, not for the control (Table 7). Therefore Cu solubility was influenced by the S^0 amendments but we found no significant difference between rhizosphere and bulk soil and between HS- and S treatment.

4.1.2.4. Zinc

Zinc concentration increased 2.2-fold in the rhizosphere of the HS- and 9.1-fold in the S treatment compared to bulk soil (Table 6). Time-dependent and time-independent tests showed a significant difference for treatments and compartments (Table 7). We found the highest Zn concentrations in the rhizosphere of the S treatment (67.2 ± 1.0mg L^{-1}, last sampling). Thus, Zn solubility was clearly enhanced locally in the rhizosphere of the willows after treating the soil with S^0.

Similar to Mn, Zn concentrations continued to increase in the rhizosphere of both sulfur treatments after day 25 and decreased in the bulk soil (Figure 6). This may indicate a change in the dominating Zn solubilisation mechanism in the rhizosphere, which was related to changes in Mn solubility. As discussed above, conditions in the rhizosphere may have shifted towards a anoxic environment and microbes may have used other TEAs such as NO^{3-} -> Mn^{4+} -> Fe^{3+} (order of electron potential). Our data suggests that, low oxygen availability and low pH in both S^0-treatments have supported reductive dissolution and co-dissolution processes, which were more pronounced in S treatment compared to HS and in the root compartments compared to bulk soil.

4.1.2.5. Cadmium

Cadmium concentrations in the rhizosphere of the treatments increased 3.5-fold in the HS- and 28.6-fold in the S treatment relatively to the control (Table 6). Time-dependent and time-independent differences were significant for treatments and compartments (Table 7). Similar

to Mn and Zn, Cd concentrations were clearly enhanced in the rhizosphere of both S^0-treatments but decreased after 25 days in the bulk soil (Figure 6).

4.1.2.6. Lead

Lead concentrations in both S^0-treatments increased throughout the experiment (Figure 6). In the rhizosphere of the treatments, Pb concentrations increased 1.7-fold in the HS- and 3.1-fold in the S treatment compared to the bulk soil (Table 6). Time-dependent and time-independent tests show a significant difference between treatment and compartment (Table 7).

Similar to Mn, Zn, Cd, we identified a rhizosphere effect for Pb. Concentrations of Pb in the rhizosphere of both S^0-treatments were higher than in bulk soil, indicating a similar mechanism of enhanced Pb solubility as described above for Zn and Cd.

Table 7: Statistical results of GLMs for Mn, Fe, Cu, Zn Cd and Pb.

	Mn		**Fe**		**Cu**		**Zn**		**Cd**		**Pb**	
	F	p	F	p	F	p	F	p	F	p	F	p
Tests of Within-Subject Effects[b,c,e] Greenhouse-Geisser												
Time	12.3	.000	16.5	.000	188.4	.000	16.0	.000	14.7	.000	37.4	.000
time*compartment	10.1	.000	2.7	**.095**	2.5	**.069**	7.6	.001	8.2	.000	10.2	.000
time*treatment	8.7	.000	5.3	.005	36.5	.000	9.9	.000	9.9	.000	17.0	.000
time*compartment*treatment	6.7	.000	2.0	**.143**	.9	**.501**	5.5	.001	6.9	.000	6.8	.000
Tests of Between-Subject Effects[c,d] Bonferroni												
intercept	86.6	.000	430.7	.000	529.2	.000	316.5	.000	239.1	.000	1302	.000
compartment	11.9	.004	4.7	.049	2.0	**.175**	32.0	.000	25.4	.000	182.3	.000
treatment	30.6	.000	44.4	.000	46.7	.000	91.6	.000	86.8	.000	290.9	.000
compartment*treatment	6.3	.011	7.0	.008	.5	**.632**	16.8	.000	18.7	.000	54.6	.000
Post-hoc test for homogenous subsets[a,d] Tukey's-b												
Control	1135.14		405.95		42.25		1987.56		23.49		69.33	
HS-treatment	33995.21		1375.85		129.99		12566.38		140		738.73	
S-treatment	89064.94		1677.17		148.3		31261.53		454.72		1217.93	

[a] grey color indicates classification in the same group
[b] Mauchly's Test on Spericity failed (p<0.05) (data not shown) therefore Greenhouse-Geisser test was used
[c] bold values are not significant (p>0.05)
[d] Between-Subject factors (Compartment; rhizo n=10, bulk n=10) (Treatment; Control n=6, HS n=8, S n=6)
[e] Within-Subject factors (Measurements 1-8)

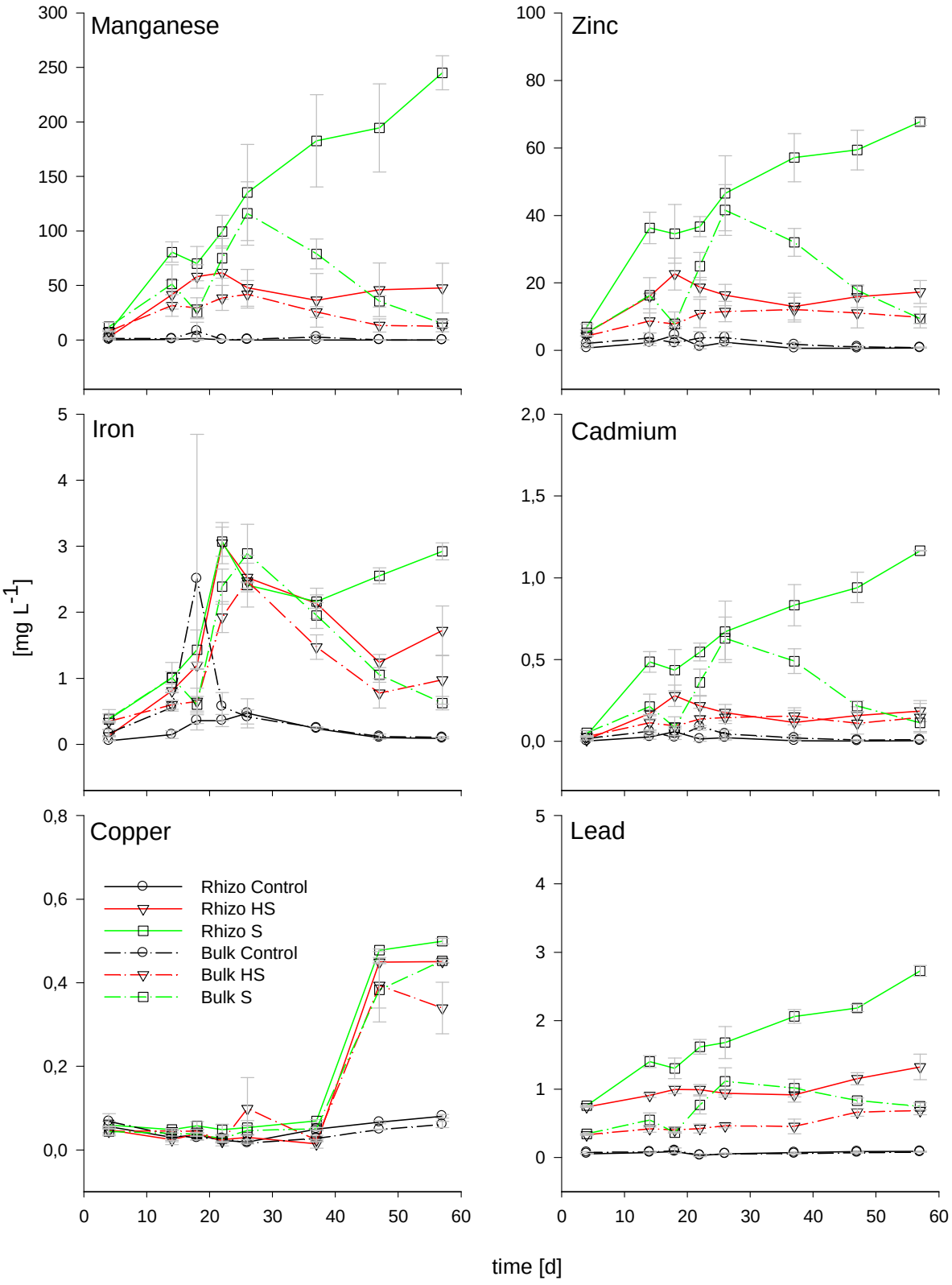


Figure 6: Soil solution concentrations of Mn, Fe, Cu, Zn, Cd and Pb (mean ±SEM) for Control (n=3), HS (n=4) and S (n=3) treatments.

4.1.3. Other anions

4.1.3.1. Nitrate

Nitrate concentrations (NO_3^-) in the soil pore water were generally higher in the control then in the S^0-treatments (Figure 7). Time-dependent and time-independent tests showed significant differences between the treatments (Table 8). Hence, S^0-addition resulted in decreased NO_3^- concentration in the soil solution. Nitrification in both sulfur treatments could have been limited due to partly anaerobic conditions as known from submerged environments e.g. rice paddy soils, were ammonium is likely to be the dominant mineral nitrogen species (Marschner and Marschner, 2012). Also the change in redox potential, indicated by high Mn concentration in both S^0-treatments, could have preceded S^0-oxidizing microbes to use NO_3^- prior or parallel to Mn as TEA. Moreover, plant uptake of NO_3^- is likely to explain the lower NO_3^- concentrations in the rhizosphere compartments observed in both S^0-amended treatments and controls (Figure 7).

