



Doctoral Thesis

Effect of soil and plant amendments on trace element uptake and distribution in crop plants

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**Effect of soil and plant amendments on trace element uptake and
distribution in crop plants**

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of
DOCTOR OF SCIENCES

presented by

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Contents

Acknowledgements	I
Summary	VII
Zusammenfassung	XI
1 Introduction	1
2 Background	7
2.1 Historical background of the experimental site	7
2.2 Genes involved in Cd detoxification	12
2.3 Auxin – a growth promoting phytohormone	14
3 Phytomanagement of metal- contaminated agricultural land using sunflower, maize and tobacco	21
Abstract	22
3.1 Introduction	23
3.2 Materials and methods	24
3.2.1 Site description	24
3.2.2 Experimental design	25
3.2.3 Soil sampling and analysis	27
3.2.4 Plant sampling and analysis	27
3.2.5 Analytical quality assessment	28
3.2.6 Statistical analysis	28
3.3 Results	29
3.3.1 Soil	29
3.3.2 Plants	32
3.4 Discussion	38
3.4.1 Soil properties	38
3.4.2 Plant concentrations	39

3.5 Conclusions	41
3.6 Acknowledgements	41
3.7 References	42
4 Phytomanagement of metal-contaminated agricultural land – follow up	45
Abstract	46
4.1 Introduction	47
4.2 Materials and methods	47
4.3 Results	49
4.3.1 Soil	49
4.3.2 Plants	51
4.4 Discussion	53
4.5 Conclusion	55
4.6 Acknowledgements	55
4.7 References	55
5 Uptake and allocation of plant nutrients and Cd in maize, sunflower and tobacco growing on contaminated soil and the effect of soil conditioners under field conditions	57
Abstract	58
5.1 Introduction	59
5.2 Materials and methods	61
5.2.1 Experimental site and design	61
5.2.2 Sampling and sample analysis	62
5.2.3 Statistical data analysis	63
5.3 Results and Discussion	63
5.3.1 Soil	63
5.3.2 Plant growth and element uptake on untreated soil	65
5.3.3 Element allocation in plants on untreated soil	70
5.3.4 Treatment effects on plant growth, element uptake and allocation	72
5.3.5 Implications for agricultural crop production	75

5.4 Conclusion	76
5.5 Acknowledgements	76
5.6 References	77
6 Expression of genes involved in cadmium uptake and detoxification in tobacco plants grown on a sulphur-amended metal-contaminated field	81
Abstract	82
6.1 Introduction	83
6.2 Materials and methods	87
6.2.1 Experimental design	87
6.2.2 Cadmium and nutrient concentrations in tobacco	87
6.2.3 Gene expression analysis	88
6.2.4 Statistical analysis	89
6.3 Results	90
6.3.1 Treatment effects on soil properties	90
6.3.2 Treatment effects on element uptake by the plants	91
6.3.3 Gene expression	93
6.4 Discussion	95
6.5 Conclusion	98
6.6 Acknowledgements	99
6.7 References	99
7 Effect of auxin and EDDS on sunflower growth and heavy metal uptake	105
Abstract	106
7.1 Introduction	107
7.2 Materials and methods	108
7.2.1 Hydroponics experiments	108
7.2.2 Pot experiments	110
7.2.3 Root growth	113
7.2.4 Metal analysis of plant and soil samples	113
7.2.5 Statistics	113

7.3 Results	114
7.3.1 Hydroponics experiments (set 1)	114
7.3.2 Hydroponics experiments (set 2)	117
7.3.3 Pot experiments	120
7.4 Discussion	123
7.5 Conclusion	125
7.6 Acknowledgements	126
7.7 References	126
8 Conclusions	129
Curriculum Vitae	133

Summary

Heavy metal contamination of large areas of agricultural soil may cause health problems to humans and animals because of the accumulation of metals in the food chain. Heavy metal stress to plants can furthermore decrease crop production. On the other hand, leaving low or moderately-contaminated land fallow may be wasting a precious natural resource, given the increasing shortage of fertile agricultural soil. Techniques to decontaminate polluted soils and to restore their fertility are in demand, therefore. Phytoextraction has been proposed as such a technique and has been investigated extensively but primarily in hydroponic and pot experiments.

In the present study, we investigated the applicability of enhanced phytoextraction in a long-term field experiment that was carried out between 2000 and 2007 on a heavy-metal (Cd, Cu and Zn) contaminated former peat soil at Witzwil in the Bernese “Seeland”. We performed a crop rotation experiment with three high-biomass crop plants (maize, sunflowers and tobacco), as high biomass is needed for effective metal extraction and established cultivation techniques with the respective machinery are available for these agricultural crop plants. In order to increase the heavy-metal uptake of the plants, the following soil amendments were tested: (I) ammonium sulphate fertilizer, (II) elemental sulphur and (III) nitrilotriacetic acid (NTA), a biodegradable chelating agent. In a fourth treatment no amendments were applied for control besides the usual fertilization.

In the first six years of the study the amendments had only minor effects on metal uptake by the three crops. The differences between years were larger than the effects of the amendments, demonstrating the role of environmental factors that are difficult or even impossible to control. This variability also shows that the extrapolation of results from a single year to estimate remediation times is very questionable. Ammonium sulphate showed no effect at all, and NTA only increased Zn uptake by maize. Elemental sulphur increased Zn uptake in all three plants, but Cd uptake only in tobacco. The Cu uptake was not affected by any of the three treatments. In the seventh and eighth year we doubled the amount of applied NTA and sulphur. NTA still showed no effect, but the additional sulphur led to an amplified increase in Zn and Cd uptake by sunflower and tobacco. The extraction potential of tobacco came close to a level required to make phytoextraction a practical option. The predicted remediation time for Cd reached less than 50 years (instead of 240 years as in the

control treatment), given tobacco could be cultivated year after year and the Cd concentration in the soil would decrease linearly.

The analysis of total plant concentrations of Cd, Cu and Zn as well as their allocation within the plants (root, stem, leaves and seeds) showed that plants or plant parts of the crops grown on the Witzwil study site may be safely used for food or fodder production. Particularly the seeds contained very low metal concentrations. The highest metal concentrations were found in the leaves, which means that there could be problems when leafy vegetables would be grown. The leaf Cd concentration of the tobacco plants grown on the untreated plots, however, were still in the range that is normal for tobacco grown on uncontaminated soil and generally used for cigarette production. The application of NTA and sulphur had only minor effects on element allocation.

The expression of tobacco gene homologues of *Arabidopsis thaliana* believed to be involved in Cd detoxification and in sulphur uptake and assimilation (*ATM3*, *HMA4*, *MRP3*, *PDR8*, *Sultr1*, *LAST*, *APR2*, *APR3*, *GSHI*, *GSHII* and *NAS3*) revealed that the sulphur treatment had an effect on metal detoxification mechanisms. Most of the investigated genes were up-regulated in the roots after sulphur application. In the shoots, we found down-regulations in the older leaves, whereas the expressions of the studied genes were not affected in the younger leaves. With one exception these gene expression results agreed well with expectations for the roots according to published data, but not for the shoots. A possible explanation is that the field grown tobacco was exposed to metal stress during the whole growth period and harvested at maturity, while metal exposure in most lab experiments lasted for only short times, generally not more than two weeks. In contrast to lab experiments, the field grown plants also experienced multiple stresses. In particular, metal stress was triggered in our case by artificial soil acidification via the application of elemental sulphur, which could have led to different responses than Cd stress alone. Nonetheless, the study showed that results from controlled lab experiments are to some extent useful predictors of gene expression effects that may be expected in the field.

Besides increasing metal accumulation, it is also important to ensure good plant growth in phytoextraction operations. We tested whether the growth of metal-stressed crop plants may be enhanced by the application of the growth promoting phytohormone auxin, a method that has been proposed only recently. For this purpose, we performed hydroponic and pot experiments with Zn and Pb stressed (hydroponics) as well as multiple metal stressed (pots with soil from the Witzwil field experiment) sunflowers. Ethylene-diamine-disuccinic acid (EDDS), a biodegradable chelating agent similar to NTA, but stronger, was applied in addition to auxin in order to increase metal solubility. Auxin alleviated the Pb and Zn stress

of the hydroponically grown plants, promoting root and shoot growth. With unchanged metal accumulation, this resulted in an increased metal extraction by the plants in some cases. Auxin also increased Zn accumulation by the test plants in combination with EDDS in comparison to plants treated with EDDS alone. The effects observed in hydroponics did not translate into similar effects in the pot experiments, however, possibly because the applied auxin concentrations were too low or because there was not sufficient heavy metal stress in the experimental soil. In contrast, EDDS increased Cu solubility in the soil as well as Cu uptake by the plants.

On the basis of this study we suggest to use one of the following two phytomanagement strategies (combination of profitable plant production with pollution control and risk reduction) for the experimental site: (I) using heavy metal excluding crop plants without addition of metal solubilising amendments, or (II) focussing on Cd extraction by means of tobacco and other Cd-accumulating high-biomass plants (e.g. *Miscanthus sinensis* or *Brassica juncea*), in combination with applications of elemental sulphur. For both strategies additional screening experiments with other plants should be performed, monitoring metal accumulation in the plant parts that are targeted for human and animal consumption. Plant products with elevated metal concentrations may be used for non-food purposes such as biofuel, fibre and wood production. Particularly in the case of sulphur addition, also the subsoil should be monitored to recognize possible metal leaching at an early stage.

Zusammenfassung

Die Belastung grosser Flächen landwirtschaftlich genutzten Bodens mit Schwermetallen kann zur Gefährdung der Gesundheit von Menschen und Tieren führen, da sich Metalle in der Nahrungskette akkumulieren. Werden Pflanzen durch Schwermetalle belastet, kann dies zudem zu Ernteeinbussen führen. Leicht bis mittelstark belastete Böden brach liegen zu lassen wäre jedoch Verschwendung einer kostbaren natürlichen Ressource, vor allem in Anbetracht der weltweit immer knapper werdenden fruchtbaren Landwirtschaftsflächen. Es werden deshalb Techniken gesucht, mit welchen solche Böden saniert und deren Fruchtbarkeit wiederhergestellt werden können. Phytoextraktion ist eine mögliche Methode, die bereits umfangreich untersucht wurde, jedoch hauptsächlich in Nährlösungs- und Topfversuchen.

In der vorliegenden Studie haben wir die Anwendbarkeit beschleunigter Phytoextraktion in einem Langzeitexperiment untersucht, welches zwischen 2000 und 2007 auf einem schwermetallbelasteten (Cd, Cu und Zn) ehemaligen Moorboden in Witzwil im Berner Seeland, durchgeführt wurde. Wir führten ein Fruchtfolgeexperiment mit drei biomasse-reichen Kulturpflanzen (Mais, Sonnenblume und Tabak) durch. Grosse Biomasse ist nötig für einen wirkungsvollen Metallentzug aus dem Boden. Zudem ist der Anbau dieser Pflanzen etabliert, und eine entsprechende Maschinerie für die Bodenbearbeitung und Ernte ist verfügbar. Um die Schwermetallaufnahme der Pflanzen zu erhöhen, wurden folgende Bodenzusätze verwendet: (I) Ammoniumsulfat-Dünger, (II) elementarer Schwefel, und (III) Nitrilotriessigsäure (NTA), ein biologisch abbaubarer Chelator. In den Kontrollfeldern wurden als vierte Behandlung nebst der üblichen Düngung keine Zusätze verwendet.

In den ersten sechs Jahren der Studie übten die Bodenzusätze nur geringfügige Wirkung auf die Schwermetallaufnahme der Kulturpflanzen aus. Die Unterschiede zwischen den Jahren waren grösser als die Behandlungseffekte, was die starke Rolle von Umwelteinflüssen aufzeigt, welche sich nur schwer oder gar nicht kontrollieren lassen. Die Variabilität zeigt gleichzeitig, dass es fragwürdig ist, Resultate aus einzelnen Jahren zu extrapolieren, um Sanierungszeiten abzuschätzen. Ammoniumsulfat hatte überhaupt keine Wirkung und NTA erhöhte nur die Zn-Aufnahme von Mais. Mit elementarem Schwefel wurde die Zn-Aufnahme aller Pflanzen erhöht, jene von Cd jedoch nur bei Tabak. Die Cu-Aufnahme wurde von keiner Behandlung beeinflusst. Im siebten und achten Jahr des Experiments verdoppelten

wir die Mengen an ausgebrachtem NTA und Schwefel. NTA zeigte wiederum keinen Effekt, der zusätzliche Schwefel jedoch verstärkte die erhöhte Aufnahme von Zn und Cd durch Sonnenblume und Tabak. Das Cd-Extraktionspotential von Tabak kam dadurch einem Niveau nahe, das Phytoextraktion zu einer praktizierbaren Sanierungsvariante für diesen Boden macht. Die geschätzte Sanierungszeit von Cd erreichte einen Wert von unter 50 Jahren (statt 240 Jahre wie in der Kontrollgruppe), unter den Voraussetzungen, dass Tabak jedes Jahr angebaut werden könnte und dass die Cd-Konzentration im Boden linear abnehmen würde.

Aufgrund der Analysen der totalen Cd-, Cu- und Zn-Konzentrationen in den Pflanzen, wie auch der Verteilung der Metalle innerhalb der Pflanzen (Wurzeln, Stängel, Blätter und Samen), können Pflanzen oder zumindest einzelne Teile der Pflanzen, die im Versuchsgebiet von Witzwil angebaut werden, ohne Gesundheitsgefährdung von Mensch und Tier für die Nahrungs- oder Futtermittelproduktion verwendet werden. Vor allem die Samen enthielten sehr tiefe Metallkonzentrationen. Die höchsten Konzentrationen wurden in den Blättern gemessen, was eventuell beim Anbau von Blattgemüse ein Problem darstellen könnte. Auf dem unbehandelten Boden war die Cd-Konzentration in den Tabakblättern jedoch im selben Bereich wie jener von Tabak, der auf unbelastetem Boden angebaut und üblicherweise zur Zigarettenproduktion verwendet wird. Die Behandlung des Bodens mit NTA und elementarem Schwefel hatte nur geringfügige Effekte auf die Allokation der untersuchten Elemente.

Die Expressionen von Tabakgenhomologen von *Arabidopsis thaliana*, die wahrscheinlich in der Cd-Entgiftung und in der Aufnahme und Assimilation von Schwefel involviert sind (*ATM3*, *HMA4*, *MPR3*, *PDR8*, *Sultr1*, *APR2*, *APR3*, *GSHI*, *GSHII* und *NAS3*), haben gezeigt, dass die Schwefelbehandlung einen Effekt auf Metallentgiftungsmechanismen hat. Die meisten der untersuchten Gene in den Wurzeln wurden durch die Schwefelbehandlung hochreguliert. Die Genexpressionen im Spross wurden bei den älteren Blättern meist herunterreguliert, während sie bei den jüngeren Blättern nicht beeinflusst wurden. Mit einer Ausnahme stimmen die Resultate bei den Wurzeln gut mit den Erwartungen gemäss publizierten Daten überein, nicht aber bei den Sprossen. Eine mögliche Erklärung ist, dass der Tabak im Feld der Metallbelastung während der gesamten Wachstumsperiode ausgesetzt war und bei der Ernte das Reifestadium erreicht hatte, während die Stressexposition im Labor meist nur während kurzer Zeit erfolgt und für gewöhnlich nicht länger als zwei Wochen dauert. Im Gegensatz zu Laborexperimenten sind die Tabakpflanzen im Feld multiplem Stress ausgesetzt. Speziell in unserem Fall wurde der Metallstress durch künstliche Bodenversauerung mit Hilfe von Schwefelapplikationen erzeugt. Dies könnte zu anderen Reaktionen geführt haben, als wenn Cd alleine gewirkt hätte. Trotzdem zeigte die Studie, dass

Resultate, die von Versuchen stammen, welche unter kontrollierten Laborbedingungen durchgeführt wurden, bis zu einem bestimmten Grad nützlich sind, um Genexpressions-effekte vorauszusagen, die im Feld erwartet werden können.

Neben der Erhöhung der Metallakkumulation ist es auch wichtig, dass Pflanzen, die für Phytoextraktion eingesetzt werden, gut wachsen. Wir haben getestet, ob das Wachstum von metallgestressten Kulturpflanzen durch die Applikation des wachstumsstimulierenden Pflanzenhormons Auxin gefördert werden kann. Dabei handelt es sich um eine Methode, die erst kürzlich vorgeschlagen wurde. Für diesen Versuch haben wir Nährlösungs- und Topfversuche mit Sonnenblumen durchgeführt. Die Pflanzen in den Nährlösungen wurden erhöhten Pb- bzw. Zn-Konzentrationen ausgesetzt, während diejenigen im Topf durch einen Mix aus Metallen (Erde aus dem Feldversuch in Witzwil) belastet wurden. Ethylendiamindibernsteinsäure (EDDS), ein biologisch abbaubarer Chelator, ähnlich wie NTA jedoch stärker, wurde zusätzlich zum Auxin appliziert, um die Löslichkeit der Metalle zu erhöhen. Auxin milderte den Pb- und Zn-Stress der Pflanzen in den Nährlösungsversuchen, indem es das Wurzel- und Sprosswachstum förderte. Da die Metallkonzentration unverändert blieb, wurde die Metallextraktion in einigen Fällen erhöht. Auxin hat die Zn-Akkumulation der Testpflanzen in Kombination mit EDDS erhöht. Die Effekte, die in den Nährlösungsversuchen beobachtet wurden, konnten in den Topfexperimenten nicht nachgewiesen werden, möglicherweise weil die Auxinkonzentrationen zu tief waren oder weil die Belastung der Pflanzen durch die Schwermetalle im Versuchsboden zu wenig stark war. Im Gegensatz hierzu hat EDDS die Cu-Löslichkeit im Boden heraufgesetzt und die Cu-Aufnahme durch die Pflanzen erhöht.

Auf der Basis dieser Studie empfehlen wir, im Versuchsgebiet eine der beiden folgenden Phytomanagementstrategien (Kombination von profitabilem Pflanzenanbau mit Bodensanierung und Risikominderung) anzuwenden: (I) den Anbau von Schwermetall ausschließenden Kulturpflanzen ohne Applikation von Schwermetall lösenden Substanzen, oder (II) den Fokus auf die Cd-Extraktion mithilfe von Tabak oder anderen Cd-akkumulierenden Pflanzen, die eine hohe Biomasse produzieren (z.B. *Miscanthus sinensis* oder *Brassica juncea*), zu richten, und den Boden zusätzlich mit elementarem Schwefel zu behandeln. Für beide Strategien ist ein zusätzliches Selektionsexperiment mit weiteren Pflanzen nötig, wo die Akkumulierung der Metalle in den verschiedenen Pflanzenteilen untersucht wird, die für die menschliche oder tierische Ernährung vorgesehen sind. Pflanzenprodukte mit erhöhten Metallkonzentrationen können für Non-Food-Zwecke wie Biotreibstoffe, Faser- oder Holzprodukte verwendet werden. Speziell im Fall der Schwefelbehandlung muss auch der Unterboden beobachtet werden, damit eine allfällige Auswaschung der Metalle in tiefere Bodenschichten in einem frühen Stadium erkannt wird.

1

Introduction

Large areas of agricultural land worldwide are contaminated with heavy metals. Their accumulation in the food chain as well as direct uptake with contaminated soil is causing health risks to humans and animals. Furthermore, crop yields on contaminated land may be decreased because heavy metal stress may negatively affect plant growth. Environmentally sound techniques are needed to clean up such soils and to restore their fertility, so that they can again be used safely for agricultural food crop production. Many authors proposed to use phytoextraction of polluting metals from soil for this purpose in the last two decades (Pilon-Smits, 2005; Salt et al., 1995).

To achieve a high metal extraction rate plants do not only need to accumulate high concentrations of heavy metals, they also need to produce a sufficiently large biomass. High-biomass plants, however, usually do not accumulate very high concentrations of heavy metals, whereas hyperaccumulators are generally plants with slow growth and small biomass (Brooks et al., 1977). The application of soil amendments that increase metal bioavailability has been proposed therefore to enhance phytoextraction by high-biomass plants, for example the application of elemental sulphur. Through the oxidation of sulphur to sulphuric acid by edaphic microorganisms (Nor and Tabatabai, 1977), acidity is produced that decreases soil pH and thereby increases the solubility of cationic heavy metals (Tichý et al., 1997). Another strategy is the application of metal solubilising chelating agents such as EDTA (ethylenediaminetetraacetic acid), NTA (nitrilotriacetic acid) and EDDS (ethylenediaminedisuccinic acid) (Blaylock et al., 1997; Huang et al., 1997; Kulli et al., 1999; Tandy et al., 2006; Wenger et al., 2002). While EDTA is very persistent in soil, NTA and EDDS are rather biodegradable and thus cause much less leaching risks (Nowack et al., 2006).

Many hydroponic as well as pot experiments have been conducted to investigate the potential of phytoextraction as a strategy to remediate metal-contaminated soil, but only few field experiments, and none of them over a longer time. Field experiments are necessary to test the applicability of promising methods in real-world situations. They are also necessary to evaluate alternative strategies such as phytomanagement. Phytomanagement aims to combine profitable plant production (biofuel, timber, fibres, fertilizer) with pollution control

and risk reduction, including the reduction of leaching and erosion risks that may originate from soil contamination (Robinson et al., 2009).

Different plant parts generally accumulate metals to different degrees (Kurz et al., 1999). Some parts are thus usually less contaminated than others and may often still be used for consumption or at least for non-food purposes even if the plant has been grown on a rather strongly contaminated soil. Seeds generally accumulate the lowest concentrations of toxic elements in a plant (Angelova et al., 2004; McLaughlin et al., 1999). Little is known about how the allocation of pollutants in plants depends on their availability in soil and other soil factors. In particular it is not known how metal solubilising soil amendments such as elemental sulphur or chelating agents affect the allocation patterns.

Plants have a variety of metal uptake, transport and detoxification mechanisms encoded in their genes to respond to metal stress (Hall and Williams, 2003; Hanikenne et al., 2005; Mills et al., 2003; Plaza and Bovet, 2008). Stress effects caused by metal-polluted soil may therefore be evaluated by studying the expression of genes involved in metal uptake and detoxification. Monitoring the expression of such genes may also help to adjust soil amendments in phytoextraction and phytomanagement schemes, e.g. the application of elemental sulphur to enhance the availability of polluting metals for plant uptake. In the case of sulphur it has to be considered that the amendment is in the same time an essential plant nutrient that is involved in the plant's detoxification mechanisms. Thus, also the investigation of genes involved in sulphur uptake and assimilation is of particular interest in this case (Kopriva and Koprivova, 2004; Sun et al., 2005; Xiang and Oliver, 1998). Gene expression analysis of Cd-stressed plants has been carried out in various hydroponic, agar and pot experiments, but not under field conditions. Because growth conditions and stress situations are much more complex in the field than in laboratory experiments, gene expression in field plants may be very different from what is predicted on the basis of laboratory studies.

The uptake of water and solutes by plant roots strongly depends on total root surface, length and branching pattern. There is a gradient in the uptake of water along the root axis, declining from the apex to the basal zones, which also affects solute uptake. The larger the total area of root tips, the higher the potential to take up solutes (Marschner, 1995). Auxin is a phytohormone that is known to promote root growth by promoting cell extension and division as well as by initiation of lateral root growth (Taiz and Zeiger, 2000) and that was found to alleviate heavy metal stress to plants through this growth-promoting ability (Israr and Sahi, 2008; Leinhos and Bergmann, 1995; Sheng and Xia, 2006). A few studies have

found that it may be used therefore to enhance phytoextraction, especially in combination with EDTA (Liphadzi et al., 2006; Liu et al., 2007).

