

## Localized Metal Solubilization in the Rhizosphere of *Salix smithiana* upon Sulfur Application

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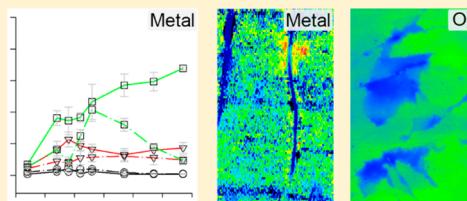
### S Supporting Information

**ABSTRACT:** A metal-accumulating willow was grown under greenhouse conditions on a Zn/Cd-polluted soil to investigate the effects of sulfur ( $S^0$ ) application on metal solubility and plant uptake. Soil porewater samples were analyzed 8 times during 61 days of growth, while DGT-measured metal flux and  $O_2$  were chemically mapped at selected times. Sulfur oxidation resulted in soil acidification and related mobilization of Mn, Zn, and Cd, more pronounced in the rooted compared to bulk soil. Chemical imaging revealed increased DGT-measured Zn and Cd flux at the root-soil interface. Our findings indicated sustained microbial  $S^0$  oxidation and associated metal mobilization close to root surfaces. The localized depletion of  $O_2$  along single roots upon  $S^0$  addition indicated the contribution of reductive Mn (oxy)hydroxide dissolution with Mn eventually becoming a terminal electron acceptor after depletion of  $O_2$  and  $NO_3^-$ . The  $S^0$  treatments increased the foliar metal concentrations ( $\text{mg kg}^{-1}$  dwt) up to 10-fold for Mn, ( $5810 \pm 593$ ), 3.3-fold for Zn ( $3850 \pm 87.0$ ), and 1.7-fold for Cd ( $36.9 \pm 3.35$ ), but had no significant influence on biomass production. Lower metal solubilization in the bulk soils should translate into reduced leaching, offering opportunities for using  $S^0$  as environmentally favorable amendment for phytoextraction of metal-polluted soils.

### INTRODUCTION

Metal-contaminated soils are a global problem of environmental quality and land use and pose risks to human health. A possible approach to manage the risks associated with metal-polluted soils is the application of suitable higher plants and associated microorganisms, that is, phytoremediation. Among the various phytotechnologies, phytoextraction aims at metal removal of the contaminants from soil. Recent field studies have shown that phytoextraction can effectively reduce total or labile metal pools.<sup>1,2</sup> Effective phytoextraction requires (i) sustained, high metal bioavailability close to the plant roots; (ii) high biomass production, metal uptake, and translocation into plant shoots; and (iii) minimization of metal leaching to the groundwater.<sup>3–7</sup> A major constraint relates to limited metal availability in soils. To address this problem, most previous research has been focusing on ligand-assisted phytoextraction.<sup>8–11</sup> However, the implementation of this approach is often limited by decreased plant growth, high risk of metal leaching to the groundwater, and toxic effects on soil bacteria and fungi.<sup>12–14</sup> Alternative, but less investigated amendments include slowly reacting soil acidifying agents.<sup>7,15,16</sup> Compared to ligand-triggered flush release of complexed metals upon chelant application, microbial oxidation of elemental sulfur ( $S^0$ ) acidifies the soil incrementally during several weeks or months,<sup>17,18</sup> depending on the rate of application and soil buffer capacity. The continued production of protons ( $H^+$ ) is deemed to sustain metal availability to phytoextraction crops while keeping the risk of leaching low.<sup>8,17,19,20</sup>

### Temporal development and spatial heterogeneity



Previous greenhouse studies investigated these processes, showing significant effects on Zn and Cd solubility upon application of  $S^0$  in acidic, neutral and calcareous soils with increased plant uptake by commonly used phytoextraction plants such as *Helianthus annuus* and *Salix viminalis*.<sup>20,21</sup> In field experiments it was shown that  $S^0$  application to a calcareous soil was effective at increasing Zn and Cd solubility at lower rates than nitrilotriacetate (NTA).<sup>17</sup> Elemental sulfur decreased the soil pH only slightly but increased metal uptake substantially while biomass production was reduced in several potential phytoextraction crops. However, the tested willow species showed increasing growth rates after the second year. In a more recent pot study, Iqbal et al.<sup>19</sup> found that metal solubilization in response to  $S^0$  application was enhanced in the root zone of *Salix smithiana* compared to unplanted controls. This finding is highly relevant as uptake would be enhanced, while the risk of metal leaching during phytoextraction may be further minimized if metal solubilization is magnified close to the site of uptake by the phytoextraction crop. As the extent of metal solubilization in  $S^0$ -amended rhizosphere soils could not be fully explained by acidification, the authors<sup>19</sup> proposed that more rapid  $O_2$  depletion due to respiration and enhanced microbial  $S^0$  oxidation<sup>22</sup> in the rhizosphere might cause a shift

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from O<sub>2</sub> to Mn (oxy)hydroxides as terminal electron acceptors (TEA) during microbial S<sup>0</sup> oxidation.<sup>23</sup> This hypothesis was supported by substantially increased Mn, Cd, and Zn concentrations in rhizosphere soil solutions.<sup>19</sup> In the study of Iqbal et al.,<sup>19</sup> only relatively few observations of metal concentrations in soil porewater over time were realized, with the first sampling only 50 days after starting the experiment. Therefore, it was not possible to monitor the earlier, probably more dynamic phases of S<sup>0</sup> oxidation and the expected shift from O<sub>2</sub> to Mn (oxy)hydroxides as TEA after O<sub>2</sub> depletion. Moreover, porewater sulfate (SO<sub>4</sub><sup>2-</sup>) concentrations were not analyzed, and redox (E<sub>h</sub>) indicators, such as O<sub>2</sub> were not directly measured.