However, our data, do not show positive or negative correlations between nitrate and sulfate (ammonium and nitrite concentrations were not measured). Nevertheless, the significantly lower nitrate concentrations in the HS- and S treatment (Table 8), could be due to increased plant assimilation, the introduction of large amounts of sulfate by S^0 oxidation (law of electroneutrality) and the response of microbes to a anoxic soil environment.

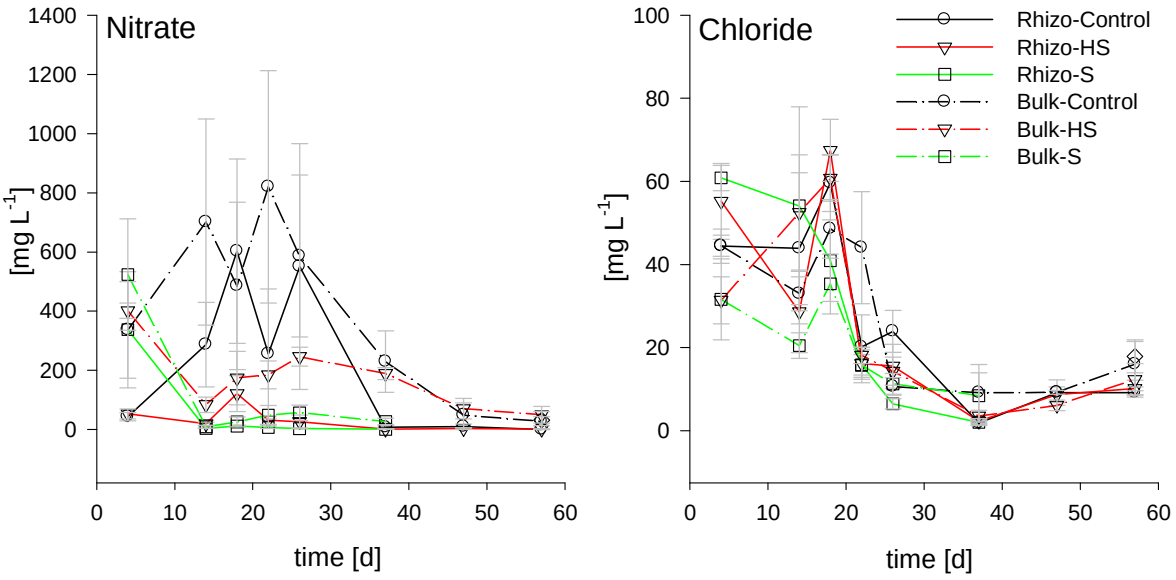


Figure 7: Soil solution concentration of nitrate and chloride (mean ±SEM) for the control (n=3), HS- (n=4) and S (n=3) treatment.

4.1.3.2. Chloride

Chloride concentrations (Cl^-) in the soil pore water declined in all treatments and compartments (Figure 7). Time-dependent and time-independent tests showed no significant difference between treatments and compartments (Table 8). Even though Cl^- can complex Zn^{2+} and Cd^{2+} and thereby increase TM solubility (Schmidt, 2003), it is not regarded to play a role in this study because concentrations were similar in both S^0-treatments and the control. Anion strength was predominantly governed by sulfate in this soil system (Figure 5).

Table 8: Statistical results of GLM for Nitrate and Chloride.

	CL-		**NO3-**	
Tests of Within-Subject Effects[b,c] Greenhouse-Geisser	F	p	F	p
Time	43.3	.000	6.5	.001
time*compartment	2.2	**.109**	1.7	**.184**
time*treatment	1.8	**.136**	4.9	.001
time*compartment*treatment	2.0	**.093**	.8	**.569**
Tests of Between-Subject Effects[d,c] Bonferroni				
Intercept	314.3	.000	25.2	.000
Compartment	1.4	**.258**	3.6	**.080**
Treatment	0.6	**.542**	5.1	.021
compartment*treatment	1.2	**.322**	.4	**.667**
Post-hoc test for homogenous subsets[a,e] Tukey's-b				
Control	22.72		311.91	
HS-treatment	25.22		103.6	
S-treatment	26.73		71.55	

[a] grey color indicates classification in the same group
[b] Mauchly's Test on Spericity failed (p<0.05) (data not shown) therefore Greenhouse-Geisser test was used
[c] bold values are not significant (p>0.05)
[d] Between-Subject factors (Compartment; rhizo n=10, bulk n=10) (Treatment; Control n=6, HS n=8, S n=6)
[e] Within-Subject factors (Measurements 1-8)

4.2. $Ca(NO_3)_2$ soil extraction

The $Ca(NO_3)_2$ soil extraction was employed as a standard procedure to compare the extractable TM concentrations in the rhizobox compartments to the results obtained from the last soil pore water sampling (Figure 8).

Concentrations of Mn, Zn, Cd and Pb declined in all treatments with increasing distance to the roots (bulk soil < rhizosphere compartment < root compartment) and in the treatments (control < HS < S) (Figure 8). This result is in line with soil pore water sampling data. However, the corresponding concentrations in the soil pore waters are generally lower as those measured in $Ca(NO_3)_2$ (Figure 6, Figure 8).

$Ca(NO_3)_2$-extractable Fe concentrations showed only small differences in the root, rhizosphere and bulk soil compartment for all treatments (Figure 8). Also, mean Fe concentrations were generally higher in the $Ca(NO_3)_2$ extraction compared to the corresponding concentrations in the soil pore water (Figure 6). We observed relatively small mean concentrations for the control in the soil pore water sampling compared to the $Ca(NO_3)_2$ extraction. Probably, $Ca(NO_3)_2$-extractions over-estimated soluble Fe concentrations.

Different to the other TMs, Cu concentrations were higher in the rhizosphere compartments of both S^0-treatments compared to the root compartments (bulks soil < root compartment < rhizosphere compartment). Also, mean $Ca(NO_3)_2$-extractable Cu was generally lower in all treatments compared to the corresponding soil pore water concentrations. The low concentrations in the root compartment of the S^0-treatments could be explained by increased uptake of Cu by *S. smithiana* (4.4.2.3). However, as mentioned in (4.1.2.3) we did not find a significant difference for the compartments in the time-independent test for the Cu concentrations (Table 7).

The results of the $Ca(NO_3)_2$ soil extraction for the target TMs are generally similar to the results from the pore water sampling. We observed differences only for soluble Fe and Cu, compared to the soil pore water sampling. The different results might be explained by methodological differences, as soluble Fe and Cu in soil pore water are generally low.