The general objectives of this study were:

- (I) to examine the potential of metal-solubilising soil amendments to enhance the phytoextraction of soil-polluting metals by high-biomass crop plants under field conditions over a time of more than one crop rotation,
- (II) to investigate the effect of metal-solubilising soil amendments on metal allocation within the plants,
- (III) to investigate the effect of metal-solubilising soil amendments on the expression of selected genes involved in metal uptake and detoxification in field grown plants, and
- (IV) to test the potential of growth-promoting phytohormones to alleviate phytotoxicity of soil-polluting metals and to increase metal phytoextraction by the combined application of phytohormones and chelating agents.

Based on the available literature, in particular the following hypotheses were proposed and tested:

- (I) The application of ammonium sulphate fertilizer, elemental sulphur and NTA to metal contaminated soil can be used to increase the uptake of Cd, Cu and Zn by crop plants grown under field conditions in a realistic agronomic setting. The effects of ammonium sulphate fertilizer and elemental sulphur are expected to increase over time.
- (II) The three soil amendments increase metal uptake predominantly in leaves and stems, but not in the seeds of field grown crop plants.
- (III) Elemental sulphur applied to a Cd-contaminated soil will induce responses in the expression of genes involved in Cd detoxification and in sulphur uptake and assimilation of tobacco plants (predominantly in the roots) grown on such soil.
- (IV) Auxin applied to nutrient solutions and soil will alleviate metal toxicity to crop plants (in our case sunflowers) and increase growth and metal uptake by the plants. This effect is also expected to work in combination with the application of metal-solubilising chelants (in our case EDDS).

To test these hypotheses the following experiments were performed and presented in detail in the following chapters:

- A field experiment extending over 8 years was carried out on a heavy-metal (Cd, Cu and Zn) contaminated former peat soil at Witzwil in the Bernese “Seeland” in which sunflower, maize and tobacco were grown in crop rotation and treated with four different soil amendments: (I) no amendment (control), (II) ammonium sulphate fertilizer, (III) elemental sulphur and (IV) NTA. Chapter 3 deals with the first six years of the experiment (2000 – 2005), focusing on metal and nutrient uptake and their year-to-year variations. Possible management strategies for the site are discussed. As the treatment effects were rather small, the experiment was extended for another two years (2006 and 2007) with the following modifications: Treatment II was not evaluated anymore and the application rates of treatments III and IV were doubled in half of the plots, in order to test the maximum phytoextraction rate achievable with the chosen plants at this particular site in practice (Chapter 4).
- In Chapter 5 we evaluate the possible use of different plant parts of sunflower, maize and tobacco grown in the previously mentioned field experiment as food, feed or other produce. We analyzed the concentrations of the nutrient elements Ca, Cu, Fe, K, Mg, Mn, P, S and Zn and the problem trace element Cd in roots, stems, leaves and seeds. The aim was to determine the allocation patterns of these elements and how they were affected by the soil amendments elemental sulphur and NTA.
- In Chapter 6 we describe and discuss the influence of sulphur applications on gene expression in roots and shoots of tobacco plants grown in the Witzwil field experiment. We determined the effect of sulphur application on gene expression by means of semi-quantitative RT-PCR. For this purpose we investigated tobacco homologues of *Arabidopsis thaliana* genes known to be involved in Cd transport and detoxification (*ATM3*, *HMA4*, *MRP3*, *PDR8*) and genes involved in sulphur uptake and assimilation as well as in Cd detoxification (*Sultr1*, *LAST*, *APR2*, *APR3*, *GSHI*, *GSHII*, *NAS3*).
- Finally, we investigated the effects of auxin, with and without EDDS, on root and shoot growth and on metal uptake by metal-stressed sunflower plants. These results are presented and discussed in Chapter 7. The first experiments were carried out with Zn and Pb in nutrient solutions in order to avoid influences of factors other than auxin and EDDS. In a subsequent experiment auxin was applied to soil from the Witzwil study site in a pot experiment with sunflowers. We applied indole-3-acetic acid (IAA, the most common auxin in plants) at different concentrations with or without simultaneous

application of the chelant EDDS. The rates of auxin applications ranged between 10^{-12} and 10^{-9} M for the hydroponics experiment and between 10^{-11} and 10^{-7} M for the soil in the pot experiment. EDDS was chosen instead of EDTA because it is readily biodegradable and thus considered much less problematic in terms of environmental risks than EDTA and other synthetic chelants.

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2

Background

The aim of this chapter is to give background information on some subjects this thesis deals with. Chapter 2.1 gives historical background information on the field site where we carried out our field experiments and where the soil for the laboratory experiments originated from. Chapter 2.2 explains the reason why which genes were chosen for the gene expression experiments in Chapter 6 and introduces into their functions. Chapter 2.3 finally gives some background information on the functions of the phytohormone auxin, which was used in the laboratory experiments in Chapter 7.

2.1 Historical background of the experimental site

Our field experiment was carried out on the estate of Witzwil (see below) in the western part of Switzerland. Witzwil is located in the Bernese “Seeland” (“lake land” in English) (Figure 2-1), which is the Bernese part of the area between the three lakes of Neuenburg, Biel and Murten. This area is also called “Grosses Moos” (“great bog” in English), indicating that it was swampy and regularly flooded before it was drained and converted to agricultural land through the first “Juragewässerkorrektion” (“correction of the Jura waters” in English) at the end of the 19th century.

During this first Juragewässerkorrektion (1861 - 1891) the Aare River was redirected into the lake of Biel, and the rivers connecting the three lakes as well as the outflow of the lake of Biel were canalised. Furthermore, the groundwater level of the whole system was drawn down by decreasing the water levels of the lakes of Neuenburg and Biel (Nast, 2006).

The costs for the corrections as well as the rights derived from them were originally planned to be shared by one third each by the Federation, the adjacent cantons (Bern, Freiburg and Neuenburg) and the municipalities of the Seeland. As the municipalities did not have sufficient means, the cantons also took over their share in costs and rights. Each canton built a prison in this area. The one of the Canton of Bern is the Witzwil prison, which

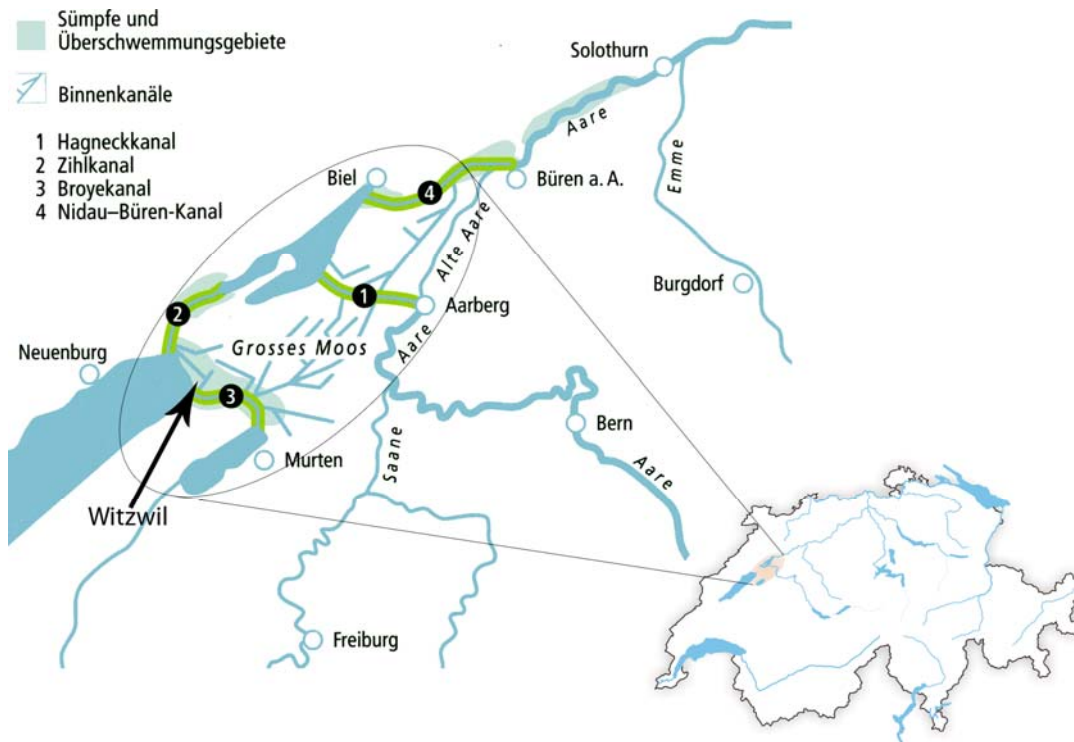


Figure 2-1: Map showing the first “Juragewässerkorrektion” (correction of the Jura waters) in the Bernese “Seeland” (lake land). Pale blue area (Sümpfe und Überschwemmungsgebiete): swamps and flood areas; blue lines (Binnenkanäle): drainage channels; 1 – 4 names of four different drainage channels. (1) Redirection of the Aare River into the lake of Biel, (2) and (3) corrections of the rivers connecting the three lakes, and (4) canalisation of the outflow of the Lake Biel. Origin of images: Map of Switzerland (http://commons.wikimedia.org/wiki/File:Karte_Seeland.png), map showing the Juragewässerkorrektion (Nast, 2006).

comprises the land where our experiment was carried out (oral communication by Peter Trachsel, head of the Witzwil prison estate, 14th May 2009).

Unfortunately major floods continued to occur frequently also after the first Juragewässerkorrektion, e.g. during the flood in 1944 (Figure 2-2), due to inappropriate proportions between inflow and outflow of the three lakes and unexpectedly strong soil subsidence (Nast, 2006). While the Witzwil estate was struggling with the problems of continuing soil subsidence and low fertility of the drained peat soil (only potatoes and sugar beet could successfully be cultivated, but no corn), it happened that the city of Bern was looking for new domestic waste disposal sites, as the gravel pits used so far for this purpose were filling up (Gemeinden des Amtes Erlach, 1974).

The idea was to plough organic waste material into the drained soil in order to stabilize it against further subsidence and to improve fertility. In 1913 a contract was made with the city



*Figure 2-2: Flood in the Bernese Seeland, Witzwil, 1944.
Origin of photos: Archives of the Witzwil prison (left), Nast (2006) (right).*

of Bern for the use of their city waste. The city was committed to deliver the whole unsorted waste. The waste included organic domestic waste and horse manure, which were both favourable, but also inorganic wastes and bottom ash from coal-fired power plants (oral communication by Peter Trachsel, 14th May 2009). The waste was delivered by railway, unloaded and inorganic compounds such as metals, glass, textiles and paper were sorted out and recycled (Figure 2-3). Thereafter it was temporarily stock-piled in big heaps for about 3 years for decomposition of the organic matter (oral communication by Peter Trachsel, 14th May 2009).

After separation and stockpiling the organic waste was loaded onto tipping lorries. These lorries were hauled into the fields of the Witzwil area by horses on mobile tracks that were temporarily laid on the land for this purpose (Figure 2-4). Layers of 15 to 20 cm of waste were distributed on the fields and ploughed into the soils. Inorganic waste materials that had not been sorted out before were collected after each tillage operation. The disposal was repeated on a given piece of land every 8 to 10 years over a period of 40 years (oral communication by Peter Trachsel, 14th May 2009).

At first, i.e. in the 1920s, the application of the wastes indeed increased yields. With time, however, the quality of the waste decreased. Even though it was sorted, more and more inorganic waste materials found their way onto the fields. Farmers started to suffer from yield losses. While the exact reason remained unclear, the yield losses were suspected to originate from the waste disposals. Besides, people living in the area complained because of the nasty smell. Furthermore it became more and more difficult to find qualified volunteers

among the prisoners to distribute the wastes on the fields (oral communication by Peter Trachsel, 14th May 2009). In 1953 the administration of the Witzwil prison withdrew from the contract, and the waste disposal was discontinued. Over the 40 years from 1913 to 1953 about 500'000 tons of waste were distributed over an area of 600 to 800 ha. On a sunny day one can still see the sparkle of broken glass all over the area even today. For this reason the area is also called "Scherbenland" (shard land).

As floods and subsidence continued to cause severe damage, the second Juragewässer-korrektion was realised in the Bernese Seeland between 1962 and 1973. Since then the



Figure 2-3: Above: Unloading and sorting of city waste by occupants of the Witzwil prison. Below: Pigs salvage part of the organic waste. Origin of photos: Archives of the Witzwil prison.





*Figure 2-4: Waste transport in tipping lorries and disposal on the field.
Origin of photo: Archives of the Witzwil prison.*

Bernese Seeland has become the most important area for vegetable production in Switzerland. The waste residues in the fields around Witzwil were not regarded as a problem, until in 1997 the construction of a new highway crossing the Witzwil area was planned. As it passed through agricultural land, land reallocation was required. As a basis for the land reallocation and for the environmental impact assessment, a soil survey was performed that led to the discovery of substantially elevated heavy metal concentrations (Cu, Zn, Pb and Cd) in the Scherbenland soils. In a more detailed follow-up survey the areal extent of the contamination was determined and in addition crop samples were analysed by the cantonal authorities. With a few exceptions the heavy metal concentrations of the plants did not exceed those normally found in crops grown on uncontaminated soil. The cantonal authorities thus declared that the consumption of these products was generally safe (Rytz, 2001). However, the information about the soil contamination led to an image problem for crops produced in the Witzwil area. One important distributor discontinued to accept agricultural products from the area for label products, resulting in substantial economic losses for the Witzwil estate and neighbouring farms.

The aim is now to find a solution for how to remediate that soil and simultaneously manage the land so that it could be used for the production of viable biomass with low risk for human and animal health. This approach is known as phytomanagement (Robinson et al., 2009).

2.2 Genes involved in Cd detoxification and sulphur metabolism

In one treatment of our field experiment we used elemental sulphur in order to increase the availability of heavy metals for plants by artificial soil acidification. In Chapter 6 we analyze the effects of this treatment on the expression of genes involved in Cd uptake and detoxification in field-grown tobacco plants. Gene expression is the process by which the information from a gene is used in the synthesis of a functional gene product, e.g. a protein. Only a small number of genes is expressed at a given time while the rest is inactive. Which genes are expressed is given by the stage of growth and by the environmental conditions. Changes in the environmental conditions may thus induce the expression of different genes so that the plant can adapt to the respective situation. In the following we give a brief account about what is known on the genes we chose to investigate and their functions in plants. All these genes have in common that they are influenced by Cd exposure and/or the plant's sulphur status (for more details see chapter 6). Monitoring gene expression can thus help to identify environmental stresses acting on a plant.

Being a macronutrient element of plants, sulphur applied to a soil may not only affect soil pH, but also the sulphur nutrient status and metabolism of the plants grown on this soil. Plants use sulphur primarily to synthesize cystein and methionine, as well as numerous essential secondary metabolites deriving from these two amino acids. Sulphur uptake by plant cells is a metabolically active process. Sulphate transporters of higher plants are divided into high- and low-affinity sulphate transporters (Leustek and Saito, 1999). High-affinity sulphate transporters (HAST), such as *Sultr1;1* and *Sultr1;2* (Yoshimoto et al., 2002), are mainly found in the roots. They are therefore thought to mediate the initial uptake of sulphate from the soil into the roots (Smith et al., 1995). Low-affinity sulphate transporters (LAST) are found in roots and leaves, suggesting that they are involved in the translocation and redistribution of sulphate in plants (Sun et al., 2005). In the assimilation of sulphate-S, sulphate is reduced in several steps to the amino acid cysteine. The sequence of reactions is shown in Formula 2-1. Adenosine 5'-phosphosulphate reductase (APS reductase, APR) was found to be the key enzyme controlling this pathway (Kopriva and Koprivova, 2004). APR catalyzes a thiol-dependent two-electron reduction of APS to sulphite (SO_3^{2-}).



Glutathione (GSH, GluCysGly) is a tripeptide composed of the amino acids glutamate (Glu), cysteine (Cys) and glycine (Gly). It is synthesized in two ATP-dependent steps. The

first step is the synthesis of glutamate and cysteine (Formula 2-2a), catalyzed by γ -glutamylcysteine synthetase (γ -ECS); the second step is then the addition of glycine to the resulting glutamylcysteine (Formula 2-2b), catalyzed by glutathione synthetase (GSHS) (May et al., 1998). The two enzymes are encoded by *GSHI* and *GSHII*, respectively (Zhu et al., 1999).



Glutathione is a precursor in the synthesis of phytochelatins (PCs), which are heavy metal-chelating compounds that play an essential role in Cd detoxification of plants (Howden et al., 1995). They form PC-Cd complexes in the cells which are sequestered in the vacuole, preventing free circulation of toxic Cd ions in the cytosol (Clemens et al., 2002). Besides PCs, also nicotianamines (NAs) are involved in heavy metal chelation, transport and detoxification (Sharma and Dietz, 2006). They are also thought to be involved in the transfer of excess metals from the roots into the shoots (Sharma and Dietz, 2006). Nicotianamine is an oligomer compound of three molecules of methionine, synthesized by means of nicotianamine synthase (NAS) and is ubiquitously present in plants (Sharma and Dietz, 2006).

Plant cells eliminate excess metal ions and toxic ions either by excreting them into the extracellular space or by their sequestration into the vacuole (Clemens, 2001). Heavy metal transporting ATPases (HMA) are important membrane enzymes in maintaining Zn and Cu homeostasis in plants and minimizing the detrimental effect of non-essential heavy metals such as Cd (Mills et al., 2003). They were found to be involved in the translocation of Cd and Zn from roots into shoots and in cytosol detoxification as efflux pumps (Courbot et al., 2007). Besides HMAs, also ATP-binding cassette (ABC) proteins play an important role in the transport of heavy metals in plants (Hall and Williams, 2003). ABC proteins are involved in the transport of a wide range of substrates including ions, sugars, lipids, peptides, pigments, xenobiotics and antibiotics. Some members of the ABC transporter family are known to confer Cd tolerance to plants: e.g. MRP3 (multidrug-resistance-related protein) (Kolukisaoglu et al., 2002), ATM3 (ABC transporter of mitochondria) (Kim et al., 2006), and PDR8 (pleiotropic drug resistance) (Kim et al., 2007). Processes in plants that are likely to depend on MRPs are the detoxification of herbicides and other organic xenobiotics, alleviation of oxidative damage, storage of endotoxins, heavy metal sequestration and

vacuolation of natural pigments (Rea, 1999). Kim et al. (2006) showed that ATM3 plays a role in plant resistance to metal stress and also presented evidence that ATM3 is involved in the regulation of cellular glutathione levels. Kobae et al. (2006) reported that PDR-like ABC transporters were first described in yeast, where they confer resistance to a multitude of toxins and organic acids. PDR8 transporters are located in plasma membranes where they may excrete cell synthesates or xenobiotic compounds. Kim et al. (2007) observed that PDR8 also contributes to Cd resistance by acting as a major Cd²⁺ efflux pump across the plasma membrane of root epidermal cells.

Most of the functions of the genes described here were found in *Arabidopsis thaliana*. We analyzed the expression of homologues of these genes (*Sultr1*, *LAST*, *APR1*, *APR2*, *GSHI*, *GSHII*, *NAS3*, *ATM3*, *MRP*, *HMA4*, and *PDR8*) in the roots and shoots of tobacco plants, assuming that these homologues fulfil similar functions as the respective *Arabidopsis* genes.

2.3 Auxin – a growth promoting phytohormone

In Chapter 7 we investigate the effect of the phytohormone auxin on plant growth and metal uptake in hydroponics and pot experiments. Phytohormones are a group of naturally occurring biochemically active organic substances in plants that control and coordinate physiological processes such as growth, differentiation and development. They act as signal messengers. Based on their structural similarities and on their effects on plant physiology, phytohormones are divided into five groups: auxins, cytokinins, gibberellins, abscisic acid and ethylene. Auxins, cytokinins and gibberellins promote growth, while abscisic acid and ethylene are growth inhibitors. Furthermore, there are steroid hormones and some other messengers such as jasmonate, salicylic acid and systemine that affect morphogenesis, pathogen resistance and herbivore defence (Taiz and Zeiger, 2000). Most physiological processes are not controlled by a single hormone, but by a complex interaction of several hormones acting together (Dörffling, 1982).

Auxin was the first phytohormone to be discovered and is often regarded as a “master” hormone because cell division, growth, maturation and differentiation are all associated with auxin regulation (Buchanan et al., 2000). There are numerous molecules belonging to the auxins. The most common and physiologically most important auxin in plants is indole-3-acetic acid (IAA, Figure 2-5). An auxin is by definition a substance with similar but not necessarily identical spectrum of effects as IAA. This includes: (1) promotion of cell elongation in isolated coleoptiles or shoot segments; (2) induction of cell division in callus

tissues in conjunction with cytokinin; (3) promotion of the formation of adventitious roots on cut shoot surfaces; (4) induction of parthenocarpic growth of tomatoes and (5) induction of ethylene synthesis. This definition was suggested by Cleland (1996) and may become refined with additional knowledge of auxin functions using new methods in molecular genetics (Taiz and Zeiger, 2000). To bind to a specific receptor, an auxin needs three main components: a planar, aromatic ring system, a carboxylic group and a hydrophobic transition group that divides the two binding sites. At neutral pH all substances with auxin activity carry a strong negative charge at the carboxylic group and a comparatively weak positively charged ring system. This separation of charges is probably a critical structure characteristic of the auxin activity. The pK_a of IAA is 4.75 (Taiz and Zeiger, 2000).

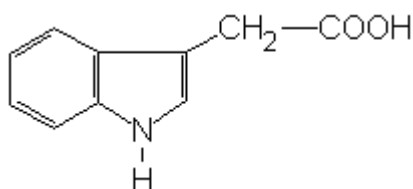


Figure 2-5: Indole-3-acetic acid (IAA)

There are two pools of auxin within a plant cell, one in the cytosol and one in the chloroplasts. The response of cells or tissues to auxin is determined by the concentration of free IAA. Normal auxin concentrations in the shoot are between 10^{-6} and 10^{-5} M, while they are much smaller in the roots (Taiz and Zeiger, 2000). Auxin concentrations that stimulate root growth range between 10^{-7} and 10^{-13} M (Salisbury and Ross, 1992), while higher concentrations inhibit plant growth. Most of the IAA present in plant cells, however, is covalently bound to other molecules such as sugars, amino acids and peptides (Hobbie, 2007). The concentration of free auxin can be controlled by factors such as synthesis and degradation of conjugated IAA, IAA-metabolism, compartmentalisation and polar auxin transport (Taiz and Zeiger, 2000).

There are several pathways of IAA biosynthesis, part of them are tryptophan-dependent (i.e. involving tryptophan as an intermediate) and others tryptophan-independent. Instead of directly applying IAA to the soil for plant treatment it is thus also possible to apply tryptophan, which is then converted into IAA by edaphic microorganisms (Martens and Frankenberger, 1993). The pathways of IAA biosynthesis are not yet completely known, but there are probably developmental and feedback regulations so that different pathways may be used at different stages or in different tissues (Hobbie, 2007). Because of the multiple and

independent auxin synthesis pathways, deficiency was not found so far. The degradation of IAA proceeds primarily through oxidation of the indole moiety or through decarboxylation (Hobbie, 2007; Taiz and Zeiger, 2000).