Here we further explore the processes controlling the differential response of rhizosphere and bulk soils to S<sup>0</sup> application. In a rhizobox experiment using the same willow clone and one of the soils of the previous study,<sup>19</sup> we monitor the effects of S<sup>0</sup> oxidation on pH, SO<sub>4</sub><sup>2-</sup> and metal concentrations in the porewater of rooted and root-free soil in high spatiotemporal resolution to investigate the metal solubilization processes in the initial compared to later phases of S<sup>0</sup> oxidation. In a rhizotron experiment we applied planar optodes (POS) to map potential depletion of O<sub>2</sub> in rooted and bulk soil with and without S<sup>0</sup> addition at high spatial resolution (~100 μm). In the same experiment we used localized solute sampling by diffusive gradients in thin films combined with laser ablation inductively coupled plasma mass spectrometry (DGT LA-ICPMS) for chemical mapping of labile metals.

## EXPERIMENTAL SECTION

If not stated otherwise, glassware and plastics were acid-washed in 2% HNO<sub>3</sub> (p.a. grade, Sigma-Aldrich, Vienna, Austria) and rinsed twice with laboratory water type 1 (0.055 μS cm<sup>-1</sup>, TKA-GenPure, Thermo Electron LED GmbH, Niederelbert, Germany). The experimental setup and analytical procedures are outlined in brief; details are stated in the Supporting Information.

**Soil and Plant Material.** The experimental soil was sampled from the topsoil of an Eutric Cambisol,<sup>24</sup> near a former metal smelter site in Arnoldstein, Austria. The topsoil was moderately contaminated by Zn, Cd, and Pb and is referred to as ARNB. A compilation of relevant soil properties, taken from Iqbal et al.,<sup>19</sup> can be found in Supporting Information Table SI-1.

The soil was amended with S<sup>0</sup> at three rates, that is, no S<sup>0</sup> amendment (C), 0.51 g S<sup>0</sup> kg<sup>-1</sup> soil (S1) and 1.02 g S<sup>0</sup> kg<sup>-1</sup> soil (S2). Elemental sulfur (NORMAPUR, VWR, Radnor, PA) was manually crushed with a plastic spatula to obtain homogeneous powder, sieved (<200 μm) and weighted. The soil was air-dried, sieved (<2 mm) and homogenized. All treatments were prepared separately. Soil and S<sup>0</sup> powder were manually mixed end-over-end in clean, sealed plastic bags for 10 min. The amount of S<sup>0</sup> for the treatments was determined to reach target pH values in the bulk soil of 4.5 (S1) and 4.0 (S2) according to the incubation experiment reported by Iqbal et al.<sup>19</sup>

As experimental plant we used *Salix × smithiana* Willd. (*S. caprea* L. × *S. viminalis* L., clone BOKU 03 CZ-001). In previous studies this clone was shown to efficiently phytoextract Zn and Cd, with foliar concentrations of >2000 mg kg<sup>-1</sup> Zn and >400 mg kg<sup>-1</sup> Cd on dry weight basis (dwt).<sup>25–28</sup> For the rhizobox experiment, fresh willow cuttings (length ~20 cm, diameter ~1 cm) were pregrown in a commercially available potting mixture for 2 weeks. For the rhizotron experiment, *S.*

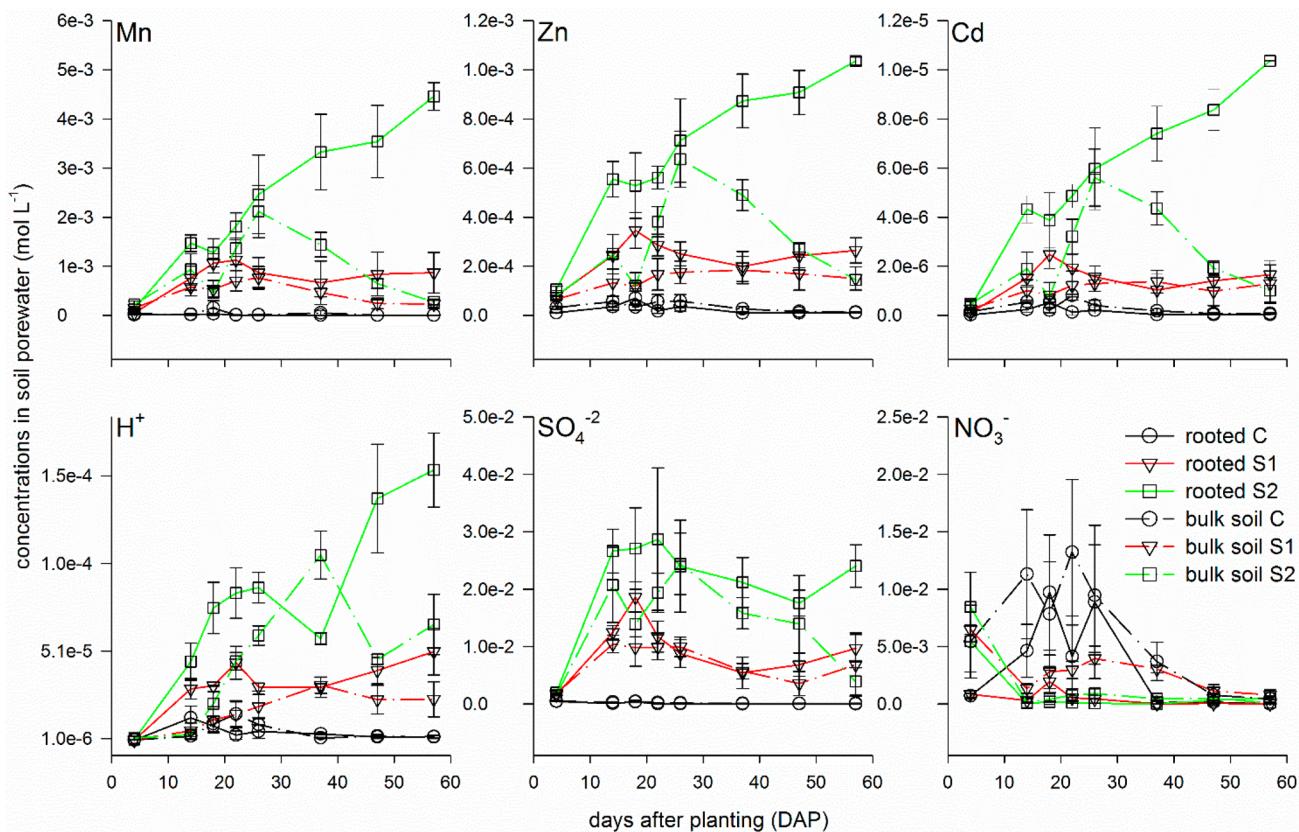
*smithiana* cuttings were kept for 7 days in tap water for sprouting and initial root development.