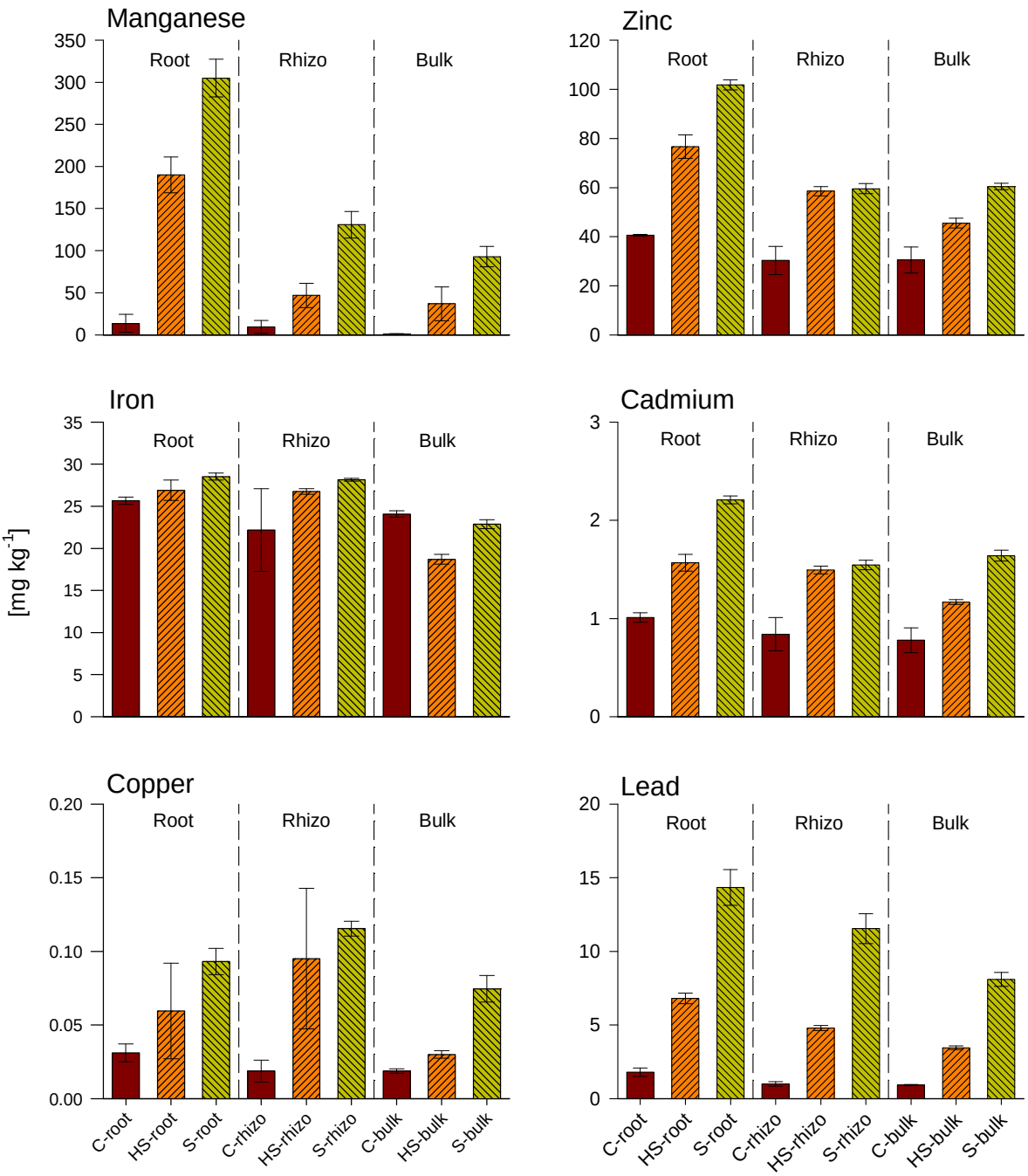


Figure 8: $Ca(NO_3)_2$ extractable concentrations of soil (ARNB-10) after termination of the experiment for root, rhizosphere, and bulk soil compartment (mean, ±SEM) for Control (n=3), HS- (n=4), S (n=3) treatment.

4.3. Rhizosphere effects on trace metal solubiliity

As shown above (Figure 6), soil pore water concentrations of Mn, Zn, Cd and Pb in the rhizosphere were significantly higher than in the bulk soil of the S^0-treatments (Table 7). The elevated TM concentrations in the rhizosphere compartments support the hypothesis that, additionally to the action of protons, rhizosphere processes such as root exudation and root-associated microbial communities were involved in the magnification of TM solubility in the soil.

Plants and microbes can control TM solubility. In the rhizosphere, root activities and microbial processes can either support or compete with each other. Roots can increase or decrease TM solubility via uptake mechanisms, properties of their root system and root activities (Wenzel, 2009). Rhizosphere-associated microorganisms can contribute to the enhanced TM solubility by mobilizing TMs through autotrophic and heterotrophic metabolisms, chelation by microbial metabolites and the release of siderophores. Microbial activity can also lead to dissolution of insoluble TM compounds e.g. minerals and oxides and desorption from exchange sites. Microorganisms can solubilize TMs by reduction and oxidation processes while TM solubilization increases with simultaneous reduction of Fe^{3+} and Mn^{4+} (Gadd, 2004).

As mentioned in (4.1.2), high Fe^{2+} and Mn^{2+} solubility in the rhizosphere is indicating a low redox potential with resulting reductive dissolution processes under anoxic conditions. Since Mn oxides are next to oxygen some of the strongest naturally occurring oxidation agents in the environment, they participate in numerous redox and sorption reactions and can control the distribution of TMs (Tebo et al., 2005). When oxygen concentrations deplete upon S^0 oxidation to SO_4^{2-} additionally, root and microbial respiration contribute to lower the redox potential locally in the rhizosphere. Thus, microbial reduction of oxidized species subsequently follows the order: O_2, NO_3^-, Mn^{3+}, Mn^{4+}, Fe^{3+} and SO_4^{2-} (Mansfeldt, 2004). Soluble Fe in soils is generally low because its redox potential is lower compared to Mn (McBride, 1994). This may explain the fact that the observed increase in Fe concentrations in the soil pore water was less distinct compared to Mn in the S^0-treatments (Figure 6, Figure 10).

In this study we did not measure redox potential directly due to experimental limitations but we were able to observe the reduction of Mn (oxy)hydroxides and the consequent effects on the biogeochemistry of Zn, Cd and Pb (Tebo et al., 2005). In both S^0-treatments, plant root activities increased TM solubility and influenced soil redox potential and pH additional to the S^0 oxidation. However, in the S treatment, rhizosphere effects were more pronounced compared to the HS treatment. We observed linear correlations between protons (H^+) and

trace metal concentrations in bulk soil and rhizosphere of *S. smithiana*. These results visualize rhizosphere effects due to the higher coefficients of determination for correlations between H^+ activity and TM concentrations in the rhizosphere compared to bulk soil. Manganese, Zn, Cd and Pb concentrations in the rhizosphere were influenced by the root activities and reductive dissolution (Figure 10).

Sulfur-oxidizing bacteria may use Mn (oxy)hydroxides as TEA for bacterial respiration and dissolves Mn and/or co-dissolve other TMs (McBride, 1994; Sposito, 1994; Iqbal et al., 2012). Both S^0-amendments enhanced TM solubility by (i) acidification and proton activity, (ii) reductive dissolution of Mn due to electron transfer during S^0 oxidation. With increasing amount of S^0, the effect was more pronounced (lower pH, higher SO_4^{2-} concentration, lower NO_3^- concentrations and higher, sustained TM solubility). These effects of S^0 oxidation, were further enhanced by rhizosphere processes lowering the redox potential by oxygen depletion extent through root respiration and microbial activities (Figure 9).

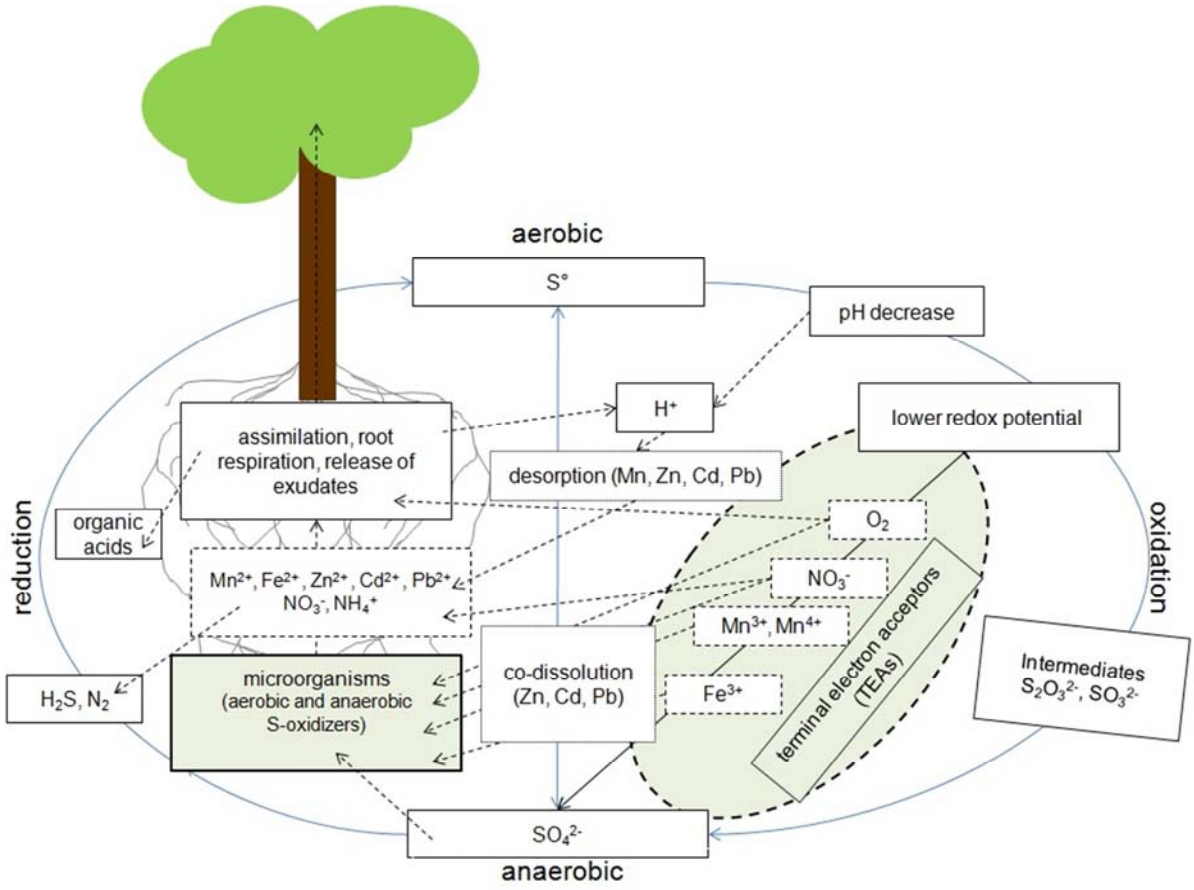
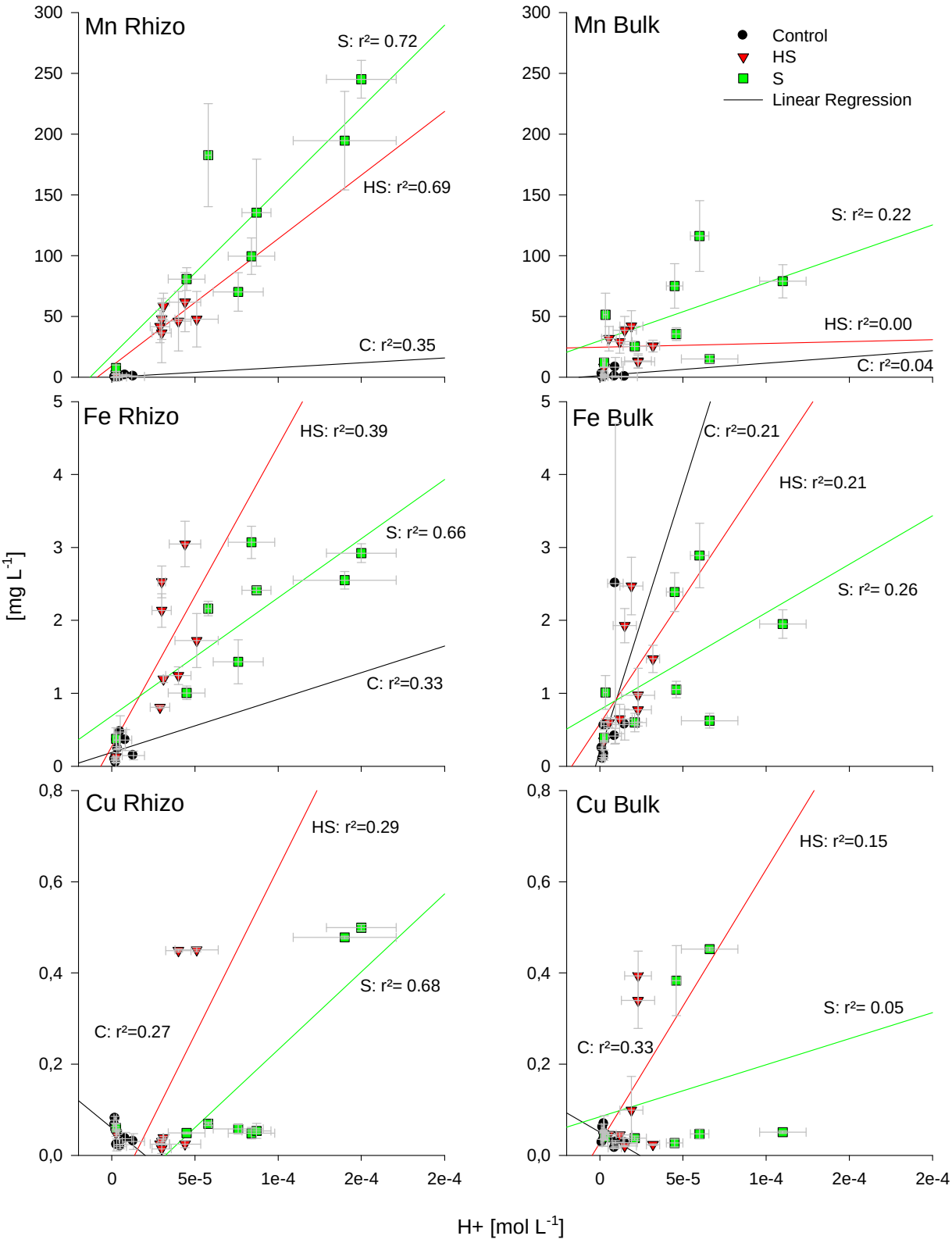