Auxin is primarily synthesized in tissues with high division activity, particularly in apical meristems of the shoot, in young leaves and in ripening fruits. It can also be synthesised in adult leaves and at the root tips. However, the rate of synthesis is usually lower in these tissues (Taiz and Zeiger, 2000). Auxin is transported from shoot to root. There are two pathways of auxin transport: a nonpolar, which proceeds via bulk flow in the phloem and is poorly understood, and a polar cell-to-cell transport, which occurs via chemiosmotic mechanisms. Three families of membrane proteins involved in auxin transport have been identified: the AUX1/LAX proteins, the PINs and the PGP/MDRs (Hobbie, 2007). They all seem to be necessary for the polar auxin transport. It is furthermore suggested that phosphorylation plays a regulatory role in auxin transport (Hobbie, 2007).

Two major effects of auxin on cells have been identified: rapid changes in protein activity and changes in gene expression. Four major groups of genes that are either induced or repressed by auxin have been identified. They are involved in cell elongation, conjugation of IAA to amino acids, biosynthesis of the plant hormone ethylene and transcriptional repression (Hobbie, 2007). The best studied auxin-affected protein is the plasma membrane H^+ -ATPase. Rapid activation of this proton pump by auxin increases the efflux of protons into the cell wall which leads to its acidification. The “acid growth hypothesis” states that the induction of cell elongation through auxin is due to this cell wall acidification. This acidification was postulated to increase the cell wall elasticity (probably by activating wall proteins called expansins), thus enabling the turgor pressure of the cell to stretch the cell (Hobbie, 2007).

Liphadzi (2006) states that the electrochemical gradient that is created by the activation of the H^+ -ATPase leads to the opening of cation channels or activates ion transport proteins in the plasma membrane, which results in the influx of cations. Plants may therefore take up more cations by this active transport in the membranes of root cells when IAA is applied. The application of IAA to plants has furthermore been shown to induce stress tolerance to plants when they are stressed with drought or heavy metals. The application of IAA increased root and in some cases also shoot growth (Israr and Sahi, 2008; Leinhos and Bergmann, 1995; Liu et al., 2007; Sheng and Xia, 2006). These properties may make auxin useful for phytoextraction or phytomanagement purposes in the way that it alleviates the metal stress by increased root and shoot growth. The larger and healthier root system may be able to take up more heavy metals, and a higher yield may lead to enhanced metal extraction.

2.4 References

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3

Phytomanagement of metal-contaminated agricultural land using sunflower, maize and tobacco

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Abstract

We investigated the long-term effectiveness of phytomanagement (the combination of profitable crop production with the gradual reduction of soil contamination by phytoextraction) to deal with moderately metal-contaminated agricultural land. In a 6-year field experiment, we grew maize (*Zea mays* L.), sunflower (*Helianthus annuus* L.) and tobacco (*Nicotiana tabacum* L.) in crop rotation. The addition of elemental sulphur (2136 kg ha⁻¹ a⁻¹) decreased the soil pH from 7.4 to 6.7, increased the Zn accumulation by maize, sunflower and tobacco by factors of 1.3, 1.4 and 1.2, respectively, and increased the Cd accumulation by tobacco 1.3-fold. Neither the addition of ammonium sulphate (129 kg ha⁻¹ a⁻¹) nor nitrilotriacetic acid (NTA, 430 kg ha⁻¹ a⁻¹) significantly increased phytoextraction. The results show that phytoextraction for soil cleansing would require centuries. However, this land could be used to generate profitable crops, including the production of safe (low Cd) stock fodder fortified with Zn, green manure for micronutrient-deficient soils, or bioenergy.

3.1 Introduction

Excessive application of low-quality fertilizers, pesticides, sewage sludge and other bio wastes has increased the concentrations of heavy metals in many agricultural soils worldwide above levels considered safe for food production. Metal leaching from contaminated soil into waters and transfer into food chains can endanger human health as well as ecosystem quality. Phytoextraction, i.e. the use of plants to extract contaminants from soils (Salt et al., 1995), is often touted as a gentle, environmentally friendly cleanup method. Theoretically, it should be suited for the treatment of large areas of agricultural land that are contaminated at low to medium levels (McGrath et al., 2002). Repeated cropping of plants that take up contaminants from soil should lower the soil's contaminant concentrations to acceptable levels, provided the harvested amounts of contaminants exceed further inputs. Each cropping would remove contaminants from the area. The metal-rich biomass would be burned, fermented or used in gasification to reduce its volume. Residual material that is rich in the contaminating heavy metals could be reprocessed to recover the metals or stored in an appropriate area, such as a contained landfill, that does not pose a risk to the environment.

Phytoextraction requires that plants accumulate large amounts of contaminants into the above-ground biomass. Hyperaccumulator plants do have this property (Brooks et al., 1977), but most of them produce little biomass so that their extraction efficiency is usually limited. In addition, agronomic cultivation techniques would still need to be developed for these plants. As an alternative strategy it has been proposed to use high biomass crop plants for which agricultural techniques exist and increase the accumulation of the contaminating metals by the application of amendments that increase their bioavailability. The addition of chelants such as EDTA can dramatically increase plant metal uptake (Blaylock et al., 1997; Huang and Cunningham, 1996). However, the formation of soluble metal-organic chelates also increases the risk of metal leaching to groundwater, in particular if the chelant is resistant to biodegradation (Nowack et al., 2006). The targeted application (e.g. the injection into the root zone) of low amounts of biodegradable compounds such as NTA or EDDS (Kulli et al., 1999; Tandy et al., 2006) may reduce risks to acceptable levels. Another possibility to solubilise metals such as Cd and Zn in soil is artificial soil acidification. In practice this can be achieved by the addition of elemental sulphur (S₈). The oxidation of elemental sulphur to sulphuric acid, which is catalyzed spontaneously through the activity of autochthonous bacteria such as *Thiobacillus*, generates acidity, resulting in a decrease in soil pH (Nor and Tabatabai, 1977). Decreasing soil pH increases the solubility of metal cations and thus also their bioavailability for plant uptake. The potential to enhance plant metal

uptake by application of elemental sulphur or biodegradable chelants has been demonstrated in pot experiments (Meers et al., 2005; Tichý et al., 1997; Wenger et al., 2002), but there is little information on how these techniques work under field conditions. Kayser et al. (2000) applied NTA and elemental sulphur for 1 year on a metal-contaminated agricultural field and found that even with artificially enhanced solubilisation of the contaminants, phytoextraction was still by far too slow to be viable in practice, unless the land could be used at the same time to produce an income that makes the whole operation profitable. The combination of phytoremediation and crop production is known as phytomanagement (Domínguez et al., 2008; Robinson et al., 2007). Potential plant products are non-food products such as biofuel, fibre, wood or, depending on the contamination level, animal feed. In order to show the viability of this approach, field experiments are necessary to test the implementation in agronomic practice. Long-term field trials are also necessary to test the effectiveness over time as climate and soil conditions vary from year to year and crop rotation is required. Therefore, the objective of this study was to investigate the potential use of sunflower, maize and tobacco in combination with the application of low amounts of biodegradable soil conditioners, such as NTA and sulphur, to remediate a heavy metal-contaminated soil and contemporaneously produce biomass that could be used as biofuel, animal feed or other purposes, depending on the levels of contaminant uptake.

3.2 Materials and methods

3.2.1 Site description

The experiment was performed on an agricultural field on a eutric anthropic regosol (FAO soil order) at Witzwil, in the Bernese Seeland, Switzerland. The Seeland area (46°58'60N and 7°2'60E, 432 m a.s.l.) has a temperate climate, with a mean annual temperature of 9 °C, and an average annual rainfall of 980 mm. It was a fen before being drained some 150 years ago and put into agricultural cultivation. To improve the soil fertility, municipal waste, including ash from coal-fired power plants, from the city of Bern was applied between 1913 and 1954 on large parts of the area. Initially, the applied waste was mainly composed of organic matter (manure, municipal refuse and green waste). However, the fraction of indecomposable materials (waste materials containing plastic, glass or metal) increased until 1954 when waste application was discontinued. As a result of these applications, the soil became contaminated with heavy metals. The Bernese soil protection agency (Rytz, 2001) reported Cd, Cu and Zn concentrations in excess of the guide (Cd, Zn)

or trigger (Cu) values of the Swiss Federal “Ordinance Relating to Impacts on the Soil” (OIS) (VBB0, 1998), indicating that this site may negatively affect ecosystem quality or human health. The guide values for total Zn and Cd concentrations in the soil are 150 and 0.8 mg kg⁻¹, respectively, and the trigger value for Cu is 150 mg kg⁻¹. Analysis of white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) samples, however, showed that contaminant uptake by these plants was minimal, indicating that the bioavailability of these contaminants may be low (Rytz, 2001). Based on the report by Rytz (2001), Cd, Cu and Zn were selected as problem contaminants to be further investigated.

3.2.2 Experimental design

Maize (cv. Magister), sunflower (cv. Sanluca), and tobacco (cv. Burley 92) were grown from 2000 to 2005 in a 3-year rotation scheme on three blocks in the experimental field. The rotation followed the order maize, sunflower, tobacco. All three crops were grown in each experimental year, but never consecutively on the same block (Figure 3-1). Such rotation is standard agronomic practice in Switzerland and elsewhere. Maize and sunflower were sown around the 10th of May. Tobacco seedlings were planted around the 20th of May. Each block was subdivided into 16 plots with a size of 12 m x 3 m each. Four treatments were applied in

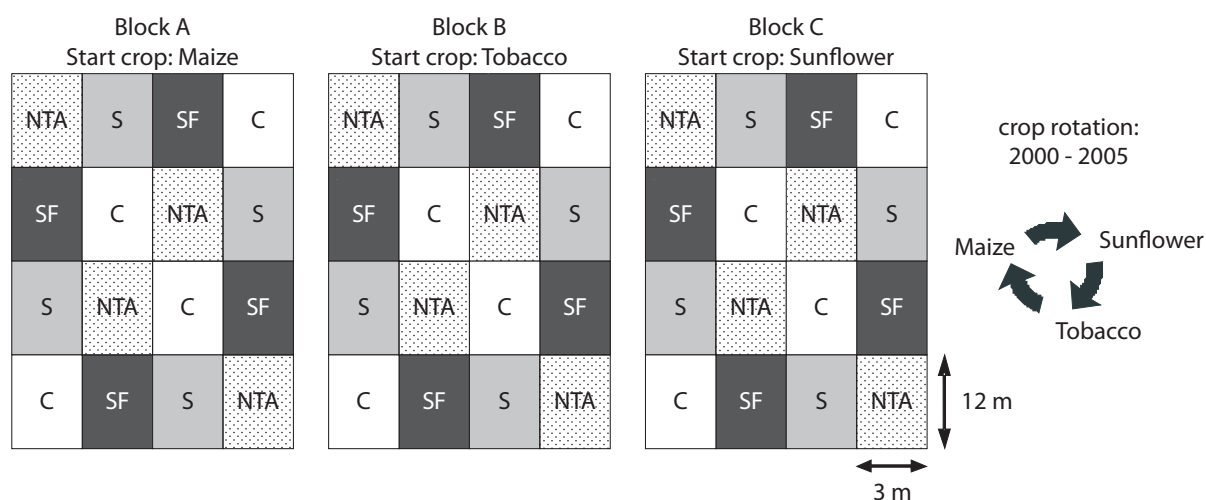


Figure 3-1: Experimental design. Each block was cultivated as a separate field. The crops grown on each block changed from year to year in the same rotation but with a phase shift between the three blocks. Treatments started in 2000. The same treatments, C = control, SF = sulphur fertilizer (ammonium sulphate), S = sulphur (elemental sulphur), NTA = nitritotriacetic acid, were repeated on the same plots year by year.

four replications to the plots of each block: (1) no amendment (control), (2) application of ammonium sulphate, (3) application of elemental sulphur and (4) application of nitrilotriacetic acid (NTA) (Figure 3-1).

The average amounts of sulphur applied in the ammonium sulphate treatment were 124, 195 and 69 kg ha⁻¹ a⁻¹ for maize, tobacco and sunflower, respectively. In the elemental sulphur treatment, we applied 2139 kg ha⁻¹ a⁻¹ sulphur with particle size of 0.5–3.0 µm to each crop. Elemental sulphur and ammonium sulphate were applied just before sowing. In the NTA treatment, 110 ml of 200 mM NTA was injected into a depth of 20 cm by a self-made syringe with four holes (pointing in the four directions) on both sides of each plant at a distance of 15 cm to the stems, corresponding to an application rate of 649, 413 and 226 kg NTA ha⁻¹ a⁻¹ for maize, sunflower and tobacco. NTA was applied 4 to 6 weeks after sowing, when the plant height was 30–40 cm. The rates of S and NTA applications were chosen on the basis of previous green house experiments (Wenger et al., 2002). The same treatments were repeated on the same plots annually for the duration of the experiment. All blocks were fertilized (N, P, K, Mg and S) according to the fertilizer recommendations of the Swiss Agricultural Research Stations (FAL and RAC, 2001). Fertilization was applied directly after sowing; N was applied a second time 2–3 weeks and a third time 4–5 weeks later (Table 3-1). Ammonium sulphate replaced ammonium nitrate as N source in the ammonium sulphate treatment. The total rates of N applied were the same in all treatments, but differed between the crops.

Table 3-1: Total applied fertilizer in kg ha⁻¹: N as NH₄NO₃ or (NH₄)₂SO₄, P as Super Triple Phosphate (Ca(H₂PO₄)₂ · H₂O), and K, Mg and S as Patentkali (K₂SO₄ and MgSO₄).

Year	Maize		Tobacco		Sunflower	
	Block	N-P-K-Mg-S	Block	N-P-K-Mg-S	Block	N-P-K-Mg-S
2000	A	110-40-190-38-120	B	170-20-200-40-127	C	90-26-330-66-209
2001	B	110-40-190-38-120	C	170-20-200-40-127	A	90-26-330-66-209
2002	C	110-40-190-38-120	A	170-20-200-40-127	B	90-26-330-66-209
2003	A	80-40-190-38-120	B	170-20-200-40-127	C	90-26-330-66-209
2004	B	100-50-200-40-127	C	170-20-215-43-136	A	90-26-330-66-209
2005	C	145-50-200-40-127	A	170-20-215-43-136	B	90-26-330-66-209

3.2.3 Soil sampling and analysis

Before the beginning of the experiment (1999) and after each harvest, 16 soil cores (0–20 cm depth) were sampled on each plot, bulked to one composite sample, dried in a convection oven at 40 °C to constant weight and sieved to 2 mm. According to Swiss standard methods, we used an extraction with boiling 2 M HNO₃ to determine pseudo-total soil metal concentrations, while the soluble fractions of Cd, Cu and Zn were estimated using a 0.1 M NaNO₃ extractant (ART and ACW, 2007). Copper and Zn were analysed using ICP OES (Inductively Coupled Plasma Optical Emission Spectrometry), S by XRF (X-Ray Fluorescence) and Cd by GF AAS (Graphite Furnace Atomic Absorption Spectroscopy). The CaCO₃ content was determined using a calcimeter (ART and ACW, 2007) and organic carbon using the potassium dichromate oxidation method (ART and ACW, 2007). Soil pH was measured in H₂O at a soil:solution ratio of 1:2.5. Soluble (plant available) P and K were extracted with CO₂-saturated water and soluble Mg with 0.0125 M CaCl₂ (FAL and RAC, 2001). Total and soluble metal concentrations were determined for the soil samples taken 1999, 2000, 2003 and 2005, soluble P, K and Mg for the samples taken in the years 1999, 2000, 2003 and 2004, while CaCO₃ and C_{org} were determined only for the samples taken 1999, 2000 and 2005. Soil pH was determined from 1999 to 2005.

In spring 2006, three plots per treatment were randomly selected for subsoil sampling. On each of the chosen plots, three soil cores (5 cm diameter) were taken down to a depth of 75 cm at increments of 25 cm using a hollow cylinder coring device (type HUMAX, Martin Burch AG, Luzern, Switzerland). The three sampling depths are designated as topsoil (0–25 cm), upper subsoil (25–50), and lower subsoil (50–75 cm) in the following. The soil samples were oven-dried at 40 °C for 5 days and sieved to 2 mm mesh size. Soil pH, concentrations of total and soluble heavy metals, and contents of S, CaCO₃ and C_{org} were determined.

3.2.4 Plant sampling and analysis

Sunflowers were harvested always in the second half of August, maize and tobacco 1 month later. To avoid edge effects, plants of the outer rows and of the first and last 2 m of each plot were not included in the sampling. All the other plants were cut about 15 cm above ground and the total above-ground biomass (stem, leaves and seeds) was immediately passed through a chaff cutter. Roots were not collected. A 2 kg sample was taken at random from the harvest of each plot, oven-dried at 105 °C and ground to 1 mm, using an impact mill (AMA 102, Amman Langenthal, Switzerland). For P, K, Ca, Mg, Zn and Cu analysis, subsamples of 2.5 g plant material were ashed in a muffle furnace (prepASH 129, Precisa

Instruments, Dietikon, Switzerland) at 600 °C for 2.5 h, dissolved in 5 ml of 6 M HCl, diluted to 50 ml with Millipore water, filtered and analysed using ICP OES. For Cd analysis subsamples of 0.5 g oven-dried plant material were microwave-digested in a mixture of 5 ml of HNO₃ (65%), 3 ml of H₂O₂ (30%), and 2 ml of H₂O. The digested samples were diluted to 25 ml with Millipore water, filtered and analysed with GF AAS. For N analysis, the Dumas combustion method was used with an elemental analyser (varioMAX CN-Analyser, Elementar Analysensysteme GmbH, Hanau, Germany), using 1 g subsamples of oven-dried plant material.

3.2.5 Analytical quality assessment

For quality assurance, we analysed ISE (International Soil-analytical Exchange, Wageningen, The Netherlands) and IAG (International Analytical Group, Linz, Austria) inter-laboratory comparison soil and plant samples together with the experimental samples. The maximal relative standard deviation (coefficient of variation) of repeated measurements of the reference samples was 7% and the respective maximal relative bias was 5% for all elements.

3.2.6 Statistical analysis

Treatment effects were determined using analysis of variance in combination with post hoc analysis by Bonferroni tests. We used the following model (3-1) with the factors plant and treatment and two normally distributed and mutually independent random effects of block and year:

$$y_{ijklm} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + \delta_{l(k)} + \varepsilon_{ijklm} \quad (3-1)$$

where y_{ijklm} is the response (i.e. dependent) variable, μ is the mean response, α_i is the effect of treatment i (1 = control; 2 = ammonium sulphate; 3 = elemental sulphur; 4 = NTA), β_j is the effect of plant species j (1 = sunflower; 2 = maize; 3 = tobacco), $(\alpha\beta)_{ij}$ represents the interaction between treatment i and plant j , γ_k is the random effect of the year k (1 = 2000, ..., 6 = 2005), $\delta_{l(k)}$ is the random effect of the block l (1 = block A, 2 = block B, 3 = block C) in year k , and ε_{ijklm} is a normally distributed independent error ($m = 1, \dots, 4$). Each metal was analysed separately. Differences with $p < 0.05$ were considered significant. Data were log-transformed to get homoscedastic residuals. As we found significant interaction effects between treatments and plants, we analysed the treatment effects on metal accumulation also separately for each plant.

3.3 Results

3.3.1 Soil

On the basis of the topsoil (0–20 cm depth) samples taken at the beginning of the experiment (1999), which on average had sand, silt and clay fractions of 57%, 24% and 19%, respectively, the soil texture was classified as a sandy loam according to the US Soil Taxonomy. The pH of the soil was 7.4, the CaCO₃ content 3.75%, and the C_{org} content 12.2%. The mean total concentrations of Cd, Cu and Zn in the 1999 samples were 1.37 ± 0.03 , 536 ± 14 , and 684 ± 16 mg kg⁻¹, respectively. Table 3-2 gives the properties of the untreated soil for the three sampling depths, topsoil (0–25 cm), upper subsoil (25–50 cm) and lower subsoil (50–75 cm), measured in 2006.

Table 3-2: Properties (mean values and standard errors in parentheses) of the untreated soil, sampled at three depths (0–25, 25–50, and 50–75 cm) in spring 2006.

Soil properties	Topsoil (0–25 cm)	Upper subsoil (25–50 cm)	Lower subsoil (50–75 cm)
pH	7.53 (0.02)	7.23 (0.04)	6.94 (0.03)
CaCO ₃ (%)	3.78 (0.48)	2.16 (0.62)	0.11 (0.03)
C _{org} (%)	10.03 (0.26)	12.92 (0.79)	18.86 (0.73)
CEC _{pot} cmol ⁺ kg ⁻¹	79.62 (1.57)	127.05 (11.55)	148.09 (9.71)
Clay (%)	30.53 (3.06)	49.15 (6.74)	38.68 (9.33)
Silt (%)	28.35 (1.17)	27.42 (0.68)	58.28 (10.00)
Sand (%)	41.12 (4.03)	23.43 (6.13)	3.03 (0.97)
Cd _{total} (mg kg ⁻¹)	1.35 (0.03)	0.69 (0.09)	0.23 (0.05)
Cu _{total} (mg kg ⁻¹)	477 (19)	244 (37)	28 (9)
Zn _{total} (mg kg ⁻¹)	652 (31)	381 (67)	39 (13)
S _{total} (mg kg ⁻¹)	171 (7)	256 (24)	582 (29)
Cd _{soluble} (µg kg ⁻¹)	1.32 (0.08)	0.90 (0.07)	0.79 (0.4)
Cu _{soluble} (µg kg ⁻¹)	469 (28)	282 (39)	28 (9)
Zn _{soluble} (µg kg ⁻¹)	81.8 (4.4)	27.5 (9.3)	21.1 (7.2)

The properties of the topsoil agree well with those of the composite samples (0–20 cm) taken in 1999 (see above), with the exception that the soil in the plots chosen for sampling in 2006 had a higher clay content (30.5%) than the average of the experimental field, indicating local variations in soil texture. The sand content decreased from 41% in the topsoil to 3% in the lower subsoil. Due to the increased silt content, the texture of the lower subsoil was

classified as silty clay loam. Soil pH and CaCO₃ content were highest in the topsoil, whereas C_{org} and S content were highest in the lower subsoil. The high CaCO₃ content of the topsoil can be explained with the input of ashes that were deposited with the city wastes in the early 20th century, while the high C_{org} content of the lower parts of the profile is due to incomplete decomposition and residues of former peat layers. The contents of plant available nutrients in the topsoil were classified as “moderate” for P, “adequate” for Mg, and “available in reserve” for K according to Swiss standards (FAL and RAC, 2001). Total Cd, Cu and Zn concentrations were highest in the topsoil, still considerably above typical Swiss background concentrations in the upper subsoil and in the range of typical background concentrations in the lower subsoil. In addition, the soluble soil Cd, Cu and Zn concentrations decreased with depth. In contrast to Cu, this drop was not proportional to the decrease in total metal concentrations for Cd and Zn. The soluble Cd concentration decreased by less than a factor of 2 with depth from 1.3 in the topsoil to 0.8 µg kg⁻¹ in the lower subsoil, while the total Cd concentration decreased by more than a factor of 5. The soluble Zn concentration decreased 4-fold from the topsoil to the subsoil, but did not decrease further with depth in the subsoil although the total Zn concentration decreased by a factor of almost 10 from the upper to the lower subsoil. The higher ratios between soluble and total Cd and Zn in the subsoil than in the topsoil correspond well to the drop in pH and CaCO₃ content from topsoil to subsoil. The depth profiles thus do not indicate that any substantial leaching of these metals occurred.