### Rhizobox Experiment and Soil Porewater Sampling.

For sampling soil porewater we used a compartmented rhizobox design (rooted, membrane, and bulk soil compartments; Supporting Information Figure SI-1).<sup>29</sup> Briefly, the S<sup>0</sup>-amended soil was filled into the compartments and was carefully compacted to a bulk density of approximately 1.4 g cm<sup>-3</sup> (~600 g soil per rhizobox). One pregrown willow cutting was transplanted into each rhizobox. In the center of the rooted and bulk soil compartments 50 mm long Rhizon samplers (Rhizosphere research products, Wageningen, Netherlands) were installed 2 cm above the bottom of the compartment for collecting soil porewater during the experiment. Constant water supply was maintained by two PE-coated glass fiber wicks (TRIPP Kristallo Rundschnur, 4 mm, IDT, Frankfurt, Germany), which were installed in the bulk soil and rooted compartments. Rhizoboxes were maintained at ~80% water holding capacity (WHC). The experiment was carried out in a greenhouse at 60% rel. humidity (day/night cycle: 16/8h). We sampled soil porewater in the rooted and bulk soil compartments eight times during the growth period (at day 4, 14, 18, 22, 26, 37, 47, 57) by applying suction to the Rhizon samplers using 10 mL syringes. The samples were measured for pH (ORION 3 Star, Thermo Scientific) and stored at -20 °C until analysis of SO<sub>4</sub><sup>2-</sup> and NO<sub>3</sub><sup>-</sup> using ion chromatography (DX-500, Dionex, Sunnyvale, CA), and Mn, Fe, Cu, Zn, Cd, and Pb on ICP-MS (Elan 9000 DRCe, PerkinElmer, Waltham, MA).

**Harvest and Chemical Analysis.** At harvest, (61 days after planting (DAP)), the willow plants were cut directly above the soil surface and separated into twigs and leaves using ceramic scissors. Twigs and leaves were washed using lab water type 1. Roots were separated from soil after partial drying in ambient conditions by gentle sieving and manual picking before washing in lab water type 1 in an ultrasonic bath. The washed plant materials were blotted on tissue paper and dried at 65 °C for 72 h. Subsamples of 0.2 g were digested in a mixture of 15.4 mol L<sup>-1</sup> HNO<sub>3</sub> (EMPARTA, ACS, Merck, Vienna, Austria) and 9.81 mol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> (TraceSELECT Ultra, Fluka, Sigma-Aldrich, Vienna, Austria) (5:1, v/v) in a closed microwave digestion system (Multiwave 3000, Anton Paar GmbH, Graz, Austria). The digests were filtered using 45 μm filter paper (150 mm diameter 14/N, Munktell, Bärenstein, Germany), collected and filled up to approximately 40 mL using lab water type 1, weighed and analyzed using ICP-MS. Mean soil moisture across all treatments and compartments was determined as 75% ± 6% WHC (w/w) at harvest. The soil pH at the time of harvest was measured in a slurry of 10 g of air-dried soil and lab water type 1 at a soil:solution ratio of 1:2.5 (w/v) after 2 h of equilibration.<sup>30</sup>

**Statistical Analysis.** For the statistical evaluation, ANOVAs with repeated measurement analysis were computed for H<sup>+</sup>, metals and anions in the soil porewater samples. Within-subject effects and between-subject effect analysis was conducted using the degrees-of-freedom-corrected Greenhouse-Geisser test<sup>31</sup> for time; time × compartment; time × treatment; and time × compartment × treatment. Time-independent between-subject effects were evaluated after Bonferroni correction for compartment; treatment; compartment × treatment. Metal accumulation in *S. smithiana* and biomass was analyzed by separate ANOVAs using Tukey's HSD as posthoc test. For all statistics, P ≤ 0.05 was adopted as probability threshold for rejecting the



**Figure 1.** Mean metal, sulfate, nitrate, and H<sup>+</sup> concentrations (mol L<sup>-1</sup>) in the rhizobox soil porewater. Error bars show standard error of the mean ( $n = 3$ ).

null hypothesis. All statistical calculations were conducted using IBM SPSS Statistics 21 (IBM Corporation, Armonk, NY).