Figure 9: Sulfur oxidation in the rhizosphere and related biogeochemical processes of TM solubilisation.



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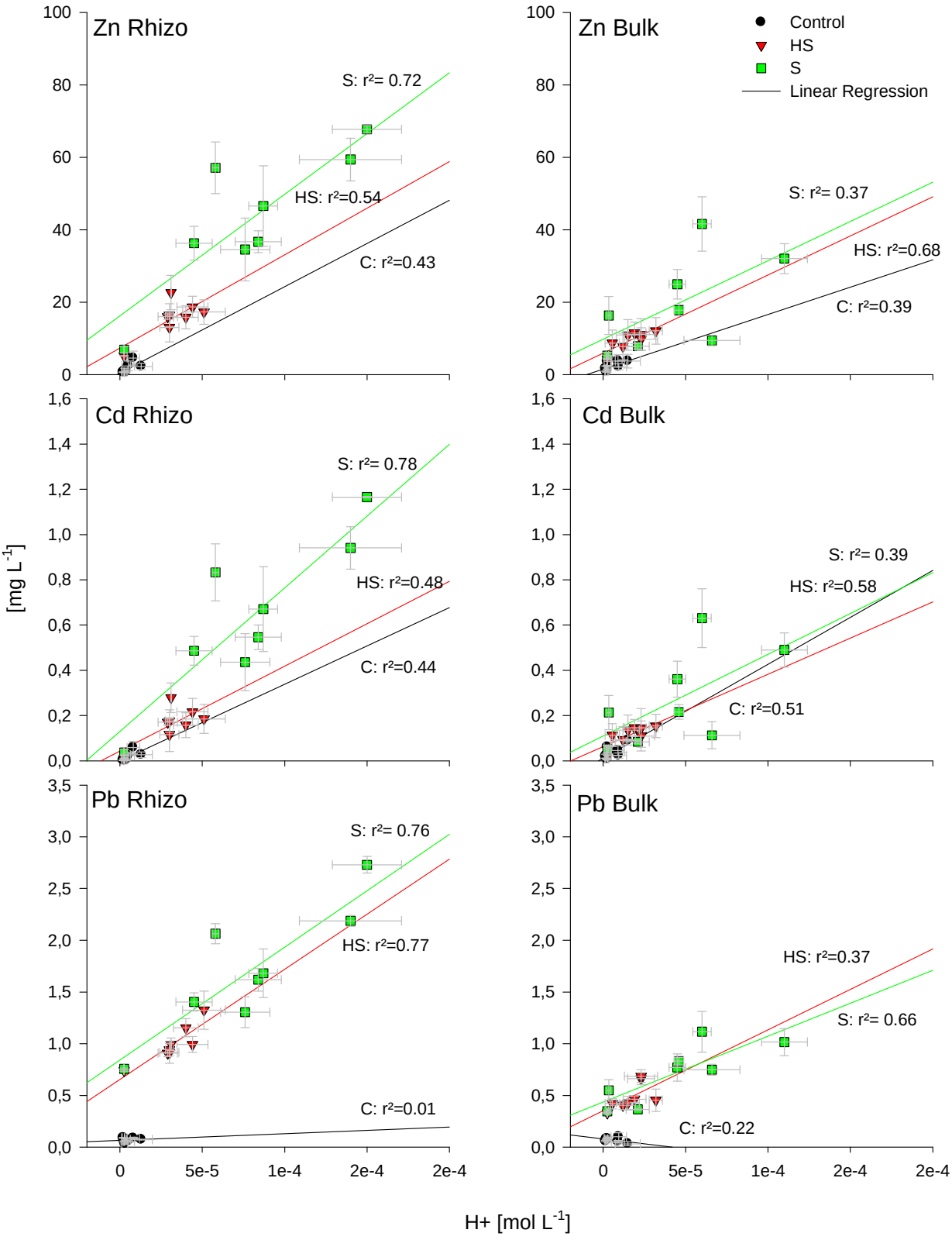

Figure 10: Correlation plots of proton activity in the soil pore water (H^+) and water-soluble TM concentrations obtained in the rhizosphere and bulk soil compartment, means and error bars ±SEM for control (n=3), HS- (n=4) and S (n=3) treatment.

4.4. Plant responses to elemental sulfur application

4.4.1. Biomass production

Plant growth during the experiment and biomass production was similar for all treatments (Figure 11). Results from the ANOVA showed no significant difference of the total biomass between the treatments. Also, test results for the separate plant parts (root, twig and leaf biomass) showed no significant differences between the treatments. We found no visible signs of Mn, Al or Cu toxicity or other negative effects due to the low pH in the HS- and S treatment.

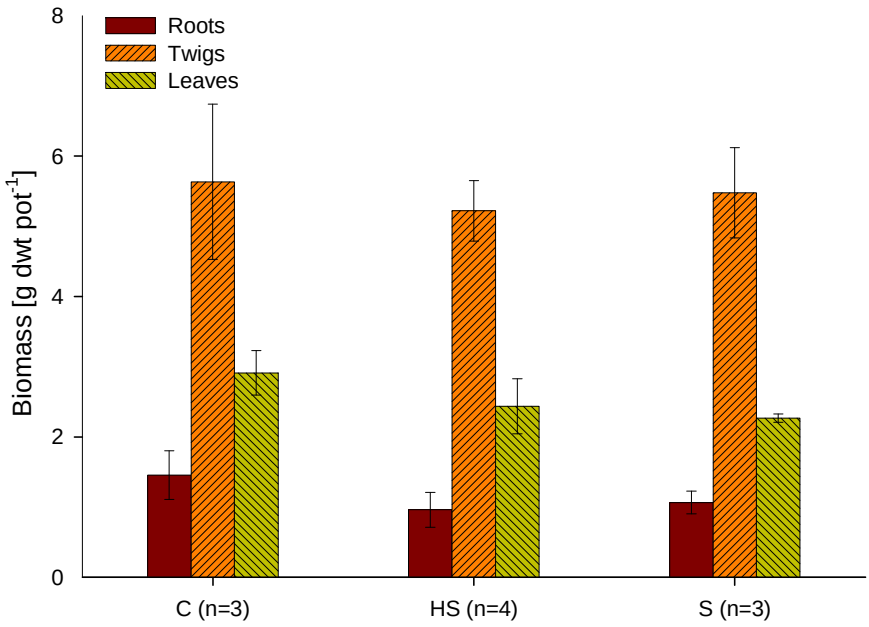

Figure 11: Biomass of *S. smithiana* after harvest for control, HS- and S treatment (mean ±SEM).