Significant treatment effects were limited to the topsoil of the elemental sulphur treatment, where the topsoil pH decreased from 7.4 to 6.7 between 1999 and 2002, and remained at this level for the rest of the experiment (Figure 3-2). The soil CaCO₃ content decreased over the experimental period in all treatments. The decrease was greater in the sulphur treatment than in the ammonium sulphate and the NTA treatments, which did not differ from the control (Figure 3-3). Conversely, soluble Mg and Cd increased over time. The sulphur treatment caused a sharp increase in soluble Zn (Figure 3-3) and a greater increase in soluble Mg than in the other treatments. There were no significant treatment effects at lower depths and no effects on the NaNO₃-soluble Cu concentration, the total soil Cd, Cu and Zn concentrations or the organic carbon content.

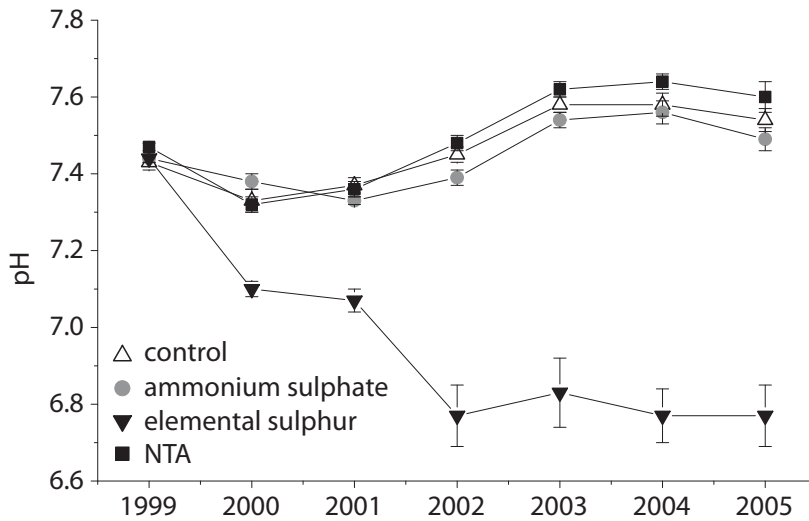


Figure 3-2: Topsoil pH (0–20 cm) in the four treatments between 1999 and 2005. The error bars show the standard errors of the means.

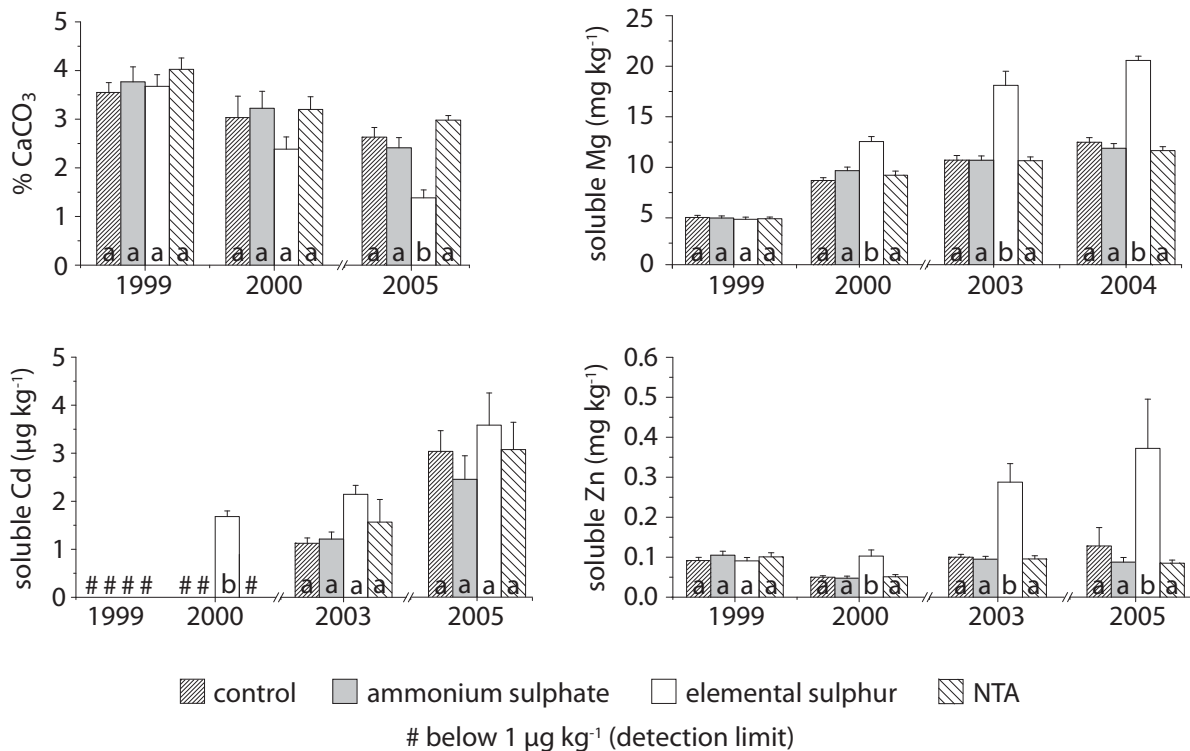


Figure 3-3: CaCO₃ content and soluble Mg, Zn and Cd concentrations of the topsoil (0–20 cm) samples taken in different experimental years as indicated in the graphs. Different letters indicate significant differences ($p < 0.05$) between the treatments of the respective year. Error bars show the standard errors of the means.

3.3.2 Plants

None of the plants showed deficiency symptoms and yields were within the usual range for these three crops in Switzerland (Walter et al., 2001) for all treatments. Compared to the concentration ranges considered to indicate sufficient supply according to Bergmann (1993), the concentrations of N, P, K and Mg were suboptimal in maize, while N was suboptimal in sunflower (Table 3-3). Furthermore, N and Ca were above and Mg below the respective ranges of mineral sufficiency for tobacco. Only the sulphur treatment had a significant effect on the accumulation of nutrients. It increased the P concentration in maize from 1.85 to 2.15 g kg⁻¹ and the Mg concentration in tobacco from 3.05 to 3.33 g kg⁻¹ (data not shown), thus bringing the concentration of these two nutrients closer to the optimal range. The absence of deficiency symptoms despite the indication of suboptimal nutrient concentrations according to the reference values given by Bergmann (1993) may indicate that these reference values do not apply to the cultivars used in our study. Bergmann (1993) mentions that variability between cultivars is common. Plant nutrient concentrations can also vary substantially with weather conditions before harvest and with the age of the plant at harvest time (Bergmann, 1993).

Table 3-3: Mean concentrations of macronutrients in the plants of the control treatment (averages for the years 2000–2005, standard errors in parentheses), in comparison to the concentration ranges considered to indicate sufficiency according to Bergmann (1993).

Nutrient (g kg ⁻¹)	Maize		Sunflower		Tobacco	
	Measured	Reference	Measured	Reference	Measured	Reference
N	15.39 (0.16)	35–50	22.78 (1.16)	30–50	29.95 (0.45)	22–25
P	1.85 (0.04)	3.5–6.0	3.05 (0.21)	2.5–5.0	2.49 (0.14)	2.5–4.5
K	10.95 (0.29)	30–45	39.29 (2.48)	30–45	36.61 (1.94)	25–45
Ca	3.43 (0.09)	3–10	20.43 (1.08)	8–20	27.93 (1.57)	13–24
Mg	1.45 (0.04)	2.5–5.0	4.13 (0.30)	3–8	3.05 (0.16)	4–8

The increase in NaNO₃-extractable soil Cd and Zn over the years in the sulphur-amended plots coincided with a significant increase in Cd and Zn accumulation in the tobacco shoots (Figure 3-4). In maize and sunflower, only Zn but not Cd concentrations increased. The application of NTA slightly increased the Zn concentration in maize, although we observed no effect on soluble Zn in the soil. None of the treatments affected the concentration of Cu in any of the three experimental crop plants, which agrees well with the corresponding lack of

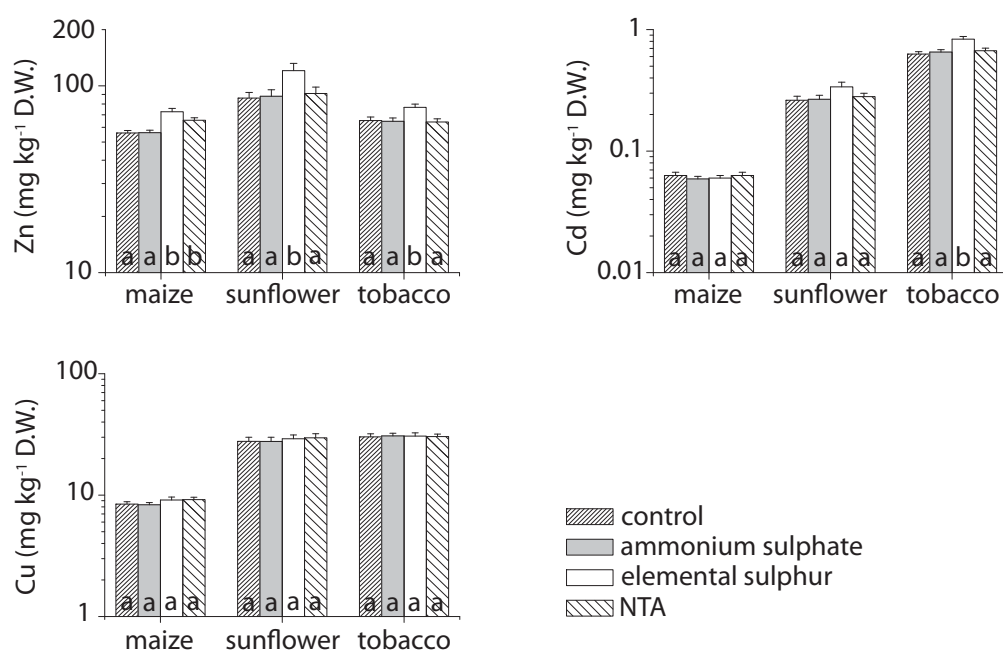


Figure 3-4: Mean concentrations of Zn, Cd and Cu in the above-ground parts of maize, sunflower and tobacco harvested between 2000 and 2005. Different letters indicate significant differences between the treatments. Error bars indicate the standard errors of the means.

effect on soluble soil Cu concentration. Compared to the controls, the sulphur treatment increased the mean Zn-to-Cd ratio in maize and sunflower by around 30% and 10%, respectively. In tobacco, the Zn-to-Cd ratio remained unaffected.

Figure 3-5 shows that the variations in yields and plant metal concentrations between years were generally larger than the differences between treatments in the same year. The variations in yield and heavy metal accumulation were not correlated to the monthly means of temperature, precipitation or evapotranspiration, at nearby weather stations (Table 3-4). Figure 3-6 shows that the effect of the sulphur treatment increased over the years in the case of Zn and Cd uptake by tobacco. The Zn concentration in maize increased, if we consider the value of 2001 to be an outlier. In sunflower, however, the effect of S on Zn uptake showed no consistent trend over the years. The increased accumulation of Zn and Cd by S-treated tobacco corresponds well with the continuous decrease in soil pH over the first three experimental years in the sulphur treatment.

Table 3-4: Climate data for the growing seasons (May–September) of the experimental years 2000–2005 recorded at weather stations close to the experimental site. Temperatures and potential evapotranspiration (ET_{pot}) values are averages of the weather stations Neuenburg (47°0'1"N, 6°57'14" E, 484 m a.s.l.) and Payerne (46°48'49"N, 6°56'33"E, 490 m a.s.l.). The ET_{pot} was calculated from data of these two stations, using the FAO Penman-Monteith equation (Allen et al., 1998). The precipitation was recorded at the weather station of Witzwil.

Parameter	Month	2000	2001	2002	2003	2004	2005
Temperature (°C)	May	15	16	13	15	13	14
	June	18	16	19	23	17	19
	July	17	19	19	21	19	19
	August	19	20	18	23	19	17
	September	16	12	14	15	16	16
Precipitation (mm)	May	94	48	127	61	52	66
	June	66	133	79	63	85	44
	July	155	112	116	73	75	39
	August	114	100	145	118	194	90
	September	56	119	57	65	28	78
ET_{pot} (mm)	May	119	120	89	106	119	105
	June	148	125	143	179	126	156
	July	127	141	135	162	138	150
	August	127	131	109	162	119	114
	September	96	68	72	98	91	83

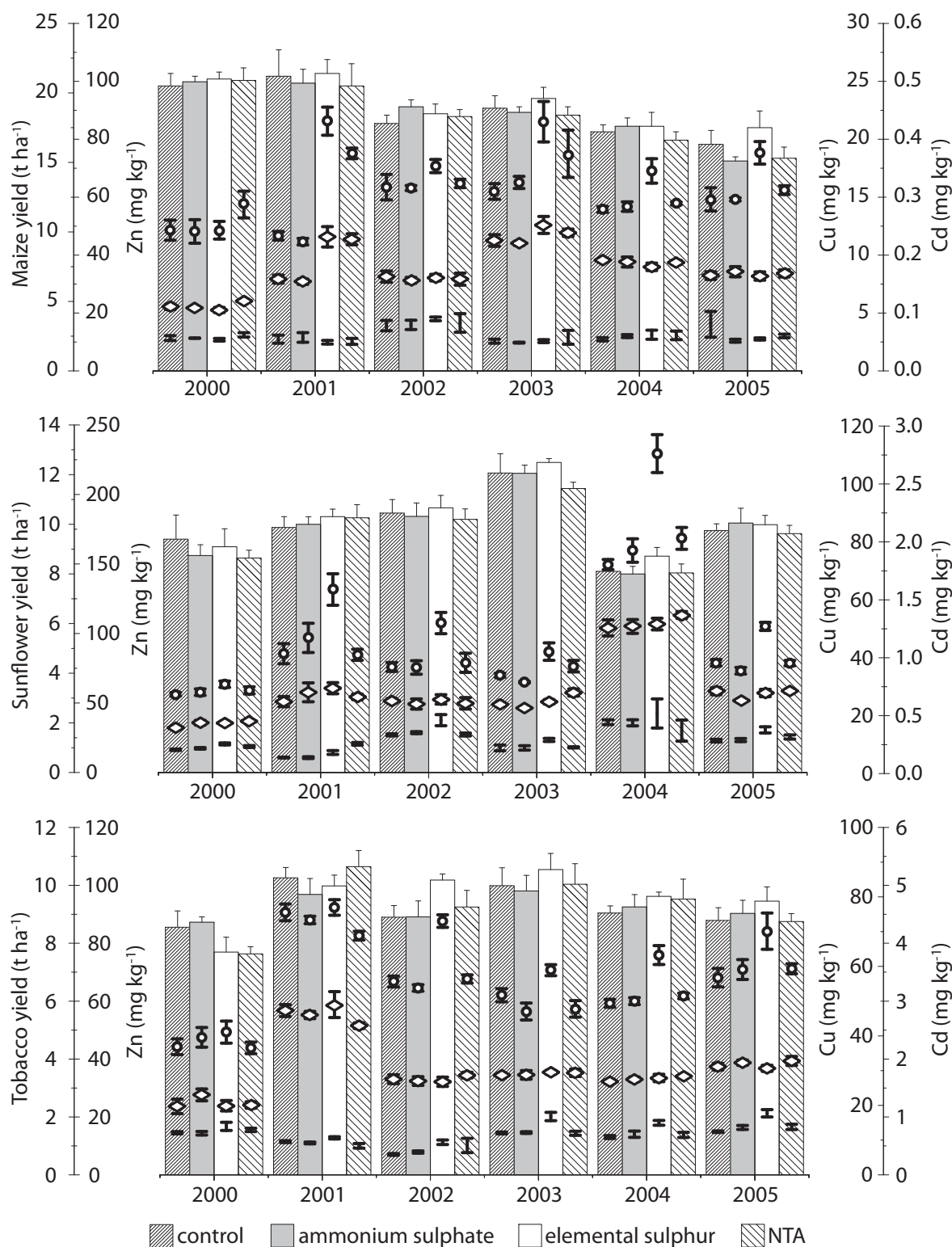


Figure 3-5: Treatment effects on the annual yield (histogram bars) and heavy metal concentrations (symbols: Zn \diamond , Cu \square , Cd \triangle) of maize (top), sunflower (middle) and tobacco (bottom). Error bars represent the standard errors of the means for the respective treatment and year.

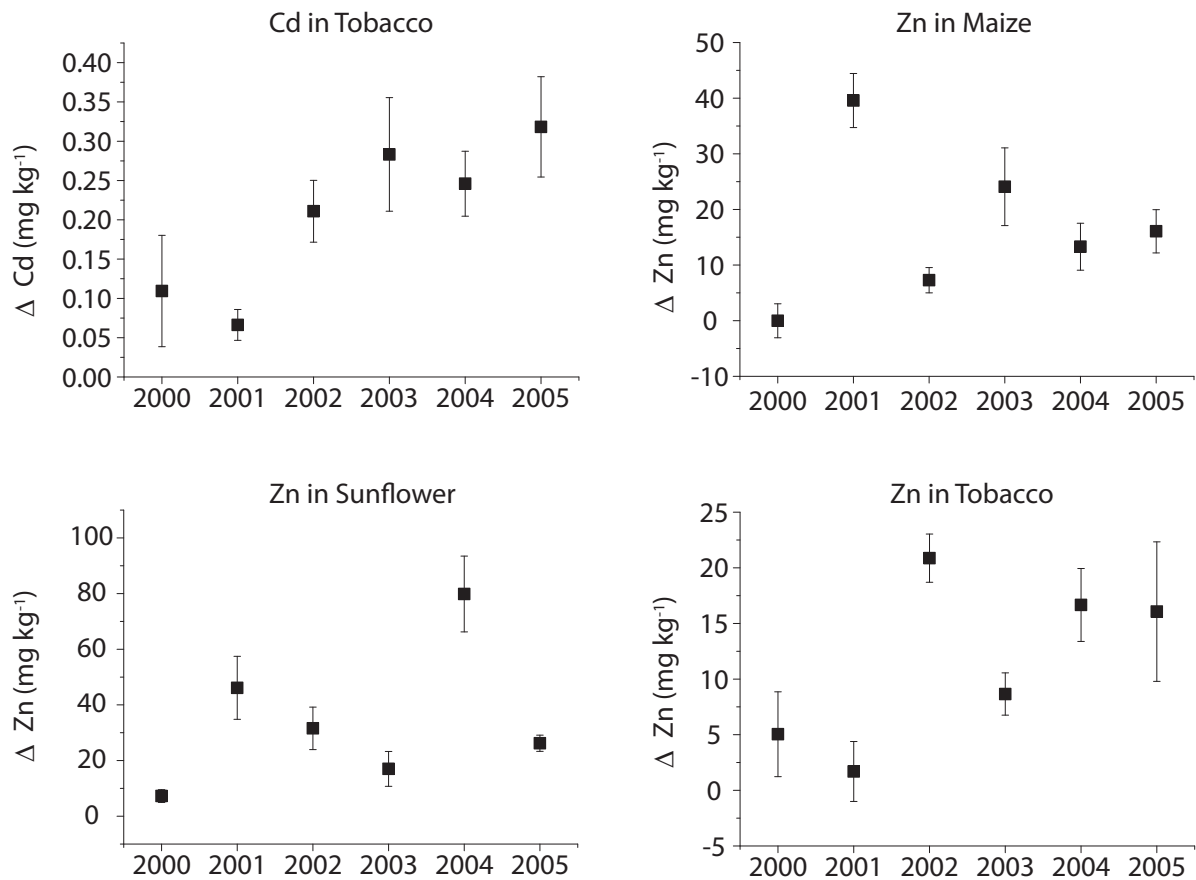


Figure 3-6: Effect of the sulphur treatment (2000–2005) on the Cd and Zn concentrations of tobacco and on the Zn concentration of maize and sunflower over time. The symbols represent the mean difference of the Cd or Zn concentration between control and sulphur treatment in the given year. The bars represent the standard errors of the means.

Given that the variations in growth and plant metal accumulation from year to year did not exhibit any close correlation, the annual extraction of metals from the soil was not constant over time. The Cu extraction rates averaged 165, 270 and 285 $\text{g ha}^{-1} \text{a}^{-1}$ for maize, sunflower and tobacco, respectively, over the 6 years of the experiment. The treatment effects on metal extraction rates corresponded to those on metal accumulation, as there was no significant treatment effect on yields. The sulphur treatment increased the Zn extraction by maize, sunflower and tobacco by 34%, 45% and 22% (Table 3-5) and the Cd extraction by 0%, 34% and 36% (Table 3-6) for the respective plants. The highest Zn extraction was obtained by the sulphur-treated sunflowers in 2004 (best-case scenario), with an extraction rate of $>2 \text{ kg ha}^{-1}$, which was more than twice the average rate of the untreated sunflowers (control), and more than three times the average Zn extraction rate of tobacco, the weakest Zn extractor of the three crops. For Cd, tobacco was the best extractor. The highest Cd

extraction rate (10.5 g ha^{-1}) occurred in 2003 with the sulphur-treated tobacco. This was twice the average Cd extraction rate of untreated tobacco, and nine times the average extraction rate of maize, the weakest Cd extractor.

Table 3-5: Zinc extraction ($\text{g ha}^{-1} \text{a}^{-1}$) from soil by maize, sunflower and tobacco, in control and sulphur treatment. Standard errors are in parentheses. Framed: best-case scenario.

Year	Maize		Sunflower		Tobacco	
	Control	Sulphur	Control	Sulphur	Control	Sulphur
2000	994 (58)	1015 (51)	531 (56)	577 (42)	378 (27)	382 (42)
2001	989 (77)	1839 (107)	836 (38)	1360 (116)	932 (45)	924 (49)
2002	1127 (72)	1309 (35)	790 (24)	1138 (50)	596 (35)	891 (6)
2003	1165 (4)	1670 (74)	845 (51)	1087 (73)	624 (52)	743 (24)
2004	961 (35)	1207 (47)	1215 (66)	2006 (150)	535 (9)	732 (36)
2005	970 (78)	1308 (44)	767 (13)	1049 (34)	595 (19)	791 (46)
Mean	1034 (33)	1392 (114)	831 (82)	1203 (175)	610 (67)	744 (72)

Table 3-6: Cadmium extraction ($\text{g ha}^{-1} \text{a}^{-1}$) from soil by maize, sunflower and tobacco, in control and sulphur treatment. Standard errors are in parentheses. Framed: best-case scenario.