**Rhizotron Experiment and Chemical Mapping.** Rhizotrons are flat growth containers in which plants are grown at an inclination and its roots therefore preferentially develop along the detachable lower front plate of the rhizotron (Supporting Information Figure SI-2).<sup>32</sup> The rhizotrons used in this study had inner dimensions of 40 × 10 × 1.5 cm (height × width × depth) and are described elsewhere.<sup>33,34</sup>

In the rhizotron experiment only two soil treatments were realized (C and S1). Five rhizotrons per treatment were packed with soil to a bulk density of ~1.35 g cm<sup>-3</sup>. To prevent the front plate from sticking to soil and plant roots, a 0.05 mm thick polytetrafluoroethylene (PTFE) foil (Haberkorn, Wolfurt, Austria) was fixed using adhesive tape before closing the rhizotrons and saturating the soil to 80% WHC. To this end, lab water type 1 was added through 14 drillings in the nondetachable back plate to homogeneously moisten the soil. After transplanting the cuttings, the rhizotrons were wrapped in aluminum foil. During plant growth, the rhizotrons were tilted to an angle of ~18°. The experiment was carried out in a growth room at 30–40% rel. humidity (day/night cycle: 16/8 h) and soil water content was kept at ~80% WHC throughout the experiment, by regularly adding water through drillings in the back plate of the rhizotron.

For chemical imaging, polyacrylamide diffuse gradients in thin film (DGT) gels containing suspended particulate reagent-iminodiacetate (SPR-IDA) resin (CETAC, Nebraska, USA) were prepared as described in previous work.<sup>35</sup> Briefly, we deployed DGT gel strips (~2 × 4 cm) 4 DAP in the region of interest (ROI) using a 10 μm thick polycarbonate filter

membrane (Nuclepore, Whatman, Maidstone, UK) between soil and gel as diffusive layer. The sampling time was 20 h, derived from a preliminary experiment, aiming at avoiding saturation of the SPR-IDA resin with metal cations. The gels were retrieved and rinsed with lab water type 1, dried using a gel dryer (Unigeldryer 3545, UNIEQUIP, Planegg, Germany) and carefully fixed onto glass plates using double-sided adhesive tape prior LA-ICPMS analysis. For oxygen imaging (17 DAP) in the rhizotron soil we used color ratiometric planar optode sensors (POS) as described in Larsen et al.<sup>36</sup> Details on DGT and POS preparation, deployment and calibration can be found in the Supporting Information.

## RESULTS AND DISCUSSION

**Metal Accumulation and Biomass in Rhizobox-Grown Plants.** Plant growth and biomass production was slightly reduced in the S1 and S2 treatments, however no significant ( $P \leq 0.05$ ) differences in total biomass or individual plant parts were found compared to C. Correspondingly, no visible signs of metal toxicity or other negative effects due to the low pH or excess Mn, Zn, and Cd concentrations in the soil porewater in S1 and S2 could be identified, although plant toxicity may occur within the observed concentration range.<sup>37</sup> This confirms the previously observed metal tolerance of *S. smithiana*.<sup>25,26</sup>

Manganese and Zn concentrations in *S. smithiana* foliar and twig biomass increased with increasing S<sup>0</sup> application, with significant ( $P \leq 0.05$ ) differences between S2 and control treatments (Supporting Information Table SI-2, Figure SI-4). In contrast to Mn and Zn, the observed increases in foliar Cd concentrations were not significant ( $P \leq 0.05$ ) in the S<sup>0</sup> treatments as compared to C, although the corresponding Cd

concentrations in roots of S2 were strongly ( $P \leq 0.05$ ) increased (Supporting Information Table SI-4). This indicates suppressed translocation from roots to foliar biomass which might have been related to competition with Mn and Zn, as Cd is often taken up via transporters of other divalent cations.<sup>38</sup>

Foliar concentrations of Mn increased 5.5-fold in S1 and 10-fold in S2 compared to C, reaching  $5810 \pm 593 \text{ mg kg}^{-1}$  dry weight (dwt) in the latter. Zinc concentrations increased in the leaf biomass 2.2-fold in S1 and 3.3-fold in S2 compared to C, reaching  $3850 \pm 87.0 \text{ mg Zn kg}^{-1}$  dwt in S2. Cadmium concentrations increased 1.7-fold in S2 compared to C in the leaf biomass accumulating up to  $36.9 \pm 3.35 \text{ mg kg}^{-1}$  dwt in S2 (Supporting Information Table SI-4). The large amounts of Zn and Cd accumulated in leaf and twig biomass of *S. smithiana* in S1 and S2 correspond to about 1.7–2.4% (Zn) and 2.5–2.8% (Cd) total removal of these elements from the experimental soil after only 61 days of growth (Supporting Information Table SI-4), which is well in line with earlier reports.<sup>19,25–28</sup>

**Temporal Changes of Soil Porewater Chemistry in the Rhizobox Experiment.** In the soil porewater (Figure 1; Supporting Information Figure SI-5),  $\text{H}^+$  activity significantly ( $P \leq 0.05$ ) increased during the experiment in both  $\text{S}^0$  treatments compared to C. The soils in S1 and S2 were acidified due to  $\text{S}^0$  oxidation to  $\text{H}_2\text{SO}_4$ , resulting in significantly ( $P \leq 0.05$ ) increased  $\text{SO}_4^{2-}$  concentrations compared to C (Figure 1). The simultaneously observed, significant ( $P \leq 0.05$ ) increases in Mn, Zn, and Cd concentrations corresponded well to the continuous increase in  $\text{H}^+$  activities in the rooted  $\text{S}^0$  compartments (Figure 1). In the  $\text{S}^0$ -treated bulk soils,  $\text{H}^+$  activities increased only until 38 DAP and leveled off subsequently, which was reflected by similar changes in the concentrations of  $\text{SO}_4^{2-}$ , Mn, Zn, and Cd (Figure 1). No such similarities in the relationships between  $\text{SO}_4^{2-}$ ,  $\text{H}^+$  and metals were found for the rooted soils. Toward the end of the experiment (57 DAP), metal concentrations were significantly ( $P \leq 0.05$ ) higher in the rooted  $\text{S}^0$  treatments, compared to the corresponding bulk soil concentrations. Until 57 DAP, Mn concentrations increased 3.5-fold in S1 and 15.3-fold in S2 relative to the corresponding bulk soil, reaching up to  $245 \pm 15.6 \text{ mg L}^{-1}$  in the rooted compartment of S2. Zinc concentrations increased up to 2.2-fold in the rooted compartment of S1 and up to 9.1-fold in S2 compared to bulk soil. As for Mn, the highest Zn concentrations occurred in the rooted compartment of S2 ( $67.2 \pm 1.0 \text{ mg L}^{-1}$ , final sampling). Similarly, Cd concentrations in the rooted compartment increased 3.5-fold in S1 and 28.6-fold in S2 relative to the bulk soil. Particularly, Mn concentrations in the rooted  $\text{S}^0$  treatments were remarkably high and corresponded to Zn and Cd solubilization (Figure 1). The  $\text{S}^0$ -induced rhizosphere effect lasted throughout the experiment and supplied *S. smithiana* with significantly ( $P \leq 0.05$ ) enhanced metal concentrations compared to C (Supporting Information Table SI-5).