As mentioned above, two plants died during the experiment and had to be removed. Possible causes may have been climatic conditions such as partly low air humidity and temporary high air temperatures in the greenhouse (Table 3).

4.4.2. Trace metal accumulation in willows

TM concentrations in *S. smithiana* tissues corresponded well to the TM solubility in the soil pore water. Plant accumulation of Mn, Fe, Zn, Cd and Pb in both sulfur treatments was enhanced compared to the controls (Figure 12). We found major proportions of Mn, Zn and Cd in the leaves while Fe, Cu and Pb accumulated predominantly in the roots. Only minor amounts of TMs were stored in the twigs.

4.4.2.1. Manganese

Manganese concentrations in leaves strongly increased in the HS- and S treatment compared to the control (Figure 12). In the HS treatment, a 5.5-fold accumulation in leaves and twigs and a 2-fold accumulation in the roots was found. In the S treatment, Mn concentrations increased about 10-fold in leaves and twigs and 5-fold in the roots (Table 9).

We found the highest Mn concentrations of 5810 ± 594 mg kg^{-1} dwt in leaves of the S treatment which is in line with the high concentrations in the soil pore water (Figure 12, Figure 6). Foliar concentrations of the control plants were 574 ± 364 mg kg^{-1} dwt, where plant toxicity is commonly found (Adriano, 2001). In plants, common Mn toxicity symptoms on leaves are Fe chlorosis and brown spots, leaf puckering, necrotic brown spots and an uneven distribution of chlorophyll in older leaves. Also browning of the roots can occur and increased Fe uptake by the plants (Kabata-Pendias, 2011). However, *S. smithiana* did not show any symptoms of Mn toxicity and seems to tolerate the high concentrations in the soil and its accumulation in plant tissues.

4.4.2.2. Iron

Iron accumulation in the leaf biomass increased 1.7-fold in the HS- and 3.0 in the S treatment compared to the control plants (Table 9). In the twigs, only small differences were found between all treatments (Figure 12). In the root biomass, Fe concentrations decreased in both S^0-treatments (Table 9). We found the highest concentrations of Fe in the roots of the control plants (5600 ± 665 mg kg^{-1} dwt). Due to the high concentrations of Fe in the roots, it is likely that Fe bioavailability was higher than the soluble Fe we were able to determine in the soil pore water, pointing to the importance of Fe replenishment from the solid phase and the action of siderophores (Ammari and Mengel, 2006; Kabata-Pendias, 2011; Marschner and Marschner, 2012).

4.4.2.3. Copper

Copper concentrations increased in the leaf biomass of the HS treatment 1.1-fold but decreased 1.4-fold in the S treatment compared to the control (Table 9). In the twigs, we found only small differences between all treatments (Figure 12). Copper concentrations in the roots increased 1.4-fold in the HS- and 2.8-fold in the S treatment compared to the control (Table 9).

Cu concentrations in the root biomass did not correspond to the concentrations in the soil pore water. Soluble Cu was generally low in all treatments of the soil pore water sampling. Copper interacts with different other TMs e.g. Mn, Fe, Zn, Cd within plant tissues and in external root media in terms of uptake and transport processes (Kabata-Pendias, 2011).

Table 9: Mean ratio between TM concentrations in plant tissues of HS- and S treatment compared to the control.

Treatment	Part of plant	Mn	Fe	Cu	Zn	Cd	Pb
HS / C	leaves	5.7	1.7	1.1	2.2	1.5	1.9
	twigs	5.4	1.5	1.1	2.1	1.3	2.6
	roots	1.9	0.7	1.4	1.4	1.0	2.2
S / C	leaves	10.1	3.0	0.7	3.3	1.7	3.5
	twigs	9.7	1.6	1.4	2.3	1.4	4.4
	roots	4.9	0.6	2.8	2.1	1.9	2.3

4.4.2.4. Zinc

Zinc concentrations increased in the leaf biomass 2.2-fold in HS- and 3.3-fold in the S treatment compared to the control (Table 9). During the experiment, *S. smithiana* accumulated mean concentrations in the leaf biomass of 1170 ± 278 mg kg^{-1} dwt in the control, 2551 ± 128 mg kg^{-1} dwt in HS- and 3846 ± 87 mg kg^{-1} dwt in the S treatment. In the twigs, concentrations in the S^0-treatments were higher compared to the control but we found only small differences between HS and S (Figure 12). In the roots, Zn concentrations increased 1.4-fold in the HS- and 2.1-fold in the S treatment compared to the control (Table 9).

In the study of Iqbal et al. (2012), Zn concentrations were lower in the leaves of the control and the comparable S^0-treatment to this study (S treatment). They found concentrations of approx. 700 mg kg^{-1} dwt in the control (ARNB, *S. smithiana*) and approx. 1000 mg kg^{-1} dwt in the sulfur treatment (ARNB + 1.02 g kg^{-1} S^0, *S. smithiana*). Leaf concentration differences between Iqbal et al. (2012) and our study may result from differences in experimental conditions. Here, the experimental duration was 61 days and total leaf biomass approx. 2.5 g dwt pot^{-1} compared to 150 days and approx. 4 g dwt pot^{-1} in Iqbal et al. (2012). We used (<2 mm) sieved soil for the rhizobox while Iqbal et al. (2012) used (<5 mm) sieved soil for the pot experiment.

Interestingly, we found even in the HS treatment, higher foliar Zn concentrations compared to the S^0-treatment in Iqbal et al. (2012). This might be explained by the high root density in the rhizobox or differences in growth conditions (temperature, air and soil moisture, etc.) and consequent differences in soluble element concentrations, leading to different uptake rates of the plant. As discussed in Puschenreiter et al. (2013), foliar concentrations of Zn and Cd in *S. smithiana* vary between comparable studies and could also be explained due to a "concentration effect" depending on total biomass production.

4.4.2.5. Cadmium

Cadmium concentrations increased in the leaf biomass 1.5-fold in HS- and 1.7-fold in the S treatment compared to the control (Table 9). Mean concentrations of Cd in *S. smithiana* leaves were 21.4 ± 5.7 mg kg^{-1} dwt in the control, 32.4 ± 2.7 mg kg^{-1} dwt in HS- and 36.9 ± 3.4 mg kg^{-1} dwt in the S treatment. Cadmium concentrations in the twigs were higher in the S^0-treatments compared to the control but we found only small differences between HS and S (Figure 12). Therefore, the amount of S^0 in the HS treatment seems to be sufficient for enhancing Cd phytoextraction. Higher amounts of S^0 seems to enhance the accumulation of the other TMs but not Cd. In contrast, in the root biomass we observed 1.9-fold concentrations in the S treatment compared to the control. Control and HS treatment had similar concentrations of Cd in the willow roots (Table 9).

In the study of Iqbal et al. (2012), Cd concentrations in the leaf biomass of the willow were approx. 40 mg kg^{-1} dwt in the control and approx. 60 mg kg^{-1} dwt in the sulfur treatment. Here, we found lower Cd concentrations in the willow leaves compared to Iqbal et al. (2012). As mentioned above, differences may result from various factors of experimental conditions. Zn solubility strongly increased in the soil pore water of both S^0-treatments and competition between Zn and Cd may have occurred in the HS and S treatment. Since Cd is a non-essential TM and mainly taken up by the same transporters as Zn and Ca, Cd uptake is strongly influenced by competing Ca^{2+} and Zn^{2+} cations and protons (Puschenreiter et al., 2013).

We cannot relate the differences in foliar Cd concentration between Iqbal et al. (2012) and our study to a specific cause, since several factors may have influenced Cd accumulation in the plant. Although, Cd solubility in the soil pore water was clearly increased in the HS and S treatments with increasing amount of S^0, accumulation in willow leaves responded differently and was not further enhanced in S compared to HS in relevant amounts.

4.4.2.6. Lead

Lead concentrations increased in the leaf biomass 1.9-fold in HS- and 3.5-fold in the S treatment compared to the control (Table 9). *S. smithiana* accumulated mean concentrations of Pb in the leaf biomass of 5.3 ± 1.1 mg kg^{-1} dwt in the control, 11.4 ± 1.7 mg kg^{-1} dwt in HS- and 21.6 ± 3.3 mg kg^{-1} dwt in the S treatment. Concentrations in the twigs also increased with increasing amount of S^0 and were generally higher in the twigs compared to the leaves (Figure 12). We found the highest concentrations of Pb in the root biomass of the S^0-treatments. Root concentrations were 485 ± 60 mg kg^{-1} dwt in the control, 1007 ± 52 mg kg^{-1} dwt in HS- and 1130 ± 124 mg kg^{-1} dwt in the S treatment.