Year	Maize		Sunflower		Tobacco	
	Control	Sulphur	Control	Sulphur	Control	Sulphur
2000	1.16 (0.09)	1.13 (0.04)	1.84 (0.16)	2.25 (0.17)	6.24 (0.46)	6.53 (0.80)
2001	1.14 (0.11)	1.05 (0.03)	1.27 (0.04)	1.79 (0.16)	5.87 (0.12)	6.36 (0.08)
2002	1.38 (0.11)	1.66 (0.02)	3.40 (0.20)	4.79 (0.42)	3.15 (0.24)	5.72 (0.28)
2003	0.97 (0.05)	1.01 (0.05)	2.53 (0.22)	3.51 (0.11)	7.26 (0.52)	10.52 (0.35)
2004	0.94 (0.05)	1.08 (0.08)	3.53 (0.23)	4.43 (0.99)	5.91 (0.31)	8.63 (0.27)
2005	1.37 (0.41)	0.97 (0.08)	2.67 (0.07)	3.65 (0.12)	6.55 (0.33)	9.96 (0.09)
Mean	1.16 (0.07)	1.15 (0.10)	2.54 (0.33)	3.40 (0.44)	5.83 (0.53)	7.95 (0.76)

3.4 Discussion

3.4.1 Soil properties

Whereas the total concentrations of Zn, Cd and Cu exceeded the respective guide or trigger values of the Swiss Federal Ordinance Relating to Impacts on the Soil (VBBo, 1998), the NaNO₃-soluble concentrations were below critical values which are 0.5 mg Zn, 0.02 mg Cd and 0.7 mg Cu per kg soil. The low metal solubility can be attributed primarily to the high pH and organic matter content of the soil. The contamination had aged for decades in the Witzwil soil (50–100 years), which may have resulted in reduced solubility of the contaminating metals over time (Tuin and Tels, 1990).

The experimental soil had an organic carbon content of 10–12% in the topsoil. Soil organic matter is an important sorbent for heavy metals, especially at a soil pH above 6.5 (Alloway and Jackson, 1991). All three contaminants are strongly bound by soil organic matter, in particular Cu (Sposito, 2008). The decrease in soil pH in the sulphur treatment was insufficient to significantly increase Cu solubility. This would have required a pH decrease below 5 (Hornburg and Brümmer, 1993). In contrast, Cd and Zn solubility start to increase strongly with increasing soil acidification already at pH values below 7 and 6, respectively (Hornburg and Brümmer, 1993). After 6 years of sulphur application, the pH of the experimental topsoil (0–20 cm) was still close to neutral (pH of 6.7). The high buffering capacity of the CaCO₃ in the soil reduced the acidifying and metal mobilizing effects of the S. Given that the carbonate content decreased to less than half of the initial concentration during the experiment, a stronger decrease in soil pH would have required another 6 years of sulphur applications at our annual dosage of 2.14 t ha⁻¹.

In contrast to the sulphur treatment, we observed no increase in metal solubility in the NTA treatment, not even for Cu, which is known to form strong complexes with amino-carboxylates such as NTA (Nowack et al., 2006). Mobilization of Cu at higher soil pH could be achieved by the formation of Cu-complexes with dissolved organic ligands. The applied dose may have been too low for the NTA to compete with the undissolved organic matter of the soil for Cu. Furthermore, the applied NTA may have degraded too rapidly to result in a measurable effect on metal mobilization in the soil when it was sampled at harvest time. Tiedje and Mason (1974) reported that up to 80% of soil applied NTA had been degraded 24 days after application. They reported that NTA degradation rates in topsoils were positively correlated with the soil's organic matter content. The high organic matter content in the Witzwil soil thus might have accelerated NTA degradation. Wenger (2000) investigated the degradation kinetics of NTA by monitoring its effect on the NaNO₃-extractable Zn

concentration in a calcareous metal-polluted soil over time. The application of 5 mmol NTA kg⁻¹ dry soil increased the soluble soil Zn concentration from 4 to 200 mg kg⁻¹, but after 20 days Zn solubility started to decrease again. With the addition of 25 mmol kg⁻¹ the mobilizing effect of NTA on soluble Zn disappeared within 63 days. The results reported by Wenger (2000) suggest that some mobilization may also have occurred in our NTA treatments, but may have been too ephemeral to be detectable still in the soil at harvest time and to significantly affect plant metal accumulation. Such a transient effect may, however, explain the small NTA effect on Zn accumulation by maize. A sustained NTA effect on soil metal solubility would require repeated applications during the growing season. However, this is impractical in larger field experiments and even less in agricultural practice.

3.4.2 Plant concentrations

The relatively low plant metal concentrations may be partly due to short-range heterogeneity in the spatial distribution of the metals in the soil. Pronounced heterogeneity in the distribution of pollutants, even over short distances, is a typical phenomenon in field soils. Potted soils are usually homogeneous, thereby giving the roots little possibility to avoid contaminants. In contrast, under field conditions they generally have more possibility to evade contaminants by growing into zones of low concentrations (Keller, 2006). Contamination does not usually extend far into the subsoil, so that deeper roots encounter fewer toxicity problems than superficial roots. The contamination of the field soil studied here was more or less limited to the upper 50 cm of the soil. Roots of sunflower can reach depths of 1.5 m (Angelova et al., 2004), indicating that only part of the root system was involved in metal extraction. Keller et al. (2003) found a maximum rooting depth of 0.75 m for tobacco with a rapid decrease in rooting density below the first 0.1 m, whereas the rooting density of maize decreased less abruptly with depth, indicating that tobacco is more suitable for phytoextraction of contaminations in the upper layers and maize for phytoextraction of deeper contaminations. We found higher Cd and Cu concentrations in tobacco than in maize. The difference in Zn accumulation, however, was only marginal.

Plant metal concentrations and yield varied considerably from year to year. This shows that predictions of the time required to remediate a soil by means of phytoextraction are unreliable if based on experimental data from a single year only. Also Keller et al. (2003) found significant year-to-year variations in root development and metal uptake of different annual plants. To calculate the remediation time (t_r) needed to reach target heavy metal concentrations as given by the guide values (c_{gv}) of the Swiss Federal Ordinance Relating to

Impacts on the Soil (VBBö, 1998), more realistic estimates will be obtained with data from experiments with a duration of several years. Using the mean annual metal extraction (c_{me}) over the 6 years of our experiment and assuming a linear decrease in soil metal concentration according to Kayser et al. (2000), beginning from the soil metal concentration in 1999 (c_0), the calculated remediation time would be around 240, 2900 and 870 years for Cd, Cu, and Zn, respectively, using equation 3-2:

$$t_r = \frac{c_0 - c_{gv}}{c_{me}} . \quad (3-2)$$

For Cd and Zn, these times would decrease by about a quarter were the soil pH maintained at 6.7. Using the value of the highest metal extraction rate achieved in our experiment, which was 2 kg ha^{-1} for Zn in 2004 with sulphur-treated sunflower and 10.5 g ha^{-1} for Cd in 2003 with sulphur-treated tobacco, then the needed remediation time would still be 360 years for Zn and 70 years for Cd. Even in this unrealistic “best-case” scenario, the time needed to reach the remediation target would still be unacceptably long. Nevertheless, the low metal accumulation rates can be advantageous, as this means that the biomass could be used for human or animal nutrition without unacceptable toxicity risks. The concentrations of Cd, Cu and Zn in our plants were in the same range as plants grown on uncontaminated land (Chaney, 1989), and well below concentrations shown to be harmful to animals (Underwood and Suttle, 1999). The Cd concentrations were also below the Swiss threshold value for undesired substances in animal fodder (FMBV, 1999), while for Zn and Cu no such threshold values exist. Sulphur application may even increase the fodder quality because of the increased Zn-to-Cd ratio. Zinc, in contrast to Cd, is an essential nutrient for plants, human and animals, and becomes toxic only at high concentrations. Zinc deficiency usually is a more serious problem in human and animal nutrition than Zn toxicity. In particular, Zn deficiency can promote the absorption and retention of Cd by human and animals (Tang et al., 1998; Underwood and Suttle, 1999). Thus increasing the ratio of Zn-to-Cd accumulation in food and fodder plants could be beneficial with respect to health risk reduction. Crop residues with elevated Zn and Cu concentrations could also be used as green manure on agricultural land where the concentrations of these two plant nutrients are low. Here, phytomanagement would not only allow a profitable agricultural use of the polluted soil, but also gradually reduce its contamination and in the same time ameliorate micronutrient-deficient soil elsewhere.

3.5 Conclusions

The uptake of Cd, Cu and Zn by the untreated plants was in the range of values found in crop plants grown on uncontaminated land. Therefore, the rate of extraction of these metals was low. The ammonium sulphate treatment had no effect on plant metal uptake. The NTA treatment was ineffective, except for a slight increase in Zn accumulation by maize. Elemental sulphur significantly increased the accumulation of Zn by all three experimental plants as well as the accumulation of Cd by tobacco. The applied elemental sulphur treatment would decrease the remediation time for the Cd and Zn contamination by about a quarter. It also improved the fodder quality of maize and sunflower by increasing the Zn-to-Cd ratio and by enhancing the nutritional status of tobacco (Mg) and maize (P). A higher rate of sulphur application would probably result in a faster and stronger decrease in soil pH and, correspondingly, in a higher Cd and Zn mobilization. However, the remediation time would still be unacceptably long. Cleansing the experimental soil by phytoextraction is thus unrealistic using the approach studied here. However, the results suggest that phytomanagement, i.e. to use the contaminated land profitably for the production of valuable biomass (animal feeding, biofortification, biofuel production and other economical purposes), would be a good strategy without running any substantial health risks for humans or animals.

3.6 Acknowledgements

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4

Phytomanagement of metal-contaminated agricultural land – follow up

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Abstract

The long-term phytomanagement experiment described in Chapter 3 (i.e. Fässler et al., 2009) showed that the applied treatments did not enhance plant metal uptake to a sufficient extent. The aim of the present study was to examine to which degree the efficiency of phytoextraction could be enhanced on this site, by doubling the elemental sulphur and NTA application rates. For this purpose, the field experiment described in Chapter 3 was continued for another two years with the following changes: (1) the ammonium sulphate treatment was not evaluated anymore and (2) elemental sulphur and NTA applications were continued at the same rates as before on half of the area of the respective plots, while the application rates were doubled on the other half-plots.

Doubling the elemental sulphur application rate (“S2-treatment”) significantly decreased soil pH in 2007 to 5.9 and the CaCO₃ content to 0.6%, whereas the values for the same parameters were 7.5 and 2.4% in the control treatment and 6.6 and 0.9% on the plots where the treatments were continued at the previous rates (“S1-treatment”). Soluble soil concentrations of Cd and Zn were increased by the S2-treatment, while the S1-treatment only increased soluble Zn. Copper solubility was decreased by both treatments. The S1-treatment significantly increased Cd and Zn uptake by sunflower and tobacco compared to the control, and the S2-treatment further increased Cd uptake by sunflower and Zn by both plants. Metal uptake by maize was not increased by the S-treatments; on the contrary, Cu concentration was even decreased in the S2-treatment. In contrast the NTA-treatments had no effect on plant metal uptake, even with the double application rates.

Tobacco in the S2-treatment gave the highest extraction rates for Cd. Could it be grown on the same field year after year and would Cd extraction remain constant, then the Cd concentration would reach the Swiss guide value of the “Ordinance Relating to Impacts on the Soil” (OIS) (VBBo, 1998) within 44 years with the S2-treatment, assuming that conditions would remain the same otherwise. As Cd is the main pollutant in the studied soil and as the levels of Cu and Zn accumulation in the investigated plants were in the beneficial range to plant nutrition, the focus could at first be exclusively on Cd extraction. We suggest testing the use of other high-biomass crop plants, such as *Miscanthus sinensis* and *Brassica juncea*, on their Cd extraction potential at the experimental site. If successful, these plants may be used in crop rotation with tobacco or could even replace tobacco, until the Cd concentration in the soil would reach a tolerable level. The experimental site could thereafter be used for the production of Zn and Cu rich biomass, which then could be used for example as biofertilizer elsewhere.

4.1 Introduction

In Chapter 3 (i.e. Fässler et al., 2009) we showed that the soil treatments applied from 2000 to 2005 (ammonium sulphate, elemental sulphur and NTA) did not enhance plant metal uptake to an extent that would be sufficient for soil clean-up by phytoextraction. Phytomanagement of the experimental site without special soil treatments, on the other hand, was found to be a reasonable option to produce viable biomass, while keeping under control the potential environmental risks that could arise from the contamination and in the same time gradually cleaning the soil. Nevertheless, the question arose if the efficiency of phytoextraction could be increased substantially if the rates of S and NTA applications were doubled, without causing intolerable losses in yield. Therefore the experiment was continued after 2005 for another two years (2006–2007) with the following modifications. The ammonium sulphate treatment was not evaluated anymore, the applications of sulphur and NTA were continued at the same rates as before on half of the area of the respective plots and doubled in rate on the other half-plots of the respective treatments. In this chapter, we present the results of this extension of the experiment, focussing on the efficiency of phytoextraction.

4.2 Materials and methods

The experiment was carried out from 2006 to 2007 on the same experimental site in Witzwil, Bernese Seeland, as between 1999 and 2005 (for details see Fässler et al., 2009). Crop rotation with maize, sunflower and tobacco was continued in the same sequence as before. Figure 4-1 shows changes in treatment design for one of the three blocks. The plots that had been treated with elemental sulphur and NTA in the previous years were divided into two sub-plots each and the applied amounts of sulphur and NTA were continued as before on one half (single dose) and doubled on the other half (double dose) of each plot. In the elemental sulphur treatment, the single dose was $2.14 \text{ t S ha}^{-1} \text{ a}^{-1}$ (“S1-treatment”) and the double dose $4.28 \text{ t S ha}^{-1} \text{ a}^{-1}$ (“S2-treatment”). In the NTA treatment, the single dose was 110 ml of 200 mM NTA (“NTA1-treatment”) and the double dose 110 ml of 400 mM NTA (“NTA2-treatment”) per injection. The solutions were injected on both sides of each plant at a distance of 15 cm to the stems. These applications corresponded to 649, 413 and 226 kg NTA $\text{ha}^{-1} \text{ a}^{-1}$ for maize, sunflower and tobacco in the NTA1-treatment, and 1'298, 826 and 452 kg NTA $\text{ha}^{-1} \text{ a}^{-1}$ for the respective plants in the NTA2-treatment. The applications of ammonium sulphate were continued in the same way as before, i.e. 124, 195 and 69 kg S

$\text{ha}^{-1} \text{a}^{-1}$ for maize, tobacco and sunflower, respectively, but the data were not evaluated with the exception of soil pH. All blocks were fertilized (N, P, K, Mg and S) according to the fertilizer recommendations of the Swiss Agricultural Research Stations (FAL and RAC, 2001), see also Fässler et al. (2009).

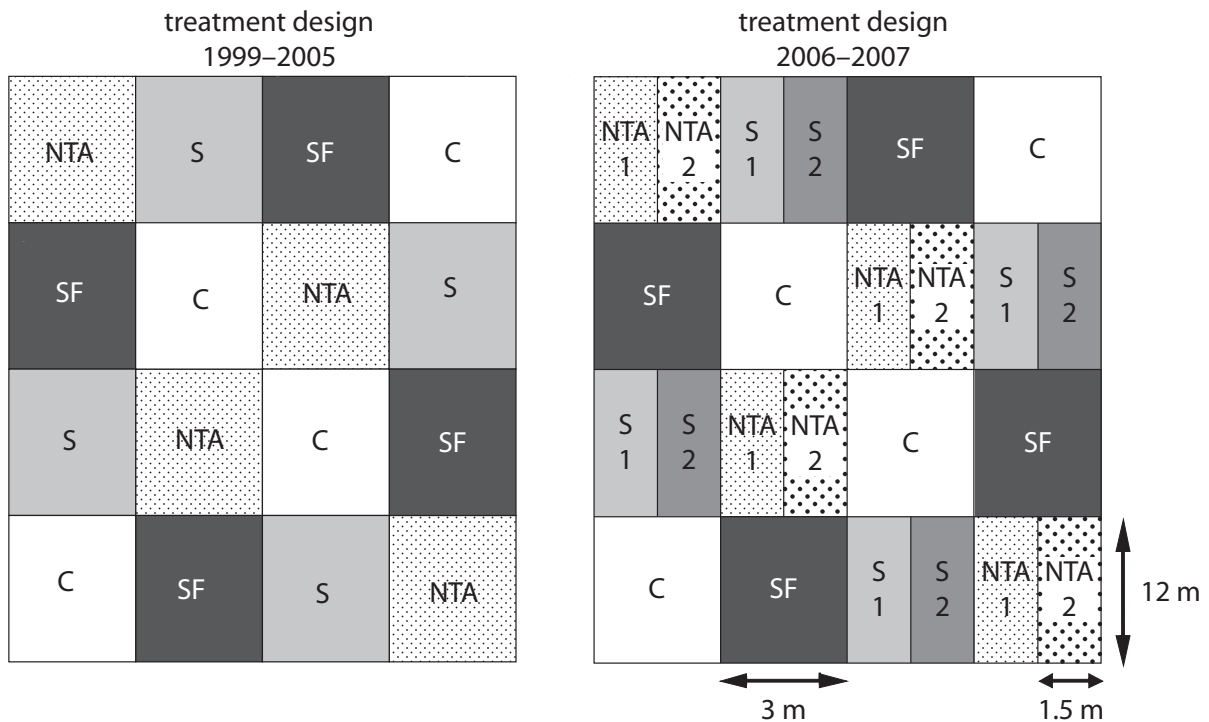


Figure 4-1: Old (1999–2005) and new (2006–2007) treatment design of a single block in the experimental field. C = control, SF = sulphur fertilizer (ammonium sulphate), S = sulphur (elemental sulphur), NTA = nitrilotriacetic acid, 1 = single dose, 2 = double dose.

Soil and plant samples were taken in both experimental years on each plot and analysed in the same way as between 1999 and 2005 (Fässler et al., 2009). We measured soil pH (H_2O), CaCO_3 , total (HNO_3 -extractable) and soluble (NaNO_3 -extractable) soil Cd, Cu and Zn concentrations, as well as plant Cd, Cu and Zn concentrations. Even though samples were taken in both years, we will focus the presentation and discussion of the results on the data from 2007, which represent the “final stage” of the experiment.

For statistical analysis the data were log-transformed to get homoscedastic residuals. Treatment effects were determined using analysis of variance in combination with post hoc analysis by Bonferroni tests. Differences with $p < 0.05$ were considered significant.

4.3 Results

4.3.1 Soil

Whereas in the S1-treatment the slight decrease in soil pH observed in the preceding years continued at the same rate, the S2-treatment markedly accelerated the decrease, resulting in a pH drop from 6.8 in 2005 to 5.9 in 2007 (Figure 4-2).

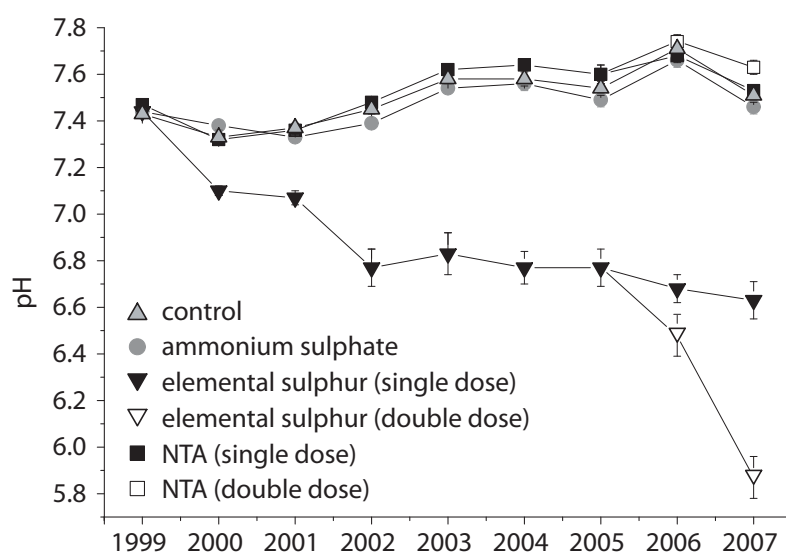


Figure 4-2: Soil pH between 1999 and 2007. Four original treatments from 1999 to 2005, and six treatments (original treatments and double doses of elemental sulphur and NTA) from 2006 to 2007. The error bars show the standard errors of the means.

In response to the doubled input of acidity, the CaCO_3 content decreased from 1.38% in 2005 to 0.6% in 2007 in the S2-treatment, while it dropped only to 0.9% in the S1-treatment (Figure 4-3). The fact that there were still substantial amounts of CaCO_3 left means that the soil pH was not in equilibrium with the buffer. The soil pH might increase again until all carbonate is consumed, and eventually even return to the initial value, if all excess acidity is buffered and no further sulphur is applied. Figure 4-3 shows that also the CaCO_3 content of the control plots had significantly decreased since the beginning of the experiment in 1999. The CaCO_3 content of the control plots was 2.4% in 2007, which is less than 2/3 of the initial content in 1999. Gradual soil acidification is a natural phenomenon in the humid and temperate zone. If the CaCO_3 content continues to decrease at a similar rate, all carbonate will disappear in about 15 years. Thereafter, a drop in pH is expected until the pH is stabilized by another buffer system. The Witzwil soil in particular still has a high buffering

capacity in form of organic matter. However, also the C_{org} content decreased substantially during the experimental period. While it was still 12.2% in 1999, it had decreased to 10.7% in 2006, i.e. by 13% in 7 years. This loss is primarily due to mineralization, as the organic matter content was still much higher than can be stabilized by a drained, oxic, arable soil in the humid and temperate zone.

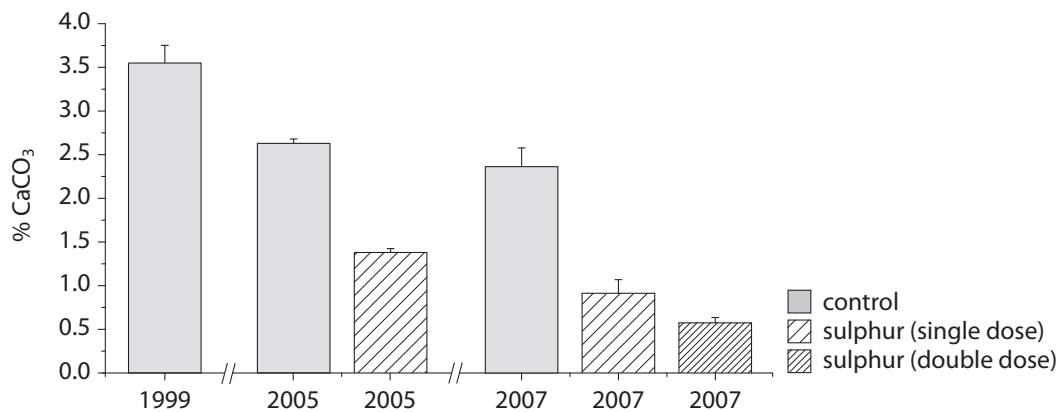


Figure 4-3: Soil CaCO₃ content of the control treatment in the beginning of the experiment in 1999 and in the experimental years 2005 and 2007, and after the application of the single (2005 and 2007) and the double amount (2007) of sulphur. The error bars show the standard errors of the means.

Whereas the S1-treatment had no significant effect on soluble Cd in the soil, the S2-treatment increased it by a factor of 3.8 (Table 4-1). The soluble Zn was increased in both treatments, i.e. by a factor of 3.7 in the S1-treatment and by 15.6 in the S2-treatment. Soluble Cu on the other hand, was slightly decreased by the application of elemental sulphur with no difference between the S2- and the S1-treatment. At pH values above 5 Cu solubility is only little affected by soil pH, as it forms organometallic and carbonate complexes. With a decrease in soil pH the fraction of organometallic complexes increases. The presence of high molecular weight organic substances can lead to a strong Cu fixation, potentially causing Cu deficiency in plants (Scheffer et al., 2002).