Iqbal et al.<sup>19</sup> observed pH-dependent solubilization of Cd and Zn in rooted and nonrooted,  $\text{S}^0$ -treated soils. They also provided evidence for additional metal mobilization, which could not be related to changes in pH and suggested comobilization of Cd and Zn from Mn oxides serving as TEA during  $\text{S}^0$  oxidation in partly anaerobic conditions.<sup>19</sup> This process appeared to be more pronounced in the rooted soils. The soil porewater samples analyzed in this previous study were collected only >50 DAP.

Here we investigated the initial effects of  $\text{S}^0$  application up to 60 DAP and found no clear evidence for codissolution of Cd

and Zn due to  $\text{S}^0$ -triggered Mn oxide reduction. Plotting metal concentrations in porewater against pH (Supporting Information Figure SI-6) for all treatments and compartments shows less differentiation between the treatments compared to Iqbal et al.,<sup>19</sup> for the period >50 DAP. Iqbal et al.<sup>19</sup> interpreted the differentiation of the pH-dependent solubility curves between rooted and bulk soil treatments as being caused by reduction of Mn serving as TEA for the oxidation of  $\text{S}^0$  to  $\text{H}_2\text{SO}_4$ . Manganese reduction releases  $\text{OH}^-$  under the presence of  $\text{S}^0$  and may counteract the acidification upon  $\text{S}^0$  oxidation. The much less pronounced differences in the present study indicate that  $\text{S}^0$  oxidation during the initial phase (<60 DAP) might predominantly occur with  $\text{O}_2$  as TEA, and  $\text{H}^+$  production appears to be the main driver of metal solubilization in the  $\text{S}^0$  treatments. Protons can exchange metal cations from mineral surfaces and enhance desorption by rendering variable charge surfaces less negative.<sup>39,40</sup> However, even during the initial phase Mn oxides might serve as TEA in microniches with lower  $\text{O}_2$  saturation, especially next to roots, as indicated by chemical imaging of  $\text{O}_2$  (Figure 3).

The role of  $\text{H}^+$  production during  $\text{S}^0$  oxidation for metal solubilization in our study becomes even more apparent from the similar temporal pattern of  $\text{H}^+$  and metal porewater concentrations in the individual treatments and compartments (Figure 1). The sustained increase in soluble metal concentrations in the rooted S2 compartment concurs with increasing  $\text{H}^+$  activities until termination of the experiment. In all other  $\text{S}^0$ -treated compartments (bulk S2, bulk S1, rooted S1),  $\text{H}^+$  activities leveled off or slightly decreased after 25–35 DAP, which is reflected by similar changes in metal solubility. Especially in the S2 treatment this resulted in remarkably larger soluble metal concentrations in the rooted compared to the bulk soil 57 DAP.

Apart from differences in the experimental setup (pot versus rhizobox experiment), the generally lower dissolved Cd and Zn concentrations observed by Iqbal et al.<sup>19</sup> as compared to our study provide evidence that the decline of dissolved metal concentrations might have continued >50 DAP. This may be explained by a combination of processes including continued metal uptake in the willow at declining rates of  $\text{S}^0$  oxidation and metal mobilization, and possibly readsorption of metals. Only the rooted  $\text{S}^0$  treatments showed Cd and Zn concentrations similar to those found at the same pH values in the corresponding S2 treatment of our study while the Mn concentrations were even larger (Supporting Information Figure SI-6).<sup>19</sup> Note that Iqbal et al.<sup>19</sup> observed larger concentrations of Mn, Cd, and Zn in the rooted  $\text{S}^0$  treatment compared to the unplanted  $\text{S}^0$ -treated soil despite the higher pH in the rooted soil, whereas here the larger Mn concentrations in the rooted S2 treatment concurred with more pronounced acidification (Figure 1).

The observed difference in  $\text{H}^+$  activities and metal concentrations in soil porewater between the rooted and nonrooted S2 treatments corresponds to a similar difference in  $\text{SO}_4^{2-}$  concentrations (Figure 1), suggesting that root activities play an important role for microbial  $\text{S}^0$  oxidation. Sulfate concentrations leveled off in the rooted compartment (25 DAP), but strongly decreased in the bulk soil. The decline of  $\text{H}^+$  activity and  $\text{SO}_4^{2-}$  concentrations in the bulk soil starting 25 DAP might be related to limited availability of carbon resources<sup>41</sup> whereas in the rooted compartment the release of root exudates such as sugars, amino acids, and carboxylates may have allowed for continued microbial oxidation of  $\text{S}^0$ .<sup>18,42,43</sup>