We found major proportions of Pb in the root biomass of *S. smithiana*. Since the main process for Pb accumulation in the roots is its deposition i.e. as Pb-pyrophosphate along the cell walls it is known that Pb accumulates predominately in the roots. Therefore, Pb translocation to above ground tissues is generally limited due to Pb deposition in cell walls outside the plasmalemma as Pb precipitates and Pb crystals (Kabata-Pendias, 2011). Our results are showing similar consequences for *S. smithiana*. Nevertheless, concentrations in roots, twigs and leaves were elevated in both S^0-treatments.

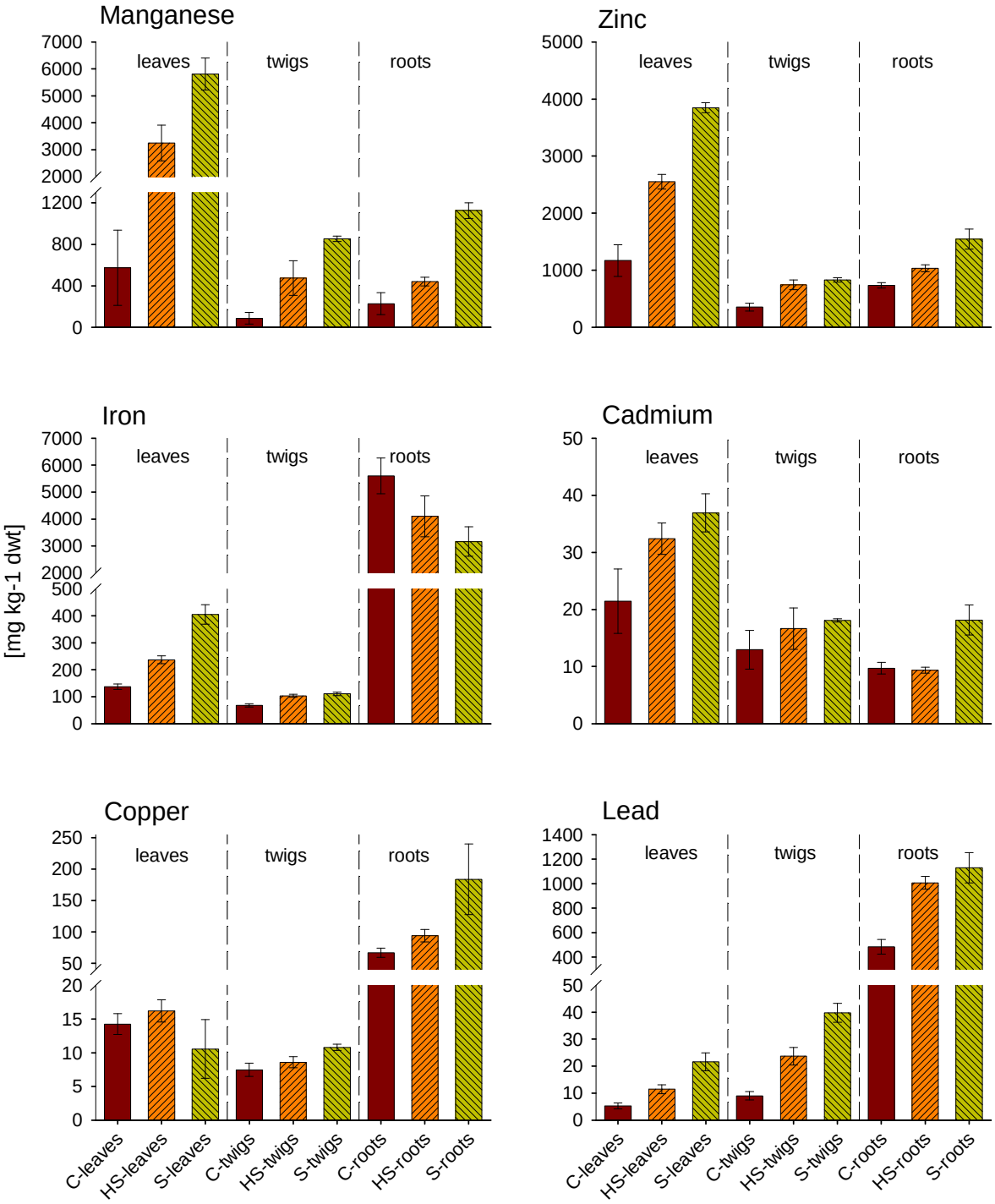

Figure 12: Trace metal concentrations (mean ±SEM) in *S. smithiana* plant tissues (leaves, twigs and roots) grown on ARNB-10, 61 days after planting in the control (n=3), HS- (n=4), S (n=3) treatments.

5. Conclusion

We found significantly lower pH in both S^0-treatments and compartments (root and bulk soil) and significantly higher SO_4^{2-} concentrations in both S^0-treatments compared to the control. TM solubility of Mn, Fe, Zn, Cd and Pb was strongly increased in both S^0-treatments compared to the control. Additionally, we found significantly higher TM concentrations in the S^0-treated rhizosphere compared to bulk soil. Continuously increasing Mn, Zn, Cd and Pb concentrations were still observed in the rhizosphere of the HS and S treatment after 25 days while concentrations in the bulk soil decreased until termination of the experiment. The rhizosphere effects on Mn, Zn, Cd and Pb solubility were more pronounced in the S treatment compared to HS.

Elemental sulfur oxidation caused (i) decreased soil pH due to acidification with H_2SO_4 and (ii) reduced redox potential due to oxygen depletion in the treatments. In the rhizosphere of *S. smithiana*, plant roots and microbial communities apparently further contributed to the reducing conditions by root respiration and uptake of nutrients e.g. NO_3^-. The reducing conditions in the rhizosphere induced dissolution of Mn oxides and a corresponding co-dissolution of Zn, Cd and Pb. Due to the low oxygen supply, (oxy)hydroxides of Mn and to a less extent also Fe served as TEA for microbial communities.

In both S^0-treatments, TM phytoextraction was clearly enhanced compared to the control. *Salix smithiana* was able to accumulate high concentrations of Mn, Zn and Cd in the leaf biomass of the HS- and S treatment without signs of toxicity while Fe, Cu and Pb were predominately stored in the roots. Soil pore water concentrations of Mn, Zn and Pb in the HS- and S treatment corresponded well to the increased plant accumulation. Interestingly, foliar Cd concentrations showed different results compared to Mn and Zn and increased to a similar extent in the HS- and S treatment compared to the control.

Expanding the findings of Iqbal et al. (2012), we were able to determine limitations in sulfate release over time in the HS treatment and decreasing NO_3^- concentrations in both S^0-treatments. Possibly, S^0-oxidizing microbes used NO_3^- prior or parallel to Mn as TEA. We also showed that the amount of S^0 influences Cd accumulation in *S. smithiana* in a different manner compared to other TMs. Our data suggests that the capacity for Cd accumulation was already saturated when applying relatively low amounts of S^0 as used in the HS treatment. However, oxygen availability, the behavior of redox sensitive elements and S^0-oxidizing bacteria needs to be investigated in more detail to further elucidate biogeochemical processes in the rhizosphere after S^0 application and their effects on TMs in more detail.

6. List of Tables

7. List of Figures

8. References

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9. Annex

The following tables show detailed information of all measured data and statistical test results used in this thesis.

Table A1: $Ca(NO_3)_2$ soil extraction analysis. Trace metal means ±SEM. Control(n=3); HS(n=4); S(n=3).

Treatment	Compartment	Mn [mg kg-1]		Fe [mg kg-1]		Cu [mg kg-1]		Zn [mg kg-1]		Cd [mg kg-1]		Pb [mg kg-1]	
		mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM
Control	Bulk	0.9	0.5	24.1	0.4	0.02	0.00	30.5	5.3	0.78	0.13	0.92	0.04
	Rhizo	9.4	7.6	22.2	4.9	0.02	0.01	30.3	5.7	0.84	0.17	1.00	0.16
	Root	13.7	10.6	25.7	0.4	0.03	0.01	40.6	0.3	1.01	0.05	1.79	0.28
HS	Bulk	37.0	20.0	18.7	0.6	0.03	0.00	45.5	2.0	1.17	0.03	3.45	0.13
	Rhizo	46.9	14.5	26.8	0.3	0.10	0.05	58.5	1.9	1.49	0.04	4.79	0.17
	Root	190.0	21.3	26.9	1.2	0.06	0.03	76.6	4.8	1.57	0.09	6.80	0.36
S	Bulk	92.9	12.0	22.9	0.5	0.07	0.01	60.4	1.3	1.64	0.06	8.10	0.48
	Rhizo	130.8	15.9	28.2	0.2	0.12	0.01	59.5	2.1	1.54	0.05	11.54	1.00
	Root	305.0	22.3	28.5	0.4	0.09	0.01	101.8	2.0	2.21	0.04	14.33	1.22

Table A2: *S. smithiana* analysis. Biomass and Trace metal means ±SEM. Control(n=3); HS(n=4); S(n=3).