No effect on metal solubility was found in the two NTA treatments (data not shown). The doubling in the NTA rates thus did not suffice to produce a significant mobilization in this soil. If NTA had had an effect on metal solubility in the soil, it had disappeared by the time when the soil samples were taken (see also Fässler et al., 2009).

Table 4-1: Mean metal concentrations 2007 in the topsoil (0–20 cm). Standard errors are in parentheses.

Metal concentrations	Control	Sulphur (single dose)	Sulphur (double dose)
Cd _{total} (mg kg ⁻¹)	1.30 (0.05)	1.37 (0.05)	1.34 (0.06)
Cd _{soluble} (µg kg ⁻¹)	1.56 (0.52)	1.74 (0.21)	5.73 (0.72)**
Cu _{total} (mg kg ⁻¹)	602 (29)	643 (29)	598 (27)
Cu _{soluble} (µg kg ⁻¹)	403 (11)	334 (11)*	358 (13)*
Zn _{total} (mg kg ⁻¹)	595 (28)	625 (30)	595 (29)
Zn _{soluble} (µg kg ⁻¹)	110 (6)	409 (60)*	1'713 (276)**

* significant difference from control

** significant difference from control and sulphur (single dose)

4.3.2 Plants

The mean dry weight of maize ($20.8 \pm 7 \text{ t ha}^{-1}$), sunflower ($8.7 \pm 0.2 \text{ t ha}^{-1}$) and tobacco ($6.4 \pm 0.3 \text{ t ha}^{-1}$) was not significantly affected by any of the treatments in the two years (values in parentheses are from 2007). Corresponding to the lack of effect on metal solubility in the soil, none of the NTA treatments affected plant metal uptake (data not shown). The sulphur applications, on the other hand, significantly increased the uptake of Cd and Zn in sunflower and tobacco (Figure 4-4). The strongest effect was found for Zn in sunflower which is consistent with the results from the previous years (Fässler et al., 2009). The S effect on Cd and Zn uptake in sunflower and tobacco also showed a significant increase with the doubling of the S dose, in agreement with the corresponding increase in the soluble Zn in the soil. The increase in Zn uptake by sunflower and tobacco, however, was not proportional to the increased Zn solubility in the soil, which was 15.6-fold in the S2-treatment relative to the control, whereas the accumulation only showed a 3.4-fold increase in sunflower and a two-fold increase in tobacco. The fact that the solubilisation effect on soil Zn did not translate to a similar degree into increased accumulation could mean that leaching risks increased disproportionately.

Compared to the S effect on soil metal solubility a disproportionately high increase in accumulation was found in tobacco for Cd. The S2-treatment increased soil Cd solubility by a factor of 3.8 compared to the control, while the Cd concentration in tobacco was 5.5-fold higher in the S2-treated than in the control plants. Although the effects were smaller in sunflower (2.7-fold increase) and maize (1.7-fold increase), they were still substantial. No significant increase in metal uptake was observed in maize. The S-treatments also had no

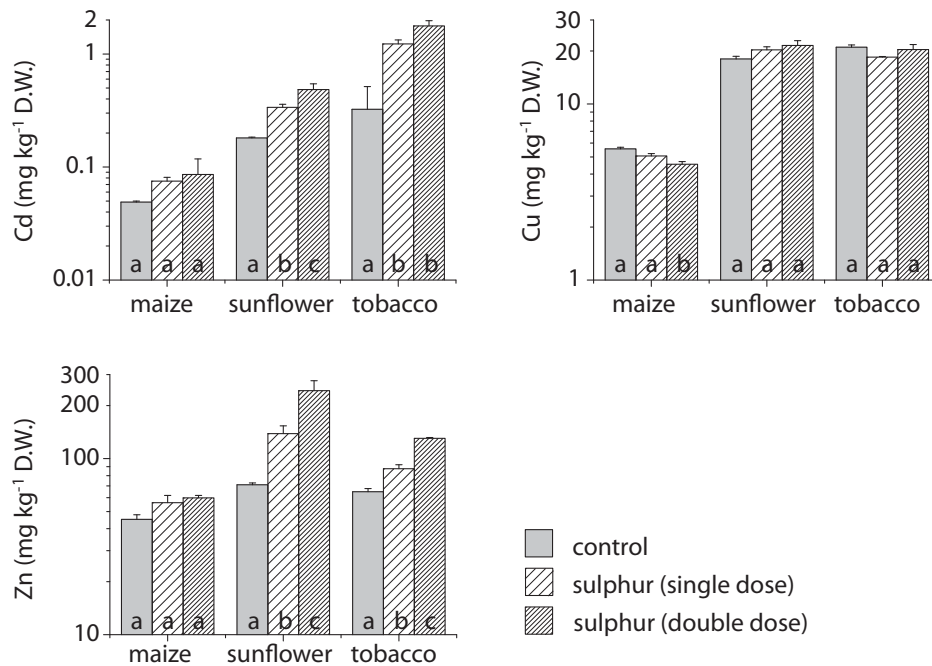


Figure 4-4: Cadmium, Cu and Zn concentrations in maize, sunflower and tobacco samples in 2007, in control plants and plants treated with single and double doses of elemental sulphur. Error bars show the standard errors of the means.

significant effect on Cu uptake by these crops, except for a slight decrease in maize at the S2 dose. These results correspond again well with the small decrease in soluble Cu in the soil.

As plant growth was not reduced by the treatments, the increased plant Cd and Zn concentrations in the S1- and S2-treatments led to equivalent increases in soil metal extraction. With the extraction rates achieved in the untreated plots in 2007 (1.46 g Cd, 129 g Cu and 619 g Zn), it would take about 380 years for Cd, 6000 years for Cu and 950 years for Zn to reach the respective Swiss guide values of the OIS (VBBo, 1998), when maize, sunflower and tobacco are grown in crop rotation. This time could theoretically be reduced to 90 years for Cd and to 400 years for Zn by the application of the S2-treatment. Using the most efficient plant every year on the same field would decrease the Cd clean-up time to 44 years (growing only tobacco), and to 270 years for Zn (growing only sunflower). This calculation is based on the assumption that the metal extraction is constant over the years and that the same crop can be grown on the same field year after year. From the study by Fässler et al. (2009) we know that plant metal concentrations as well as crop yields vary considerably between years. Thus, extrapolation of rates obtained in a single year is questionable. Comparing the data from 1999 to 2005 with those from 2007 shows that metal extraction in the control treatment was lower in 2007 than on average in the previous years.

The S1-treatment was in the same range as in the previous years, whereas the S2-treatment led to higher extractions.

4.4 Discussion

Assuming that tobacco could be grown year after year on the same field and that the rate of Cd extraction would be the same each year independent of the remaining pollution level, the concentration of Cd in the soil would decrease linearly with time and would reach the Swiss guide value of the OIS (VBBö, 1998) within 44 years. If Cd extraction could be further increased, then the remediation time for this metal would come close to what would be required to refer to it as “phytoextraction”. Phytomanagement of the experimental site could be carried out in two steps. First, the focus could be exclusively on Cd extraction, ignoring the two other metals, as their accumulation at current concentration levels in the investigated plants was still in the beneficial range (Bergmann, 1993). Of those plants studied only tobacco took up enough Cd to be suitable for the purpose of gradual Cd extraction. In order to have alternatives for crop rotation, more high-biomass plants should be tested on the site for their potential to extract Cd. It would be desirable that they could be cultivated in crop rotation with tobacco and that their biomass could be used for bioenergy production. As soon as the Cd concentration in the soil would reach a level at which it becomes unproblematic also for other crops, attention could be turned to the production of Zn and Cu biofortified crops on the study site. These crops could then be safely used as organic Zn and Cu fertilizers for producing Zn and Cu enriched food or animal feed. Possible alternative high-biomass plants are *M. sinensis*, which gives an average yield of 20 t ha⁻¹ in Switzerland (FAL and RAC, 2001), and *B. juncea*, which produces a biomass of about 18 t ha⁻¹ (Bhargawa, 1991) and has been reported to accumulate heavy metals such as Cd, Cu, Ni, Zn, Pb and Se (Blaylock et al., 1997; Kumar et al., 1995; Raskin et al., 1997; Salt et al., 1995). Table 4-2 gives Cd concentrations in *M. sinensis* and *B. juncea* found by various authors in hydroponic, pot and field experiments. *Miscanthus sinensis* is a perennial plant. Therefore, no crop rotation would be required, reducing the labour costs. Assuming an accumulation of 18.3 mg kg⁻¹ Cd, as found by Ardurini et al. (2004), the annual Cd extraction would average 366 g ha⁻¹. This may be a rather optimistic estimation. Kalembasa (2006) measured the Cd concentrations of 25 *M. sinensis* plant samples used for bioenergy production in Poland and found a mean Cd concentration of 11 mg kg⁻¹, which would lead to a Cd extraction of 220 g ha⁻¹ a⁻¹ if multiplied with the average Swiss yield. Unfortunately,

Kalembasa (2006) does not give the Cd concentrations of the soils on which the plants were grown. *Brassica juncea* was found to accumulate high Cd concentrations in pot experiments (Table 4-2). Kayser et al. (2000), however, observed much lower Cd accumulation by *B. juncea* under field conditions, resulting in a mean Cd extraction rate of only 6.59 g ha⁻¹ (Keller et al., 2003). As plant metal uptake strongly depends on growth conditions, soil properties and soil metal concentrations, on-site tests are necessary to evaluate the local Cd extraction potential of these as of any other candidate plants.

Table 4-2: Cadmium concentrations of *M. sinensis* and *B. juncea*, obtained in various experiments according to the literature.

Plant species	Cd in plant D.W.	Experimental design	Reference
<i>M. sinensis</i>	18.3 mg kg ⁻¹	Hydroponics, Cd 0.25 mg l ⁻¹ , 93 d plant growth	(Arduini et al., 2004)
<i>M. sinensis</i>	11 mg kg ⁻¹	25 samples of field crops used for energy production	(Kalembasa, 2006)
<i>B. juncea</i>	25 mg kg ⁻¹	Pots, greenhouse, Cd _{tot} 5 mg kg ⁻¹ , 90 d plant growth	(Pinto et al., 2008)
<i>B. juncea</i>	1.0 mg kg ⁻¹	Field, Cd _{tot} 2.5 mg kg ⁻¹ , Cd _{sol} 2.2 µg kg ⁻¹ , 126 d plant growth	(Kayser et al., 2000; Keller et al., 2003)
<i>B. juncea</i>	17 mg kg ⁻¹	Pots, 40 mg kg ⁻¹ , 50 d plant growth	(Quartacci et al., 2006)

The application of sulphur has shown to increase soil metal solubility by a much higher factor than plant metal uptake. Such mobilization therefore bears the risk of metal leaching into the groundwater. However, in the previous study mentioned before we found no significant differences in neither total, nor soluble soil metal concentrations between treated and untreated plots at a depth of 50–75 cm in the soil profile (Fässler et al., 2009). Groundwater samples should still be taken from time to time to monitor the situation and to control risks, in particular if sulphur applications would be continued so that soil pH could further decrease.

NTA had no significant effect, neither on soil metal solubility nor on plant metal uptake. While Zn concentrations in maize were significantly increased in the previous study (Fässler et al., 2009), no effects were found in 2007 in both NTA treatments. Possible reasons for the missing effects such as rapid degradation or insufficient application rates were discussed by Fässler et al. (2009). Another reason might be that the NTA competes with high

concentrations of soil-borne chelating compounds in DOC (dissolved organic compounds) in the Witzwil soil. Chelating agents with higher stability constants for the complexation of Cu, Zn and Cd may be more efficient in solubilising these metals and making them available for plant uptake. A possible alternative would be EDDS (ethylene diamine disuccinic acid), which is biodegradable like NTA, but binds more strongly to all three metals than NTA.

4.5 Conclusion

While the application of NTA did not enhance metal extraction, doubling the rate of sulphur application led to a rate of Cd extraction by tobacco that comes close to an efficient phytoremediation. By testing potential Cd extractors such as *B. juncea* or *M. sinensis* on the study site, other high-biomass plants might be found that could be used in crop rotation with or instead of tobacco, until the Cd concentration in the soil is low enough to use it safely for the production of feed or food. Phytomanagement in combination with elemental sulphur application could thus be a viable strategy to control and gradually reduce the metal contamination of the soil and in the same time use the land profitably for crop production.

4.6 Acknowledgements

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5

Uptake and allocation of plant nutrients and Cd in maize, sunflower and tobacco growing on contaminated soil and the effect of soil conditioners under field conditions

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Abstract

Contaminated land may in many cases still be used for agriculture, provided that crops are chosen appropriately, as the accumulation of contaminants varies greatly among cultivars and also plant parts. We aimed to determine whether maize (*Zea mays*), sunflower (*Helianthus annuus*) and tobacco (*Nicotiana tabaccum*) grown on a heavy-metal contaminated soil containing cadmium ($1.4 \text{ mg Cd kg}^{-1}$), copper ($540 \text{ mg Cu kg}^{-1}$) and zinc ($680 \text{ mg Zn kg}^{-1}$) could be used to gradually remediate the soil, while producing valuable biomass. The soil was treated with either a normal fertiliser regime (control), elemental sulphur (S), or the biodegradable chelant NTA (nitrilotriacetic acid), to test how soil acidification or chelating organic compounds would affect the uptake and allocation of selected elements (Ca, Cd, Cu, Fe, K, Mg, Mn, P, S and Zn).

The highest concentrations of Cd, Cu and Zn occurred in the leaves and/or roots, while seeds and grains contained much lower concentrations of these elements. All these concentrations, however, were still in the ranges considered normal for the respective plant parts grown on uncontaminated soil. While sunflower and maize could be safely used as food and feed, tobacco would better be used for bioenergy than for cigarette production because of its relatively high foliar Cd concentration. The two treatments (S and NTA) had only slight effects on the uptake and allocation of plant nutrients and Cd. Thus, there was little benefit of these treatments for phytoextraction purposes at this site.

5.1 Introduction

Heavy metal contamination of agricultural soils is a worldwide problem. In particular, the deposition of industrial and traffic emissions, excessive use of low-quality fertilizers, and the application of biowastes as fertilizers have substantially increased soil metal concentrations on large areas of agricultural land. Using contaminated land for the production of food or feed crop plants bears the risk that the contaminants are transferred into food and feed stuffs and ultimately create health risks for humans and animals. On the other hand, leaving low- or moderately-contaminated land fallow may be wasting a precious natural resource, given the increasing shortage of fertile agricultural soil.

Remediating such large areas of land may be prohibitively expensive or even impossible. Baker et al. (1994) and Chaney et al. (1997) suggested that using plants to extract contaminants from such soil (phytoextraction) may be a low-cost and gentle remediation option that restores the fertility of the soil. Unfortunately, phytoextraction is slow and may take centuries to reach regulatory clean-up standards, even for relatively mobile trace metals such as Cd or Zn (Kayser et al., 2000). However, the treatment time would be less important if the soil was used simultaneously to produce profitable crops, an approach also known as phytomanagement (Domínguez et al., 2008). Phytomanagement aims to combine the production of valuable biomass, such as timber, bioenergy or stock fodder, with the gradual removal of the contaminants, and simultaneously mitigates risks by preventing the off-site movement of contaminants via leaching, runoff or erosion. Studies have shown that the accumulation of contaminants or nutrients by plants varies greatly among crops and cultivars and also differs between different parts of a plant (Kurz et al., 1999; Liu et al., 2007). Seeds and fruits generally accumulate metals at lower concentrations than leaves or roots (Angelova et al., 2004; Liu et al., 2007; McLaughlin et al., 1999; McLaughlin et al., 1996; Wenger et al., 2002a). For example, maize seed produced on contaminated land may be suitable for animal feed (Wenger et al., 2002a), while the stems and leaves could be used for non-food purposes such as bioenergy production (Amon et al., 2007; Licht and Isebrands, 2005; Meher et al., 1995).

We had tested such a phytomanagement approach in a 6-year field experiment that began in 2000 on a heavy-metal contaminated agricultural soil that had developed from a peat soil after artificial drainage in the late 19th century (Fässler et al., 2009). From 2000–2005, maize, sunflower and tobacco were cultivated in this field experiment in a 3-year crop rotation scheme. Four treatments were applied: (1) elemental sulphur (S), (2) nitrilotriacetic acid (NTA), and (3) ammonium sulphate fertilizer ((NH₄)₂SO₄) to increase soil metal availability

for uptake by plants (Kayser et al., 2000; Kulli et al., 1999; Wenger et al., 2002b), and (4) control (no amendment). As expected, the elemental sulphur applications decreased soil pH and CaCO_3 content, increased soil Cd and Zn solubility, and enhanced the uptake of Zn by all three plant species as well as the uptake of Cd by tobacco. NTA only increased the accumulation of Zn in maize. No NTA effect was found in the soil. The $(\text{NH}_4)_2\text{SO}_4$ showed no effect on soil and plant metal uptake. None of the treatments affected crop yield. While the results indicated that soil cleansing via phytoextraction would require centuries, the plant concentrations were such that it may be viable to use the plant material for fodder or bioenergy, while contemporaneously stabilising the soil and extracting some contaminants.

If the plants are to be used for food or stock fodder, then information on the metal allocation within the plants and how it is affected by potential amendments is critical, as allocation determines the potential human or animal exposure to contaminants. There is a lack of knowledge on the effects of S or chelant application on the distribution of metals in crop plants at harvest time. Studies on soil amendments focused on the soil-to-root and root-to-shoot transfer of contaminating metals, rather than on metal allocation in harvested plant parts.

There are few data on the impact of such soil treatments on the nutritional status of crop plants (e.g. Kayser, 2000). Some studies dealing with S-applications investigated S-deficient plants. Nasreen and Huq (2002) showed that S-application to an S-deficient soil increased concentrations of N, P, K and S in sunflower to maximum values at a dose of 80 kg S ha^{-1} , while further additions caused a subsequent decrease in the plant concentrations of these macronutrients. Plant yield was positively correlated with plant macronutrient concentrations. Çimrin et al. (2008) reported similar results with lentils with an optimal dose of 120 kg S ha^{-1} . However, neither of these studies investigated the effects of the treatment on soil pH. At a soil pH between 5.5 and 6.5 most nutrients are readily available for uptake by plants (Brady and Weil, 2002). Changes in soil pH to values below or above this range may cause nutrient imbalances and reduce growth (Marschner, 1995).

In this study, our objective was to (1) determine the allocation of the contaminating elements Cd, Cu and Zn, and several plant nutrients, in maize, tobacco and sunflower plants grown on the above mentioned field, (2) investigate how this allocation is affected by soil amendments with S and NTA and (3) evaluate the possibility of using the land for a profitable production of these crops. For this purpose the field experiment described by Fässler et al. (2009) was extended for another year. The applied S and NTA dosages were doubled in comparison to the preceding years in order to produce stronger effects.

5.2 Materials and methods

5.2.1 Experimental site and design

The experiment was conducted on a field in the metal-contaminated area of Witzwil in the Bernese Seeland, Switzerland. The area is located between the lakes of Neuchâtel, Biel and Murten at an altitude of 432 m a.s.l. It has a mild and warm climate (9 °C annual mean temperature) with an annual average rainfall of 980 mm. The sandy loam soil prevailing in this area had developed from a former peat soil that had been drained for agricultural use in the late 19th century. The soil contamination originated from the disposal of primarily organic city waste between 1913 and 1954, a practice originally also employed in order to increase the fertility of the reclaimed soil. While this objective was achieved, increasing amounts of contaminants including in particular Cd, Cu and Zn, were introduced into the soil in the same time, as the quality of the wastes decreased with time. The concentrations of these metals in the ploughed topsoil were approximately 1.4 mg kg⁻¹ for Cd, 540 mg kg⁻¹ for Cu, and 680 mg kg⁻¹ for Zn (Fässler et al., 2009).

Three blocks, each comprising 16 rectangular plot areas of 3 m × 12 m size were cultivated since 2000 in a 3-year crop rotation scheme of maize (cv. Magister), sunflower (cv. Sanluca), and tobacco (cv. Burley 92). The rotation was phase-shifted between the three blocks, so that all three crops were grown every year, but shifting to another field each year. All plots were fertilized according to the fertilizer recommendation of the Swiss Agricultural Research Stations (FAL and RAC, 2001). Four treatments were applied in the years 2000–2005 in four replications on the sixteen plots within each block: (1) S, (2) NTA, (3) (NH₄)₂SO₄, and (4) no amendment (control treatment). Each treatment was repeated on the same plot every year.

For the present study the experiment was extended one further year in 2006. For this purpose, S, NTA and control treatments were applied in spring 2006 on the same plots as previously, but the rates of S and NTA application were doubled in comparison to the years before. Thus, in the S treatment 0.427 kg m⁻² sulphur were applied, resulting in a cumulative total addition of 1.71 kg m⁻² over the entire experimental time from 2000–2006. In the NTA treatment 110 ml of a 400 mM NTA solution (instead of 200 mM as in the years before) were injected on both sides of each plant at a distance of 15 cm from the stem. For reasons of feasibility, NTA was applied before the plants exceeded 40 cm in height, which was the case approximately 1.5 months after sowing.

5.2.2 Sampling and sample analysis

Sunflower was harvested on the 15th of August, maize and tobacco on the 19th of September 2006. Three plants per treatment (S, NTA and control) and species were chosen at random for analysis. Roots were excavated and separated from the adhering soil by washing. Shoots were divided immediately into stems, leaves, seeds (maize and sunflower) or flowers (tobacco) and “remainder” (sunflower heads without kernels, maize cobs without grains and male flower of maize). Tobacco seeds were not fully developed at the time of sampling. Thus, it was not possible to separate them from the flowers. The samples were packed into plastic bags and immediately transported into the laboratory. Here, they were well washed with tap water, cut into pieces, using a chaff cutter (Wintersteiger, LH 120) for the tobacco stems and secateurs for the other material, and then oven-dried for 5 days at 60 °C. The dried maize grains and tobacco flowers were ground to 0.5 mm using a centrifugal mill (Retsch, ZM1). The dried sunflower seeds were ground using a coffee grinder (Trisa 6200). All root samples as well as the tobacco and maize stems were pre-ground to 5 mm using a knife mill (Fuchs, 180S) and then ground to 0.75 mm using a heavy-duty cutting mill (Retsch, SM1). All other plant material was directly ground to 0.75 mm using the Retsch mill. Subsamples (0.5 g) were microwave-digested in 5 ml of HNO₃ (65%), 3 ml of H₂O₂ (30%), and 2 ml of H₂O. The digests were diluted to 25 ml with Millipore water and filtered.

Calcium, Cu, Fe, K, Mg, P, S and Zn were analysed by Inductively Coupled Plasma Optical Emission Spectrometry (ICP OES), Cd and Mn by ICP MS (Mass Spectrometry). Certified material of Virginia tobacco leaves (CTA-VTL-2) was digested and analyzed together with the samples for quality assurance. The maximal relative standard deviation of repeated measurements of the reference samples was 7% and the respective maximal relative bias was 5% for all elements.