In the rooted compartment,  $H^+$  production, as indicated by the increasing  $H^+$  activity, appeared to continue, suggesting further microbial oxidation of  $S^0$  to sulfuric acid ( $H_2SO_4$ ). However,  $SO_4^{2-}$  concentrations remained almost unchanged (Figure 1). This as well as the decrease of  $SO_4^{2-}$  concentrations in all other  $S^0$ -treated compartments may be explained by increasing  $SO_4^{2-}$  removal from soil porewater while the production rate of  $SO_4^{2-}$  likely decreased due to the decline in  $S^0$  and microbial substrates. Sulfate removal from soil porewater could have occurred through adsorption<sup>44,45</sup> to increasingly protonated mineral surfaces due to proton production during  $S^0$  oxidation and immobilization of S in organic matter including sulfate esters.<sup>18</sup> Reduction to sulfide is unlikely to explain our data as indicated by the rather low solubility of Fe, suggesting that the  $E_h$  was not low enough.<sup>46</sup> According to Kertesz et al.,<sup>18</sup> immobilization of  $SO_4^{2-}$  in organic matter is microbially mediated and the highest oxidized organic S pool in the soil is considered to be ester sulfate-S. It is expected that microbial communities varied between rooted and bulk soil compartments since substrate availability and microenvironmental conditions are different.<sup>41</sup> Enhanced microbial  $S^0$  oxidation is considered to occur under 20–30 °C and is mainly mediated by early exponential growth of *Thiobacillus* spp. and aerobic heterotrophic  $S^0$  oxidizing bacteria. In the longer term, *Thiobacillus* spp. may disappear again because of substrate limitations<sup>47</sup> which could have, simultaneously, diminished the  $S^0$  oxidation rates in the S2 bulk soils.

For identification of possible Mn solubility controls, we calculated the log  $Mn^{2+}$  activities in soil porewater using Visual MINTEQ (Supporting Information Figure SI-7). Initially (4 DAP), pH values were elevated in all treatments and compartments compared to the soil characterization (pH 5.6; Supporting Information Table SI-1) and the soil porewater pH reported for the untreated control by Iqbal et al.<sup>19</sup> In the rooted compartments, pH increased to 7.17 (C), 6.85 (S1), and 6.33 (S2), respectively. In the bulk soil compartments the corresponding pH values were 6.59 (C), 6.38 (S1) and 5.91 (S2). Manganese solubility appeared to be shifted initially to levels above the thermodynamic equilibrium with pyrolsite or Manganite (Supporting Information Figure SI-7).<sup>48</sup> The observed anomalies in pH and Mn solubility after setup of the experiment may indicate soil physicochemical re-equilibration effects due to soil rewetting, changing the initial soil pH and Mn solubility as suggested in earlier studies.<sup>49</sup> This was unavoidable, since soil preincubation would have initiated  $S^0$  oxidation in the  $S^0$  treatments prior to the start of plant growth. The solubility of redox sensitive metals like Mn can easily increase up to 10-fold due to reduction of Mn oxides through electron transfer from new organic surface groups such as phenolic acids exposed during the drying process, and re-equilibration may last for several weeks after rewetting.<sup>47,49,50</sup> In C and the  $S^0$ -treated bulk soils, Mn solubility returned to values slightly above the Manganite line (Supporting Information Figure SI-7) within 2 to 6 weeks. In the rooted  $S^0$  treatments, rewetting effects on pH were less apparent as they were confounded by the rapid kinetics of  $S^0$  oxidation, related  $H^+$  production and metal solubilization (Figure 1).

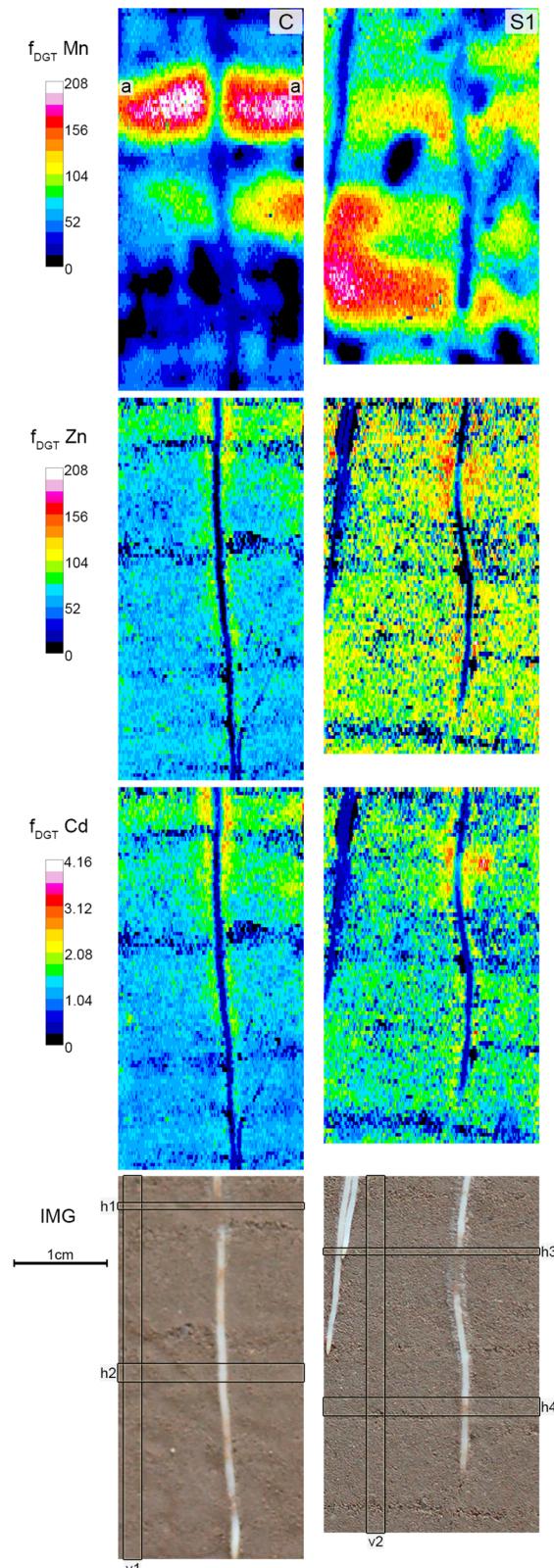
**Chemical Imaging of DGT-Available Metals along the Root Axis of Rhizotron-Grown Willows.** High-resolution (120 × 400 μm) imaging of DGT-measured metal fluxes around single *S. smithiana* roots was conducted to obtain insight into the spatial variability and potential  $S^0$ -induced solubilization hotspots, metal depletion and accumulation in