Treatment	Plant tissues	Mn [mg kg^{-1}]		Fe [mg kg^{-1}]		Cu [mg kg^{-1}]		Zn [mg kg^{-1}]		Cd [mg kg^{-1}]		Pb [mg kg^{-1}]		Biomass [g dwt pot^{-1}]	
		mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM
Control	roots	228.8	106.0	5600.0	665.4	66.7	7.5	736.6	48.1	9.7	1.0	485.2	60.1	1.46	0.35
	twigs	87.9	55.6	67.7	5.8	7.5	1.0	354.3	71.1	12.9	3.4	9.0	1.6	5.63	1.11
	leaves	574.1	363.5	136.9	9.8	14.2	1.5	1169.0	278.6	21.4	5.7	5.3	1.1	2.91	0.32
HS	roots	442.1	41.9	4104.8	756.1	93.9	10.1	1034.3	63.7	9.4	0.5	1006.7	51.5	0.96	0.25
	twigs	474.9	167.1	103.4	5.4	8.6	0.8	744.6	85.3	16.6	3.6	23.7	3.3	5.22	0.43
	leaves	3248.6	662.1	236.6	14.8	16.2	1.6	2550.6	128.7	32.4	2.7	11.4	1.7	2.44	0.39
S	roots	1126.8	76.0	3166.7	543.6	183.7	56.3	1545.2	175.5	18.1	2.6	1129.9	124.0	1.07	0.16
	twigs	854.2	27.5	110.6	6.4	10.8	0.5	828.9	37.1	18.1	0.3	39.8	3.5	5.48	0.64
	leaves	5808.6	593.5	405.0	36.0	10.5	4.3	3845.9	87.0	36.9	3.4	21.6	3.3	2.27	0.06

Table A3: Soil pore water analysis. Trace metals, pH and anions means ±SEM. Control(n=3); HS(n=4); S(n=3).

Treat-ment	ID	Time [d]	Mn [mg L-1]		Fe [mg L-1]		Cu [mg L-1]		Zn [mg L-1]		Cd [mg L-1]		Pb [mg L-1]		pH		CL- [mg L-1]		NO3- [mg L-1]		SO4- [mg L-1]	
			mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM
Control Rhizo	R1C	4	0.43	0.05	0.05	0.00	0.06	0.00	0.69	0.02	0.00	0.00	0.06	0.01	5.60	-	44.63	3.93	43.58	13.87	43.58	3.56
	R2C	14	0.64	0.40	0.15	0.06	0.03	0.02	2.25	0.84	0.03	0.01	0.07	0.00	5.42	0.70	44.12	18.06	286.7	142.5	11.61	5.36
	R3C	18	1.58	0.68	0.36	0.14	0.04	0.01	4.54	1.92	0.06	0.03	0.09	0.01	5.34	0.41	61.83	7.11	625.7	311.5	35.38	13.52
	R4C	22	0.27	0.22	0.36	0.09	0.02	0.01	1.14	0.64	0.01	0.01	0.03	0.01	5.71	0.43	15.38	9.66	257.3	219.6	5.07	2.24
	R5C	26	0.20	0.08	0.47	0.22	0.02	0.00	2.39	1.33	0.02	0.01	0.06	0.01	5.61	0.39	24.54	5.19	564.8	414.8	5.04	4.03
	R6C	37	0.04	0.00	0.24	0.00	0.05	0.00	0.62	0.08	0.00	0.00	0.07	0.01	5.44	0.07	1.96	0.69	7.46	5.59	0.51	0.36
	R7C	47	0.07	0.01	0.10	0.02	0.07	0.00	0.61	0.02	0.00	0.00	0.09	0.00	5.81	0.19	9.20	3.04	9.58	8.93	2.32	1.38
	R8C	57	0.08	0.02	0.09	0.01	0.08	0.00	0.66	0.04	0.00	0.00	0.09	0.00	5.73	0.16	9.15	0.49	0.10	0.07	0.03	0.01
HS Rhizo	R1HS	4	2.57	0.24	0.13	0.01	0.05	0.01	5.15	0.13	0.01	0.00	0.74	0.01	5.60	-	57.96	9.61	55.34	16.54	155.3	12.0
	R2HS	14	41.60	13.28	0.81	0.02	0.02	0.00	16.00	0.74	0.17	0.01	0.91	0.03	4.56	0.09	29.66	9.41	21.51	6.46	1201	112
	R3HS	18	58.28	10.70	1.19	0.05	0.04	0.00	22.65	4.74	0.28	0.07	0.99	0.06	4.51	0.00	70.25	9.17	127.33	60.70	1788	254
	R4HS	22	61.83	24.37	3.05	0.31	0.02	0.01	18.69	2.90	0.22	0.06	0.99	0.08	4.38	0.08	16.71	3.40	30.86	13.80	1129	260
	R5HS	26	47.89	16.88	2.53	0.22	0.03	0.01	16.38	3.18	0.18	0.05	0.94	0.06	4.52	0.03	16.12	5.40	26.24	22.62	843	233
	R6HS	37	36.43	24.46	2.14	0.23	0.01	0.01	13.01	3.99	0.12	0.07	0.92	0.10	4.55	0.09	2.41	0.76	1.19	0.68	521	261
	R7HS	47	46.11	24.46	1.24	0.12	0.45	0.00	15.85	3.22	0.16	0.05	1.15	0.09	4.43	0.09	8.69	1.88	3.50	2.35	657	197
	R8HS	57	47.74	22.81	1.72	0.37	0.45	0.00	17.29	3.39	0.19	0.06	1.32	0.19	4.35	0.13	10.17	2.05	1.07	0.72	931	233
S Rhizo	R1S	4	7.45	2.69	0.38	0.15	0.06	0.01	6.89	0.87	0.04	0.01	0.76	0.01	5.60	-	61.59	3.71	378.23	196.77	190.5	28.2
	R2S	14	80.70	9.35	1.01	0.09	0.05	0.00	36.28	4.68	0.49	0.06	1.40	0.09	4.37	0.10	54.13	23.80	3.77	0.52	2562	366
	R3S	18	70.14	15.75	1.43	0.30	0.06	0.01	34.53	8.70	0.44	0.13	1.30	0.15	4.14	0.09	41.90	8.94	11.76	3.48	2602	684
	R4S	22	99.53	15.03	3.07	0.22	0.05	0.01	36.67	2.99	0.55	0.05	1.62	0.11	4.09	0.08	16.33	2.56	7.43	3.87	2759	1191
	R5S	26	135.32	44.00	2.41	0.05	0.05	0.02	46.54	11.10	0.67	0.19	1.68	0.23	4.06	0.04	6.75	1.32	2.38	1.15	2305	768
	R6S	37	182.68	42.30	2.16	0.10	0.07	0.00	57.11	7.14	0.83	0.13	2.06	0.10	4.24	0.01	2.04	0.55	0.63	0.35	2034	415
	R7S	47	194.58	40.41	2.55	0.12	0.48	0.00	59.37	5.88	0.94	0.09	2.19	0.05	3.88	0.09	22.12	10.98	19.90	16.63	1687	217
	R8S	57	245.09	15.59	2.92	0.13	0.50	0.01	67.72	1.03	1.17	0.00	2.73	0.08	3.82	0.06	17.92	3.96	31.92	29.04	2309	356

Table A3 is continued on the following page.