After the plants had been harvested, 16 soil cores (0–20 cm depth) were sampled on each plot area, bulked to one composite sample, dried in an oven with air circulation at 40 °C to constant weight, sieved to 2 mm and divided into subsamples for the following analyses. One 4-g subsample per sample was mixed with 0.9 g of wax and pressed into tablets under a pressure of 15 tons. The total element concentrations of Ca, Cd, Cu, Fe, K, Mg, Mn, P, S, and Zn were determined using X-Ray Fluorescence (XRF) spectrometer. A second subsample was digested using boiling 2 M HNO₃ to determine pseudo-total soil metal concentrations and the extract was analysed for the same elements as the first subsample except for S (ART and ACW, 2007). The same elements as in the HNO₃ extract were also analysed in extracts obtained from a third series of subsamples using 0.5 M CH₃COONH₄

and 0.03 M EDTA (ART and ACW, 2007) to determine the plant available fraction. The fourth subsample was extracted using 0.1 M NaNO₃ solution to determine soluble Cd, Cu and Zn concentrations (ART and ACW, 2007).

Cadmium was analyzed by GF AAS (Graphite Furnace Atomic Absorption Spectroscopy), the other elements by ICP OES. Soil pH was measured in H₂O at a soil:solution ratio of 1:2.5. The CaCO₃ content was determined with a calcimeter (ART and ACW, 2007). Samples of certified sandy clay soil (no. 992, period 2005.2, obtained from the International Soil-analytical Exchange (ISE) program, Wageningen University) were analyzed together with the experimental samples for quality control. The maximal relative standard deviation of repeated measurements of the reference samples was 7% and the respective maximal relative bias was 5% for all elements.

5.2.3 Statistical data analysis

Treatment differences in the sample concentrations of the analyzed elements were determined by ANOVA and a post hoc Bonferroni test, using SPSS (version 15). The data were log-transformed in order to normalize frequency distributions. Differences were judged significant if the error probability was 5% or less ($p < 0.05$).

5.3 Results and Discussion

5.3.1 Soil

The pH of the untreated topsoil was 7.7, the CaCO₃ content 2.7%, and the soil organic carbon (C_{org}) content 10.7%. In 1999 the average soil pH had been 7.4, the CaCO₃ content 3.6%, and the soil organic carbon content 12.3% (Fässler et al., 2009). Our measurements here confirm the trends already observed in our previous study: a slight tendency of the soil pH to increase over the years, and a marked decrease in CaCO₃ and C_{org} contents. According to the fertilisation recommendations of the Swiss Agricultural Research Stations FAL and RAC (2001), the Witzwil soil was adequately supplied with K and P and had a good stock in Mg (data not shown).

The concentrations of HNO₃-extractable Cd, Cu, and Zn (Table 5-1) exceeded the respective guide values (Cd: 0.8 mg kg⁻¹, Cu: 40 mg kg⁻¹, Zn: 150 mg kg⁻¹) of the Swiss Federal “Ordinance Relating to Impacts on the Soil” (OIS) (VBBo, 1998). These guide values indicate a pollution level at which the agricultural and ecological quality of a soil may

be adversely affected in the long term according to Swiss environmental legislation (USG, 1983). The HNO_3 -extractable Cu also exceeded the respective trigger value for this element (150 mg kg^{-1}), indicating potential endangerment to exposed humans or animals (VBBO, 1998). The NaNO_3 -extractable (“soluble”) metal concentrations, however, were not found to exceed the respective OIS guide values (Cd: 0.02 mg kg^{-1} , Cu: 0.7 mg kg^{-1} , Zn: 0.5 mg kg^{-1}). These results indicated that the availability of these metals for uptake by plants did not exceed legally tolerable limits.

As a result of the S treatment over 7 consecutive years, the soil pH decreased by around one unit to a value of 6.5 at harvest time in 2006. The decrease in soil pH would have been greater had it not been buffered by the dissolution of CaCO_3 . In the S treatment the CaCO_3 content decreased from approximately 3.6 to 1.4% over the same 7 years. The high organic matter (OM) content added to the buffering capacity of the soil. Soil OM has its highest buffer effect at intermediate pH values (5–7) (Brady and Weil, 2002).

The S treatment did not significantly change the total concentration of any of the analyzed soil elements, except for the concentration of S itself, which was more than doubled (data not shown). However, it increased the EDTA-extractable Fe and Mn concentrations as well as the NaNO_3 -extractable Zn concentration, whereas it decreased the NaNO_3 -extractable Cu and the EDTA-extractable Ca and K concentrations (Figure 5-1). The only significant effect of the NTA treatment was to increase EDTA-extractable P from 0.06 to 0.08 g kg^{-1} .

Table 5-1: Mean concentrations of selected elements in the untreated soil. Standard errors of the means are in parentheses.

Element	XRF (g kg^{-1})	HNO_3 extraction (g kg^{-1})	EDTA extraction (g kg^{-1})	NaNO_3 extraction (mg kg^{-1})
Ca	37.63 (0.60)	33.15 (1.74)	16.65 (0.47)	n.d.
Cd	n.d.	0.0014 (0.0001)	0.0013 (0.0000)	0.0017 (0.0004)
Cu	0.59 (0.03)	0.53 (0.04)	0.28 (0.01)	0.381 (0.011)
Fe	39.33 (0.84)	20.42 (1.11)	0.74 (0.00)	n.d.
K	10.44 (0.10)	0.74 (0.06)	0.18 (0.01)	n.d.
Mg	4.86 (0.07)	2.95 (0.14)	0.28 (0.01)	n.d.
Mn	1.05 (0.02)	1.00 (0.05)	0.13 (0.00)	n.d.
P	2.48 (0.06)	1.69 (0.09)	0.06 (0.00)	n.d.
S	3.13 (0.08)	n.d.	n.d.	n.d.
Zn	0.80 (0.03)	0.68 (0.04)	0.14 (0.01)	0.081 (0.014)

XRF: X-ray fluorescence
n.d.: not determined

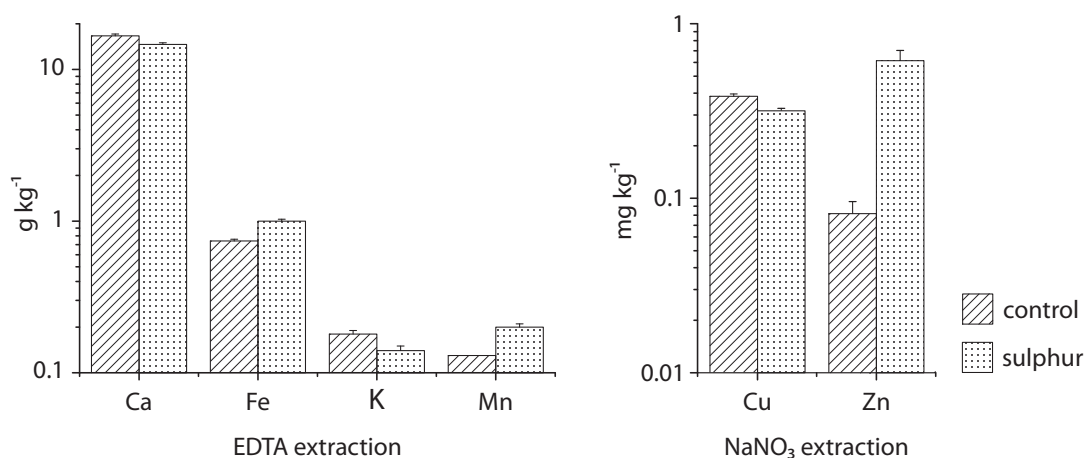


Figure 5-1: The effect of S on soil metal solubility in EDTA extracts (left) and NaNO₃ extracts (right). All depicted element concentrations were significantly changed by the S treatment. Error bars represent the standard errors of the means.

5.3.2 Plant growth and element uptake on untreated soil

Plant growth appeared to be normal. There were no visible symptoms of deficiency or toxicity. The yield was 20.5 t ha⁻¹ for maize, 9.2 t ha⁻¹ for sunflower, and 7.8 t ha⁻¹ for tobacco, which was in or above the normal range for these crops in Switzerland (Walter et al., 2001). The total above-ground biomass of single plants was 0.25 ± 0.02 kg, 0.21 ± 0.01 kg, and 0.32 ± 0.05 kg for maize, sunflower, and tobacco, respectively. Figure 5-2 shows the biomass ratios between different parts of the three plant species. Maize grains amounted to 46%, sunflower kernels to 26% and tobacco flowers only to 3–4% of the total dry weight (including roots) of the respective plants.

Judged by the respective lower and upper threshold concentrations given by Bergmann (1993) to indicate the ranges of sufficient nutrient supply for different crop plant species, the maize plants should have been considered slightly deficient in Cu, Ca, Fe, Mg and P, and even more clearly deficient in K and Mn (Table 5-2). Potassium only reached 1/4 and Mn 1/8 of the lower sufficiency threshold. Potassium and Mn concentrations were also below the respective lower threshold values in sunflower. In tobacco, Mg, Mn and P concentrations were deficient, while Cu was slightly above the respective threshold values. All other elements were within the respective ranges of sufficiency.

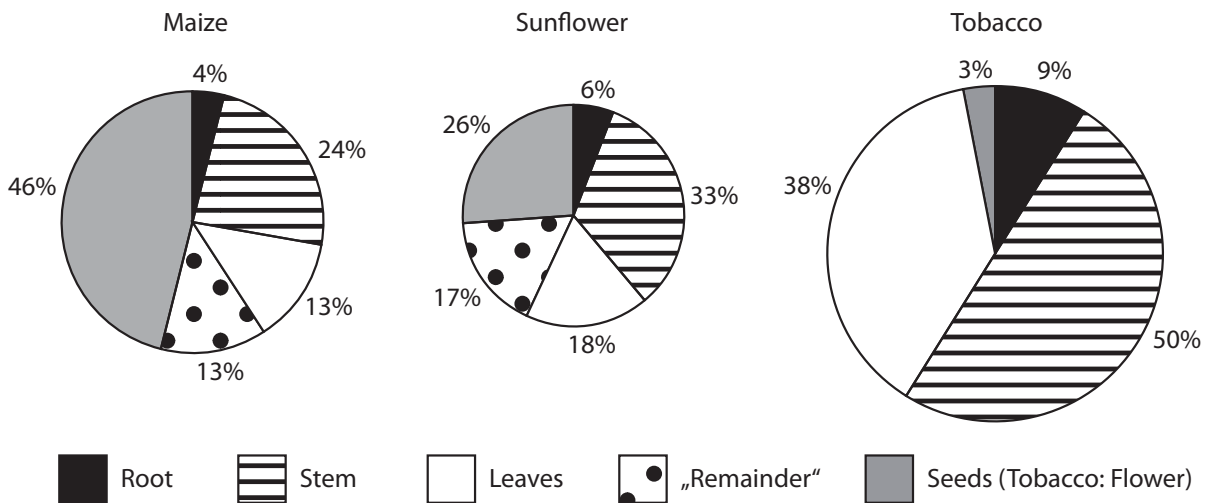


Figure 5-2: Dry weight proportions of maize (left), sunflower (middle) and tobacco (right) parts of the plants of the control treatment. „Remainder“ consists of cobs (without grains) and male flower in maize and heads without kernels in sunflower. The areas of the circles are proportional to the total dry weights of the plants (0.25 ± 0.02 kg, 0.21 ± 0.01 kg, and 0.32 ± 0.05 kg for maize, sunflower and tobacco, respectively).

That no deficiency symptoms were seen, even though the concentrations of some elements in each plant species were below the sufficiency threshold given by Bergmann (1993) for the respective species, indicates that not all of these values may apply to the cultivars used in the present study. Bergmann (1993) states that substantial variation between cultivars is common. Furthermore, the published sufficiency threshold values may not fully apply to the particular edaphic and climatic conditions of the study site. Both soil and climate can have substantial influence on nutrient uptake (Bergmann, 1993).

Plant Cd concentrations did not exceed values typically found on uncontaminated land. Copper and Zn concentrations were within the respective sufficiency ranges, indicating sufficient supply of these elements as micronutrients according to Bergmann (1993), with the exception that Cu just slightly exceeded the upper threshold of sufficiency for this element in tobacco (Table 5-2). The low uptake of these metals despite of their high total concentrations in the soil can be attributed to the alkaline soil pH and the high OM content. Low bioavailability of all three metals is also indicated by the rather low NaNO_3 -extractable metal concentrations (Table 5-1) as compared to the respective guide values.

Table 5-2: Elemental concentrations of tobacco, sunflower and maize shoots grown on the untreated contaminated soil in comparison to reference values given by Bergmann (1993). Standard errors of the means are in parentheses.

Element	Maize		Sunflower		Tobacco	
	measured	reference	measured	reference	measured	reference
Ca (g kg ⁻¹)	2.38 (0.04)	3–10 ^a	16.63 (0.10)	8–20 ^a	22.80 (0.93)	13–24 ^a
Cd (mg kg ⁻¹)	0.04 (0.00)	0.2–3.0 ^b	0.28 (0.00)	0.2–3.0 ^b	0.80 (0.02)	0.2–3.0 ^b
Cu (mg kg ⁻¹)	3.16 (0.07)	7–15 ^a	18.21 (0.33)	10–20 ^a	18.58 (0.72)	8–15 ^a
Fe (mg kg ⁻¹)	42.00 (2.88)	50–250 ^c	64.60 (8.07)	50–250 ^c	76.20 (7.18)	50–250 ^c
K (g kg ⁻¹)	7.54 (0.37)	30–45 ^a	25.84 (2.10)	30–45 ^a	27.38 (2.18)	25–45 ^a
Mg (g kg ⁻¹)	1.13 (0.05)	2.5–5.0 ^a	3.38 (0.05)	3–8 ^a	2.67 (0.04)	4–8 ^a
Mn (mg kg ⁻¹)	4.80 (0.14)	40–100 ^a	22.80 (0.43)	25–100 ^a	17.90 (0.81)	50–120 ^a
P (g kg ⁻¹)	1.51 (0.04)	3.5–6.0 ^a	2.70 (0.20)	2.5–5.0 ^a	2.01 (0.04)	2.5–4.5 ^a
S (g kg ⁻¹)	1.01 (0.02)	1–5 ^d	1.63 (0.02)	1–5 ^d	3.38 (0.09)	1–5 ^d
Zn (mg kg ⁻¹)	39.60 (1.50)	30–70 ^a	61.40 (1.25)	30–80 ^a	63.10 (1.63)	25–70 ^a

^a sufficient range of mineral nutrients in the specific crop plant (tobacco, sunflower or maize)

^b normal range of heavy metal concentration in plants, grown on uncontaminated soils

^c sufficient range of Fe concentration in plants

^d range of S concentration in plants, grown under normal conditions

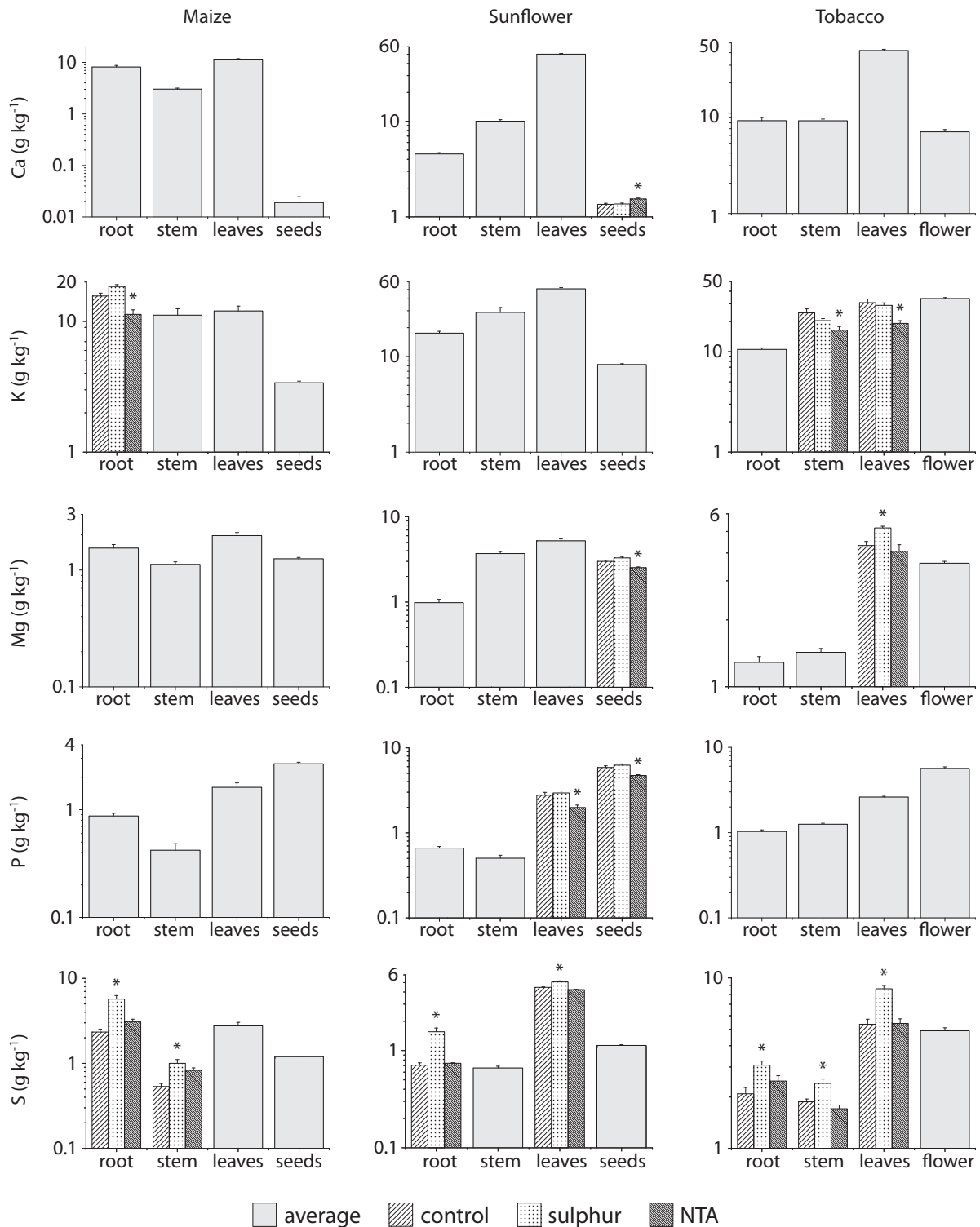


Figure 5-3: Allocation of the macronutrients Ca, K, Mg, P, and S in maize (left), sunflower (middle), and tobacco (right). The wide grey bars show the mean nutrient concentrations for those parts where no treatment effect was found. Stars indicate significant differences between treatment and respective control. Error bars represent the standard errors of the means.

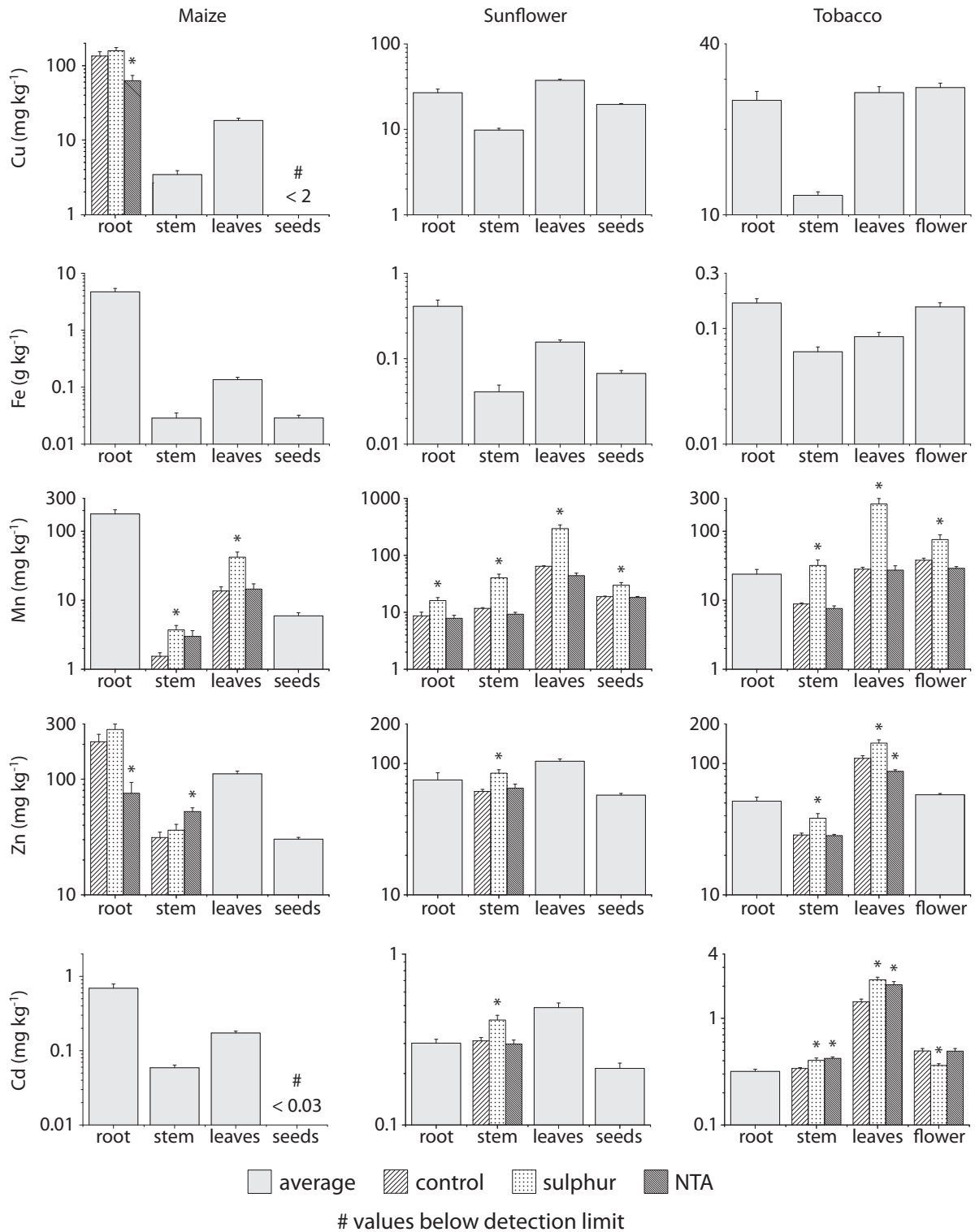


Figure 5-4: Allocation of the micronutrients Cu, Fe, Mn, and Zn, and of Cd in maize (left), sunflower (middle), and tobacco (right). The wide grey bars show the mean concentrations for those parts where no treatment effect was found. Stars indicate significant differences between treatment and respective control. Error bars represent the standard errors of the means.

5.3.3 Element allocation in plants on untreated soil

The highest concentrations of the macronutrients S, Ca, K, and Mg occurred in the leaves of the three investigated species (Figure 5-3). In maize, the concentrations of these elements differed less among roots, stems and leaves than in tobacco and sunflower. In particular, there were no significant differences in S, Ca, K and Mg concentrations in the leaves and roots of maize. The concentration of Ca in the seeds of maize and sunflower was significantly lower than in the other parts of these two species. This may be attributed to the low phloem mobility of this element (Marschner, 1995).