the rhizosphere of *S. smithiana* in response to the C and S1 treatment. Figure 2 shows the DGT-measured metal fluxes (proportional to the DGT-available fraction) of Mn, Zn and Cd around the willow roots, comparing treatment C and S1. Iron, Cu, and Pb maps can be found in Supporting Information Figure SI-8.

Similar to the rhizobox experiment, we found clearly increased Mn, Zn, and Cd concentrations in S1 compared to C (Figure 2). The chemical images revealed detailed spatial information on Mn, Zn, and Cd availability near single *S. smithiana* roots in the early stage of  $S^0$  oxidation (4 DAP). Hot spots of soluble Mn, Zn, and Cd were found along the root axis of S1 and might be associated with the root-hair zone (Supporting Information Figure SI-9, h3). Also in C, we found slightly increased DGT-measured Zn and Cd fluxes along the willow root, however much less pronounced compared to S1. The enhanced DGT-measured Cd and Zn fluxes in C and S1 were identified to occur at the soil–root interface (Supporting Information Figure SI-9, h1, h3). While Mn concentrations were reduced up to 1.5 mm distance from the root surface, depletion zones for Zn and Cd were absent (h1—next to root tip) or within sub-mm range (h3—root-hair zone) (Supporting Information Figure SI-9). We hypothesize that this depletion was resulting from Mn, Zn, and Cd uptake by *S. smithiana*. Compared to Zn and Cd, Mn showed higher spatial variability in DGT-measured flux (Figure 2), which probably relates to its readiness for participation in soil redox processes. The observation of distinct peaks of DGT-measured Zn and Cd fluxes (Supporting Information Figure SI-9) close to the root surface at the microscale strongly supports the hypothesis derived from the rhizobox experiment that root exudates and differences in the microbial habitat may be key factors in explaining this phenomenon. We hypothesize that enhanced  $S^0$  oxidation and related metal solubilization in the  $S^0$ -amended root compartments may be partly linked to strains of microbial  $S^0$  oxidizers colonizing the volume of soil around willow roots. This close association with the roots might be explained by specific physical (habitat) conditions and—as discussed above—sustained resource availability due to root exudation.

In the bulk soil, Zn and Cd concentrations were slightly higher in S1 compared to C. In S1 we found colocalized hotspots of Zn and Cd flux close to the root-hair zone, supporting that local, reductive microniches or root exudates may have contributed to enhanced metal solubilization in the rhizosphere. The results clearly show a more pronounced metal solubilization in S1 compared to C, supporting our concept of localized metal solubilization in the rhizosphere of the phytoextraction crop. In practical terms, the narrow zone of metal mobilization along the willow roots further refines the concept of reduced metal leaching from bulk soil in  $S^0$ -assisted phytoextraction technologies.

Apart from plant- or treatment-induced changes, a zone with high DGT-measured Mn fluxes was found in C, where concentrations were expected to be lower compared to S1. This elevated zone in C, (Figure 2, a) visually matched a soil layer in the photographic image, indicating decreased soil  $E_h$ , probably due to higher soil compaction, textural discontinuity and/or different water matrix potential. Manganese reduction consumes  $H^+$  and  $e^-$  in the absence of  $S^0$ , (e.g.,  $MnO_4^{2-} + 8H^+ + 4e^- \rightarrow Mn^{2+} + 4H_2O$ ).<sup>51</sup> As Cd and Zn are not directly participating in redox reactions, in the absence of  $H^+$  production a mobilization of these metals is not expected, or

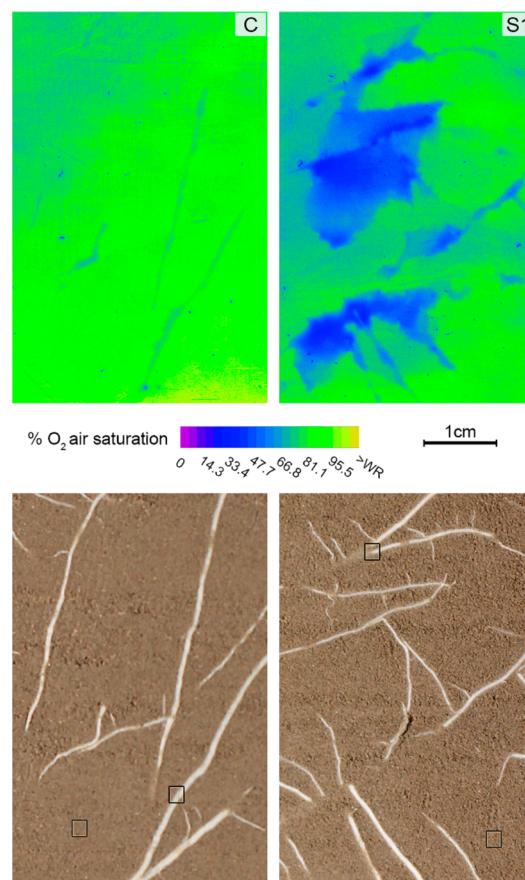


**Figure 2.** High resolution two-dimensional mapping of Mn, Zn and Cd in the rhizosphere of *S. smithiana*, 4 days after planting on C and S1, obtained by 20h DGT gel application. Metals are shown as DGT-measured metal fluxes  $f_{\text{DGT}}$  ( $\text{pg cm}^{-2} \text{s}^{-1}$ ). Root cross sections (h1, h2, h3, h4) and vertical soil profiles (v1, v2) are framed in boxes and refer to Supporting Information Figure SI-9 and SI-10. Excess Mn concentrations in C labeled with (a) are referring to a soil “reduction patch”, an experimental artifact.

if the metals are cosolubilized during Mn oxide reduction, they may be readily readsorbed to other constituents of the soil matrix. Since the remaining bulk soil area showed lower DGT-measured Mn fluxes it can be interpreted as a locally restricted, experimental artifact. A similar “reduction patch” reflected by increased Mn and Fe solubility in DGT imaging was observed in a preliminary rhizotron experiment.