Treat-ment	ID	Time [d]	Mn [mg L-1]		Fe [mg L-1]		Cu [mg L-1]		Zn [mg L-1]		Cd [mg L-1]		Pb [mg L-1]		pH		CL- [mg L-1]		NO3- [mg L-1]		SO4- [mg L-1]	
			mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM	mean	±SEM
Control Bulk	B1C	4	1.56	0.42	0.17	0.06	0.07	0.02	2.03	0.45	0.02	0.01	0.08	0.01	5.60	-	44.7	2.6	336.6	163.8	49.2	5.3
	B2C	14	1.21	1.06	0.56	0.05	0.04	0.02	3.67	1.54	0.06	0.01	0.08	0.01	6.35	0.79	34.0	4.3	720.3	348.4	31.9	2.8
	B3C	18	8.16	8.09	2.51	2.19	0.03	0.00	2.17	0.78	0.02	0.01	0.10	0.04	5.82	0.97	50.5	6.1	494.7	283.0	41.3	18.6
	B4C	22	0.45	0.28	0.57	0.21	0.03	0.01	3.70	1.85	0.09	0.01	0.03	0.02	5.45	0.80	44.6	13.8	820.3	392.8	21.7	1.5
	B5C	26	0.57	0.40	0.41	0.11	0.02	0.00	3.78	1.73	0.05	0.02	0.05	0.01	5.25	0.37	10.7	2.1	586.8	273.3	15.4	4.7
	B6C	37	2.67	2.61	0.25	0.02	0.03	0.01	1.69	0.71	0.02	0.01	0.06	0.01	5.90	0.14	9.5	7.2	236.1	103.7	10.9	3.7
	B7C	47	0.15	0.09	0.12	0.04	0.05	0.00	1.01	0.35	0.01	0.00	0.07	0.00	5.72	0.23	9.3	0.5	47.9	42.4	7.4	4.5
	B8C	57	0.08	0.02	0.10	0.02	0.06	0.01	0.83	0.15	0.01	0.00	0.08	0.00	5.84	0.25	16.0	5.5	28.1	15.3	10.7	4.0
HS Bulk	B1HS	4	8.12	0.21	0.35	0.01	0.04	0.01	4.27	0.08	0.03	0.00	0.33	0.01	5.60	-	31.4	5.7	401.5	25.9	176.9	14.0
	B2HS	14	31.70	9.98	0.60	0.07	0.05	0.01	8.70	3.59	0.11	0.05	0.42	0.05	5.79	0.41	54.5	13.7	90.3	26.0	1004.9	146.3
	B3HS	18	29.22	9.55	0.65	0.20	0.04	0.00	7.74	3.66	0.09	0.06	0.40	0.04	5.24	0.32	69.2	6.3	204.9	90.1	943.0	312.4
	B4HS	22	38.61	11.41	1.93	0.23	0.02	0.00	10.93	4.21	0.14	0.07	0.43	0.07	5.07	0.30	19.1	2.2	189.7	47.1	949.2	208.5
	B5HS	26	41.99	12.68	2.47	0.39	0.10	0.07	11.49	2.99	0.15	0.04	0.46	0.03	4.83	0.19	14.7	3.6	249.1	32.4	955.6	161.9
	B6HS	37	25.68	4.88	1.47	0.19	0.02	0.00	12.07	3.67	0.15	0.05	0.45	0.11	4.51	0.06	3.9	1.4	197.5	20.5	550.6	129.4
	B7HS	47	13.37	5.95	0.77	0.22	0.39	0.05	11.05	4.38	0.11	0.07	0.66	0.06	4.73	0.18	6.2	1.3	70.3	34.6	344.4	204.0
	B8HS	57	12.78	5.22	0.97	0.37	0.34	0.06	9.80	3.13	0.15	0.09	0.69	0.06	4.81	0.25	12.4	3.8	49.9	28.1	659.2	530.8
S Bulk	B1S	4	12.14	3.92	0.39	0.07	0.04	0.01	5.19	0.92	0.05	0.02	0.35	0.01	5.60	-	32.9	9.1	586.8	188.0	151.5	14.1
	B2S	14	51.39	17.61	1.01	0.23	0.04	0.01	16.29	5.24	0.21	0.08	0.55	0.10	5.57	0.23	20.9	2.9	10.1	4.5	1994.5	626.6
	B3S	18	25.37	4.44	0.60	0.13	0.04	0.00	7.83	0.77	0.08	0.01	0.37	0.01	4.75	0.20	36.0	6.9	29.0	16.9	1336.3	210.9
	B4S	22	75.03	18.26	2.39	0.27	0.03	0.01	24.94	4.06	0.36	0.08	0.77	0.13	4.35	0.05	14.8	3.5	50.8	32.8	1861.0	327.4
	B5S	26	116.15	29.16	2.89	0.44	0.05	0.01	41.59	7.49	0.63	0.13	1.12	0.20	4.23	0.04	12.2	2.5	70.9	26.3	2345.1	514.7
	B6S	37	78.96	13.65	1.95	0.20	0.05	0.00	32.01	4.11	0.49	0.08	1.02	0.13	3.98	0.06	8.2	5.3	30.1	11.0	1519.4	261.4
	B7S	47	35.53	5.18	1.05	0.11	0.38	0.08	17.78	1.58	0.22	0.03	0.83	0.04	4.34	0.03	12.1	8.5	29.5	10.8	1343.8	807.1
	B8S	57	14.97	3.28	0.62	0.10	0.45	0.00	9.41	1.45	0.11	0.06	0.75	0.05	4.21	0.11	7.9	2.6	10.1	2.6	377.7	232.0

Table A4: Statistical results from general linear modeling of soil pore water data using repeated measures. SPSS output from test results.

		Mn		Fe		Cu		Zn		Cd		Pb		H+		CL-		NO3-		SO4-	
		F	p	F	p	F	p	F	p	F	p	F	p	F	p	F	p	F	p	F	p
Tests of Within-Subject Effects[a,c,f]																					
Greenhouse-Geisser Test result	time	12.3	.000	16.5	.000	188.4	.000	16.0	.000	14.7	.000	37.4	.000	30.4	.000	43.3	.000	6.5	.001	9.7	.000
	time*compartment	10.1	.000	2.7	.095	2.5	.069	7.6	.001	8.2	.000	10.2	.000	10.1	.000	2.2	.109	1.7	.184	1.7	.173
	time*treatment	8.7	.000	5.3	.005	36.5	.000	9.9	.000	9.9	.000	17.0	.000	13.4	.000	1.8	.136	4.9	.001	3.8	.002
	time*compartment*treatment	6.7	.000	2.0	.143	.9	.501	5.5	.001	6.9	.000	6.8	.000	6.0	.000	2.0	.093	.8	.569	.8	.560
Tests of Between-Subject Effects[a,e]																					
Bonferroni confidence interval adjustment Test result	intercept	86.6	.000	430.7	.000	529.2	.000	316.5	.000	239.1	.000	1302	.000	203.6	.000	314.3	.000	25.2	.000	115.3	.000
	compartment	11.9	.004	4.7	.049	2.0	.175	32.0	.000	25.4	.000	182.3	.000	16.6	.001	1.4	.258	3.6	.080	3.5	.082
	treatment	30.6	.000	44.4	.000	46.7	.000	91.6	.000	86.8	.000	290.9	.000	57.3	.000	0.6	.542	5.1	.021	35.6	.000
	compartment*treatment	6.3	.011	7.0	.008	.5	.632	16.8	.000	18.7	.000	54.6	.000	6.0	.013	1.2	.322	.4	.667	1.6	.235
Post-hoc test for homogenous subsets[a,b]																					
Tukey's-b	Control	1135.14		405.95		42.25		1987.56		23.49		69.33		4.9E-06		22.72		311.91		18.25	
	HS-treatment	33995.21		1375.85		129.99		12566.38		140		738.73		2.4E-05		25.22		103.6		800.55	
	S-treatment	89064.94		1677.17		148.3		31261.53		454.72		1217.93		6.2E-05		26.73		71.55		1711.15	
Test of Equality of Error Variances[a,f]																					
Levene's Test result	Measurement1	6.3	.003	10.5	.000	2.1	.133	4.1	.017	2.9	.052	.7	.643	12.0	.000	4.2	.015	4.9	.009	2.9	.056
	Measurement2	4.5	.012	5.7	.005	6.8	.002	3.0	.046	3.2	.038	3.6	.028	4.3	.013	2.6	.072	15.0	.000	6.1	.003
	Measurement3	2.9	.053	14.9	.000	2.6	.070	3.5	.029	3.5	.029	2.7	.063	3.6	.027	0.2	.947	3.4	.031	4.0	.018
	Measurement4	9.0	.001	0.5	.771	2.7	.065	1.0	.444	1.5	.264	3.6	.027	2.3	.106	4.7	.010	10.3	.000	9.4	.000
	Measurement5	6.6	.002	2.7	.063	5.3	.006	4.6	.010	5.5	.005	7.5	.001	5.5	.005	1.5	.267	10.0	.000	7.6	.001
	Measurement6	5.0	.008	2.4	.087	6.9	.002	3.3	.034	4.1	.017	2.8	.062	3.7	.025	8.8	.001	9.3	.000	3.6	.027
	Measurement7	6.4	.003	2.2	.114	7.5	.001	3.1	.043	3.6	.026	3.0	.049	11.4	.000	9.2	.000	16.5	.000	8.5	.001
	Measurement8	4.9	.008	3.7	.024	486.3	.000	4.3	.015	3.4	.032	4.0	.018	4.4	.013	2.3	.105	21.9	.000	3.2	.041

[a] alpha=0.05
[b] grey color indicates classification in the same group
[c] Mauchly's Test on Spericity failed (p<0.05) (data not shown) therefore Greenhouse-Geisser test is used
[d] Tests the null hypothesis that the error variance of the dependent variable is equal across the groups
[e] Between-Subject factors (Compartment; rhizo n=10, bulk n=10) (Treatment; Control n=6, HS n=8, S n=6)
[f] Within-Subject factors (Measurements 1-8)

Table A5: Statistical results from *S. smithiana* biomass ANOVA. SPSS output from test results.

		pH		EC		roots		twigs		leaves	
		F	p	F	p	F	p	F	p	F	p
Analysis of variance (ANOVA)[a,d]		145.7	.000	47.6	.000	.95	.432	.09	.916	.96	.428
Homogenity of Variances[a,c,d]			.045		.070		.483		.271		.001
Post Hoc Test for homogenous subsets[a,b,d]											
Tukey-b test	Control	5.71		75.76		1.46		5.63		2.91	
	HS-Treatment	4.86		713.25		1.07		5.22		2.44	
	S-Treatment	4.19		1302.30		0.96		5.48		2.27	

[a] alpha=0.05
[b] grey color indicates classification in the same group
[c] Levene's Test results
[d] Sample size: Control n=3, HS n=4, S n=3