### Response of Soil Oxygen to $\text{S}^0$ Oxidation and Root Growth.

Seventeen DAP we deployed planar optode sensors (POS) onto rooted soil of treatment C and S1 to explore changes in  $\text{O}_2$  levels in response to  $\text{S}^0$  oxidation at the microscale. In C,  $\text{O}_2$  was depleted from  $90.5 \pm 2.75\%$  in the bulk soil to  $82.8 \pm 5.23\%$  air saturation at the very surface of some, but not all, roots (Figure 3). Treatment S1 showed a



**Figure 3.** Two-dimensional mapping of oxygen concentration in rooted soil of *S. smithiana* in C (left images) and in  $\text{S}^0$ -amended soil (S1, right images), 17 days after planting and 48 h after POS application. Framed areas in the photograph indicate the areas selected for calculating the mean  $\text{O}_2$  concentration and standard deviation in rhizosphere and bulk soil ( $n = 2500$ ); WR denotes the upper limit of the working range.

much more pronounced depletion in  $\text{O}_2$  throughout various soil patches, which were partly located around single roots and partly in soil areas where several roots were present. Note that in addition to the roots visible in the photograph, roots covered with a thin soil layer that are not visible might have contributed to the observed  $\text{O}_2$  depletion pattern. While the maximum  $\text{O}_2$  level in S1 at locations far away from roots was similar to C with  $86.9 \pm 4.69\%$ , the minimum  $\text{O}_2$  level was  $44.6 \pm 5.92\%$ , much lower than the depletion along the single roots in C (Figure 3). This considerable, localized  $\text{O}_2$  depletion in S1

rendered several soil patches partially anaerobic and might thereby have contributed to a lower  $E_h$  in micro niches next to roots. Later measurements (72 h after application) showed a similar pattern where  $O_2$  depletion in several soil patches progressed in S1 (Supporting Information Figure SI-11). The decreased  $O_2$  concentrations in these patches supports our hypothesis that in the subsequent phases of  $S^0$  oxidation (>60 DAP), reductive dissolution of Mn (oxy)hydroxides and codissolution of Zn and Cd may—compared to the initial phase investigated here—have become a more dominant process of metal solubilization.<sup>19</sup>

**Environmental Implications.** The presented soil porewater data confirm the magnification of Zn and Cd solubility in the willow root zone in response to  $S^0$  amendments.<sup>19</sup> Repeated porewater sampling in the first few weeks after  $S^0$  application showed that after an initial high rate of  $S^0$  oxidation,  $SO_4^{2-}$  and  $H^+$  production as well as related metal solubilization level off in bulk soils. Sustained acidification and metal solubilization throughout the experiment could only be observed in the rooted compartments, especially at the higher  $S^0$  application rate (S2). Metal mapping along roots of *S. smithiana* by DGT-LA-ICP-MS showed that zones of high metal solubilization were largely confined to root surfaces with particular hotspots possibly in the root-hair zone. Our findings at macro- and microscale support the hypothesis that microbial oxidation of amended  $S^0$  is strongly enhanced close to root surfaces and can be sustained for longer periods, probably because of favorable physical habitat conditions (root surface) and continued supply of organic substrates through root exudation.

Using POS, we further demonstrate the occurrence of  $O_2$ -depleted zones along willow roots. While—apart from some microniches next to root surfaces—the  $O_2$ -depletion was probably not sufficient to induce reductive dissolution of Mn oxides in the  $S^0$ -amended soils (<60 DAP). This mechanism of metal solubilization might have become more important in the long term if  $O_2$  depletion would have progressed.<sup>19</sup> Based on our findings we suppose that during the early phase (<60 DAP) after  $S^0$  application,  $H^+$  production during the oxidation of  $S^0$  to  $H_2SO_4$  might be the key process triggering metal solubilization. Management of metal-polluted soils using phytoextraction can benefit from  $S^0$  amendments by localized, continuous enhancement of Mn, Zn, and Cd solubility and related plant uptake. With increasing amounts of  $S^0$ , effects in the soil porewater were more pronounced. Accumulation of Mn and Zn in willow shoots corresponded to concentrations in soil porewater and the amount of  $S^0$  added (Figure 1; Supporting Information Figure SI-4). However, foliar Cd levels in *S. smithiana* showed no significant ( $P \leq 0.05$ ) enrichment in the  $S^0$  treatments. The use of  $S^0$  amendments could potentially facilitate the processes controlling metal solubility in metal-polluted soils due to its distinct effects on metal solubilization in the willow rhizosphere compared to bulk soil. This technology represents an environmentally favorable option for enhanced metal removal in the rooted soil volume with reduced initial metal flushing from the bulk soil compared to chelant assisted phytoextraction enhancement techniques.

## ASSOCIATED CONTENT

### Supporting Information

Detailed information on soil characteristics and plant metal concentrations, rhizobox and rhizotron setup, rhizobox soil porewater data and statistical results, and results from DGT-

chemical imaging for Fe, Cu, and Pb can be found in the Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

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### Notes

The authors declare no competing financial interest.

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