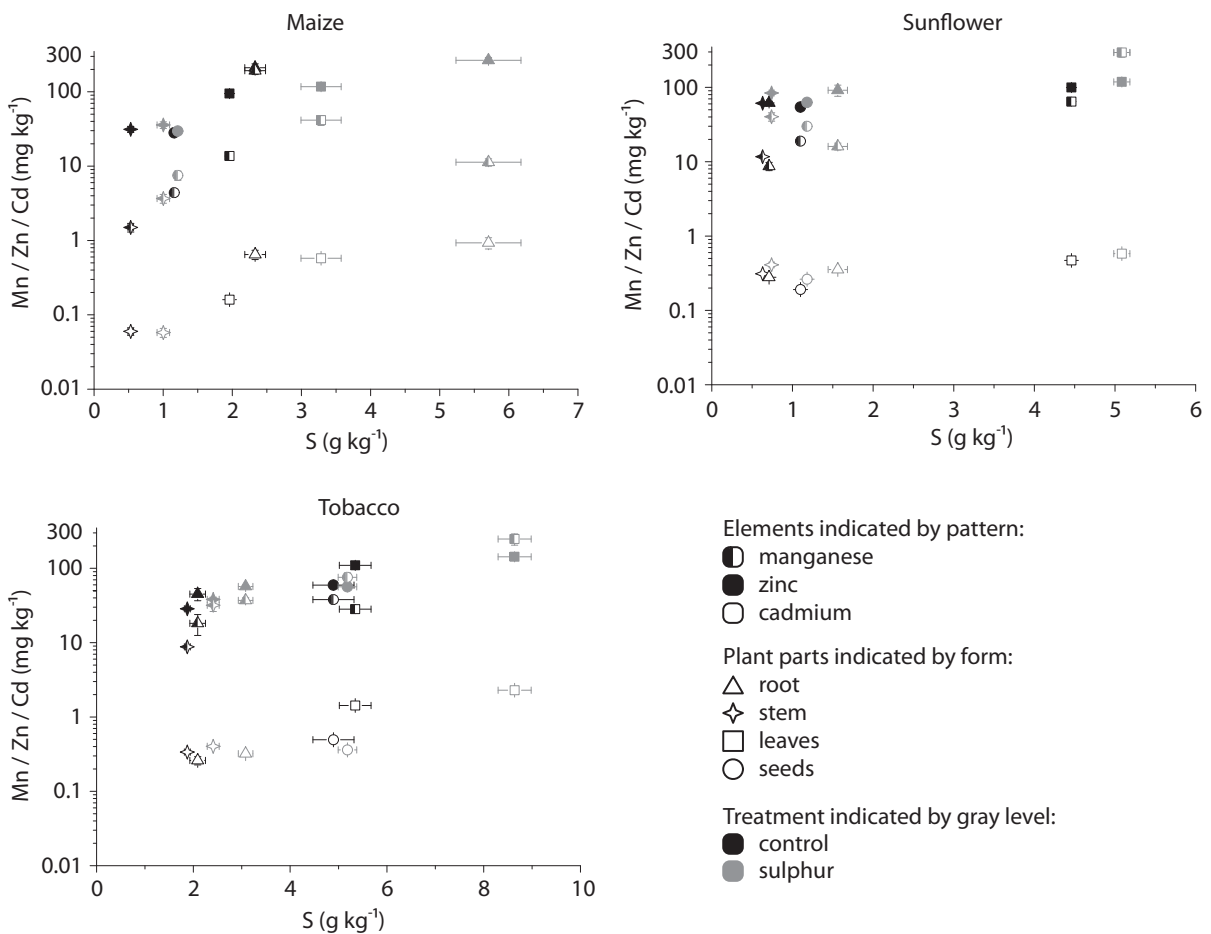


Figure 5-5: Relations between S and Mn, Zn and Cd concentrations in plant material, with and without S treatment. Error bars represent standard errors of the means.

In contrast to the other nutrients, the highest P concentrations were found in the seeds of maize and sunflower and in tobacco flowers. In seeds, most phosphate is bound as phytate (Marschner, 1995). In cereal grains, nuts and legume seeds, phytate-P may represent up to

80% of the total seed P. Phytate plays an important role in pathogen resistance of seedlings, which is a major factor for seedling survival and growth (Frossard et al., 2000).

The allocation patterns of the micronutrients Cu, Fe, Mn, and Zn showed similar diversity as those of the macronutrients (Figure 5-4). The allocation patterns of Cd were most similar to those of S, in particular for the vegetative parts, and resembled those of Mn and Zn (Figure 5-5). In maize, the micronutrient concentrations were always highest in the roots and lower in stems and seeds than in the leaves. The Cd and Cu concentrations of maize seeds were below their respective detection limits. Maize is known as a shoot excluder of Cd, meaning that the root-to-shoot translocation of Cd is strongly restricted (Marschner, 1995). In contrast, sunflower and tobacco translocated Cd, Cu, Fe, Mn and Zn efficiently into their upper parts. In these two plant species, Cd and Zn preferentially accumulated in the leaves. In sunflower this was also the case for Mn.

Given that Cd preferentially accumulates in leaves compared to other above-ground plant parts, growing leafy vegetables may be riskier than producing grains for food or feed on Cd-contaminated soil (Alloway and Jackson, 1991). However, for many crops it is the seeds that are consumed. A comparison of the element concentrations of maize and sunflower seeds with reference values given in the literature for human and animal nutrition is given in Table 5-3. Sunflower seeds contained normal concentrations of Ca, Fe and Zn. Copper, K, and Mg were slightly above the normal mean values. Maize had Ca and Cu concentrations far below the respective mean concentrations, whereas Mg and K exceeded reference values. The high K concentration of the maize grains is further evidence that the maize plants were not deficient in K, even though the average K concentration of the whole plant was below the respective reference value (as discussed above). There are no Swiss threshold values for concentrations of K and Mg in plant raw materials used for food or feed. Underwood and Suttle (1999) reported that the tolerable limit of Mg concentrations for ruminants was 5 g kg⁻¹ dry matter. There are no reported threshold values for K, however, a concentration of 60 g K per kg fodder led to a decline of appetite and growth in calves (Underwood and Suttle, 1999). Our measured Mg and K concentrations in maize grains of 1.18 g kg⁻¹ and 3.18 g kg⁻¹ should not negatively affect animal health. Moreover, grain Fe, Mn, S were in the normal range, while grain Zn was slightly above.

The mineral nutritional value of seeds depends on the bioavailability of the nutrient elements to humans and animals, rather than just on the total concentrations. In particular, excessive levels of P in seeds, when it is primarily present in form of phytate, may reduce the bioavailability of nutrients as well as contaminants. In our case, the P concentration was slightly above the normal range for sunflower seeds (Table 5-3). The concentration of Cd,

the main problem element in our soil with respect to food chain transfer, was below the threshold values in both, sunflower and maize seeds. Thus, they could be safely used at least as feed for animals.

Table 5-3: Elemental concentrations of maize and sunflower seeds grown on the untreated contaminated soil and reference values for human or animal nutrition given in the literature. Standard errors of the means are in parentheses.

Element	Maize		Sunflower	
	measured	Reference	measured	reference
Ca (g kg ⁻¹)	0.02 (0.00)	0.5 ^b	1.35 (0.03)	1.6 ^b
Cd (mg kg ⁻¹)	< 0.031	0.1 ^c / 1 ^d	0.19 (0.01)	1.6 ^c / 1 ^d
Cu (mg kg ⁻¹)	< 2	9 ^b	18.93 (0.31)	14 ^b
Fe (mg kg ⁻¹)	26.76 (5.47)	25 ^b	62.09 (0.61)	68 ^b
K (g kg ⁻¹)	3.18 (0.09)	0.8 ^a	8.64 (0.26)	6.4 ^a
Mg (g kg ⁻¹)	1.18 (0.03)	0.2 ^a	3.00 (0.07)	3.87 ^a
Mn (mg kg ⁻¹)	4.39 (0.39)	5–8 ^e	18.86 (0.29)	
P (g kg ⁻¹)	2.47 (0.09)	3.4 ^b	5.87 (0.22)	4.5 ^a
S (g kg ⁻¹)	1.16 (0.03)	1 ^e	1.10 (0.02)	
Zn (mg kg ⁻¹)	28.07 (0.87)	21 ^b	54.32 (1.05)	48 ^b

^a tables of nutritional values of food stuffs (values of sunflower seeds and polenta (“Maisgriess”)) (SwissFIR, 2008)

^b tables of nutritional values of swine forage (values of sunflower seeds and maize corn) (ALP, 2004)

^c threshold value for human nutrition (sunflower: value of oilseed, maize: value of cereals (“übrige Getreide”)) (FIV, 1995)

^d threshold value for undesired substances in plant raw materials for animal feeding (FMBV, 1999)

^e typical content in cereal grains (Underwood and Suttle, 1999)

5.3.4 Treatment effects on plant growth, element uptake and allocation

Neither the S nor the NTA applications significantly affected the biomass production of the plants (data not shown). They also had little effect on the uptake and allocation of the investigated macro- and microelements (Figures 5-3 and 5-4). Where it had an effect, the S treatment generally resulted in a slightly increased concentration of the affected element. Not surprisingly, the S treatment increased S concentrations in the plants. The greatest increase occurred in the roots of all three crops, but the S concentrations increased also in maize and tobacco stems as well as in sunflower and tobacco leaves, while no significant effects were found in the seeds and flowers.

Apart from S itself, Cd, Mn, and Zn responded most strongly to the S treatment (Figure 5-5). These elements showed similar allocation patterns and similar relationships to the S concentration in the various plant parts. Most conspicuous was the increase in Mn accumulation, which was observed in all three plant species, primarily in the stems and leaves, and in sunflower roots and seeds as well as in the flowers of tobacco (Figure 5-4). Sulphur applications were also found by other authors to increase plant Mn concentrations (Çimrin et al., 2008; Juste and Solda, 1988). In sunflower and tobacco, Zn was slightly increased in the stems, but not in the seeds or flowers, respectively. An S effect on leaf Zn was only observed in tobacco. The S treatment effects on Cd concentrations were similar to those on Zn in sunflower and tobacco, with the exception of the rather unexpected result that Cd was slightly decreased in tobacco flowers. Magnesium was increased in tobacco leaves (Figure 5-3).

For a comparison with the results obtained in the previous study on the same experimental field (Fässler et al., 2009) we calculated the mean element concentrations for the whole above-ground biomass from the values of the individual plant parts of the above-ground biomass. In the previous study, the S treatment had led to increased Cd and Mg concentrations in tobacco and an increased Zn concentration in all three plants, which is consistent with what we obtained in the present study (data not shown). The doubling of the S application in 2006 had little additional effect on plant metal uptake. The results of the S treatment are also in good agreement with those of Kayser (2000), who investigated the response of maize and tobacco upon the addition of elemental S in two different soils (a carbonate rich silty clay loam with pH 7.4 and a low carbonate sandy loam with pH 6.8). While S applications decreased the pH of both soils, plant accumulation of S and Mn was only increased in the soil with low CaCO₃, indicating that not the pH effect but the increased availability of S was the dominating factor for the increase in Mn uptake. In agreement with our findings here, Kayser (2000) reported that the responses of plant Cd and Zn concentrations were similar to those of plant S and Mn concentrations.

The observed similarities in the accumulation and allocation patterns of Cd, Mn and Zn suggest that transport mechanisms common to all three of these elements played a role. Cadmium is taken up into plant cells among others through transporters belonging to the ZIP and Nramp families, which also transport Zn and Mn (Guerinot, 2000; Pittman, 2005). The similarity in Zn and Cd allocation has been reported in other species. In *Arabidopsis halleri* it was not only found at the level of whole plant organs, but also of single cells (Küpper et al., 2000). Ma et al. (2005) also found that Cd and Zn had similar subcellular localisation in the leaves of *Thlaspi caerulescens*. The similarity of the two metals also results in competition

for binding sites. This is one reason why not only positive correlations are found between Cd and Zn uptake by plants, but also antagonistic effects (Cosio et al., 2004). However, plants discriminate between Cd and Zn by producing ligands that are particularly specific for one of them. Studying Cd-stressed *T. caerulescens* plants Küpper et al. (2004) found that histidine was the main ligand for Zn, while SH-bearing ligands, such as phytochelatins, predominantly bound Cd. Phytochelatins have a particularly high binding affinity for Cd compared to other metal ions and are known to be involved in the detoxification of Cd in plants (Cobbett and Goldsbrough, 2002; Satofuka et al., 2001). As phytochelatins are rich in S, the increased S accumulation observed in the crop plants on the S-treated plots may not only have been a response to higher S-availability in the soil but also to increased Cd stress due to the decrease in soil pH. This is consistent with the observation of an increased shoot S concentration in Cd-stressed *A. halleri* plants by Küpper et al. (2000).

Competition in plant uptake was not only reported to exist between Cd and Zn (Cosio et al., 2004) but also between Cd and Mn. Hernandez et al. (1998) treated hydroponically grown *Pisum sativum* and *Z. mays* with 50 μM Cd and found that Mn uptake was almost completely inhibited, while Peng et al. (2008) found that the addition of 9.1–5000 μM Mn to Cd-treated (10–50 μM) *Phytolacca americana* plants significantly reduced Cd accumulation and alleviated Cd toxicity in a hydroponic experiment. The increase in plant Mn concentrations on the S-treated plots may thus have counteracted a stronger increase in Cd uptake in our study.

In the few cases, where we observed an NTA effect on the uptake or allocation of the investigated elements, it consisted of a slight decrease, with few exceptions. There were decreases in shoot concentrations of K and Zn in tobacco and P in sunflower (Figures 5-3 and 5-4), while Zn was increased in maize stems. The decrease in shoot K accumulation of tobacco led to a concentration below the sufficiency range given for this plant by Bergmann (Table 5-3). In contrast to the effects of S applications, NTA had no effects on Mn concentrations, but decreased the K, Cu and Zn concentrations in maize roots. The decrease in root Zn combined with the increase in stem Zn in maize may indicate an enhanced translocation of Zn from root to shoot with the NTA treatment. In sunflower seeds, NTA decreased Mg and P, and increased Ca. Also, in contrast to the observed S effects, NTA effects on Cd differed from those on Zn. The accumulation of Cd was increased in tobacco leaves and stems by NTA, but was unaffected in all other cases. While the increased Zn concentration in maize stems is consistent with the results obtained in previous years (Fässler et al., 2009), the increased Cd accumulation in tobacco was new and may be due to the doubling of the applied NTA concentration compared to the latter study.

5.3.5 Implications for agricultural crop production

Although the HNO₃-extractable soil Cd and Zn concentrations exceeded the respective OIS guide values and in the case of Cu, the trigger value, the concentrations of these elements in edible parts of all the plants in all treatments were below the values considered problematic for consumption by humans or animals (Tables 5-2 and 5-3). Plant metal concentrations exceeding regulatory values were only observed in the case of tobacco, where leaf Cd concentrations exceeded the Swiss threshold value of 1 mg kg⁻¹ for undesired substances in raw materials for animal feeding (FMBV, 1999) in all treatments.

Although tobacco is not used as a food plant, the accumulation of Cd in tobacco may exacerbate the harmful effects of smoking, which is the main source of Cd intake by humans (Bernhard et al., 2005). Leaf concentrations of tobacco used for cigarette production are reported to vary between 0.5 and 5 mg kg⁻¹ on uncontaminated soil (Lugon-Moulin et al., 2004). The leaf Cd concentrations found in our experiment on the untreated soil were in the lower part of this range and were still in this range after S or NTA treatments. To be on the safe side, however, tobacco plants may be used for energy production instead (Meher et al., 1995). The unexpected finding that Cd accumulation in the tobacco flowers was slightly decreased in the S treatment indicates that testing the effect of S amendments on the seeds and fruits of related plants, such as tomato (*Solanum lycopersicum*) or chilli peppers (*Capsicum* sp.) may be an interesting avenue for future research.

The elevated total soil metal concentrations are still relevant for crop contamination risks arising from the deposition of suspended soil particles on plant surfaces due to wind erosion or rain splash. We compared our plant concentrations with data obtained from plants grown on the same field but harvested in usual agricultural manner, i.e. they were passed through a chaff cutter directly on the field without being washed (data not shown). The Cu concentrations were significantly lower in all three washed compared to the unwashed crops (maize 52%, sunflower 12% and tobacco 22%). Zinc concentrations in maize and sunflower were also significantly lower in the washed plants (maize 16%, sunflower 22%). No washing effect was found for Zn in tobacco and for Cd in all three plants. If economically feasible, washing the plants before feeding them to animals would thus reduce the animals' exposure to Cu and Zn. As the amount of suspended soil particles on the plant surface certainly decreases with the distance to the soil surface, an increase in the cutting height might also reduce the contamination.

Our results show that moderate concentrations of contaminating metals do not necessarily render agricultural soils unsuitable for the production of food or fodder plants. However,

when planting crops on contaminated soil, it is important to monitor and control the availability of the soil contaminants to potential crop plants, thereby taking particular account of the allocation of potentially toxic elements in the harvested plant parts. Heavy metal concentrations in plant tissues may increase over time in response to changes in acidity, organic matter and other soil properties. In the case of the soil investigated here, all three tested crops are realistic options to be used in a sustainable phytomanagement of the land, with or without S treatment.

5.4 Conclusion

The studied soil contaminants at Witzwil were predominantly accumulated in the leaves and/or roots, rather than in the seeds. Thus sunflower seeds or maize grain could be safely used as food or animal feed. In contrast, the production of leafy vegetables may result in these toxic elements presenting a health risk to humans or animals. Besides the increase in Mn accumulation by all three tested crop plants after S application, both treatments (S and NTA) had only slight or no effects on the uptake and allocation of plant nutrients and Cd. Thus, there is little benefit of these treatments for phytoextraction purposes at this site, even though the application doses were doubled compared to the previous years. The use of sunflower, maize and tobacco without treatment, on the other hand, provides valuable biomass while the environmental risks posed by the soil contaminants are gradually reduced.

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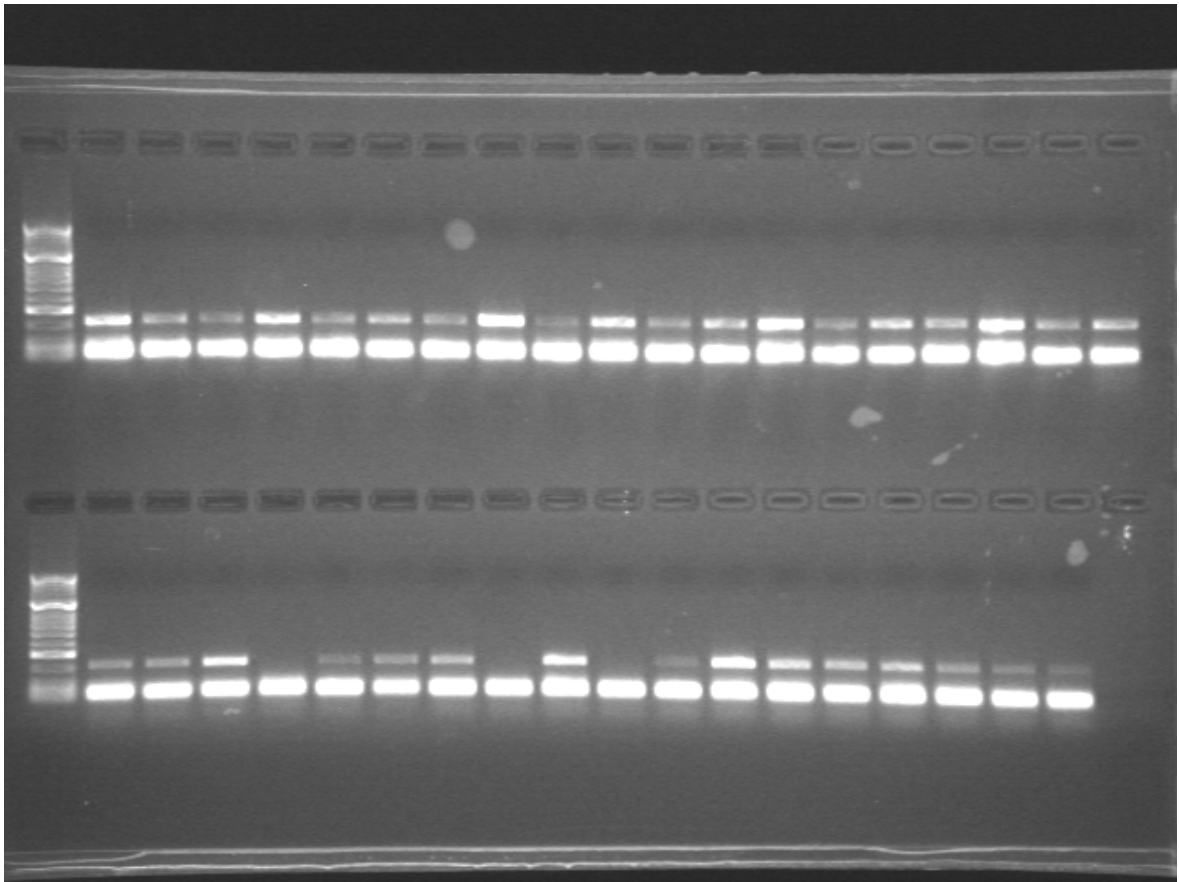
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6

Expression of genes involved in cadmium uptake and detoxification in tobacco plants grown on a sulphur-amended metal-contaminated field

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Abstract

We investigated the effect of a Cd solubilising soil treatment on the expression of genes regulating Cd uptake and detoxification in field-grown tobacco plants. Tobacco plants were grown on a heavy-metal contaminated soil to which elemental sulphur was applied to increase the phytoextraction of Cd. The expression of tobacco gene homologues for *Arabidopsis thaliana* *HMA4*, *MRP3*, *PDR8*, *ATM3*, *Sultr1*, *LAST*, *APR2*, *APR3*, *GSHI*, *GSHII*, and *NAS3* was assayed by RT-PCR.

In agreement with expectations, an increase in root and shoot Cd concentration resulted in the up-regulation of the putative Cd transporters and the genes involved in sulphur assimilation in root tissues. In contrast, most of the tested genes were unaffected in the younger leaves and down-regulated in the older leaves.

Differences to the expected results may be due to the more complex stress situation that plants experienced here under field conditions. Moreover, our plants were sampled at maturity and not in the seedling stage. Our results indicate that hydroponic or agar experiments are useful predictors of effects that may be expected in the field. However, there is a need for studies investigating gene expression in response to multiple stresses representative for field conditions at later developmental stages.

6.1 Introduction

Cadmium (Cd) is a trace element that is not essential for plants, animals and humans. It is highly toxic at elevated concentrations. Large areas of agricultural land are contaminated with Cd, which is readily taken up by plants and thus can easily enter food chains. Phytoextraction has been proposed to clean up such land (Chaney et al., 1997). Tobacco is a crop plant that produces a large biomass and accumulates relatively high concentrations of Cd (Lugon-Moulin et al., 2004). While the latter property creates health problems for smokers, the combination of large biomass and high Cd accumulation also makes tobacco a candidate plant to be used for the remediation of agricultural soils with low to moderate Cd contamination.

The expression of genes that are involved in the uptake, distribution and detoxification of Cd is affected by the exposure of plants to this element in the rhizosphere. Table 6-1 shows a selection of Cd-related genes and their response to elevated concentrations of Cd in roots and shoots. Cadmium is taken up by plants due to its chemical similarity with other cations that are essential for plants such as Ca and Zn and due to lack of specificity of the uptake and distribution systems for these elements (Clemens et al., 2002). Genes that are involved in Cd-detoxification notably include heavy-metal transporting ATPases (HMA) and ATP-binding cassette (ABC) proteins (Hall and Williams, 2003; Hanikenne et al., 2008; Mills et al., 2003; Plaza and Bovet, 2008). Courbot et al. (2007) suggested a role of *AhHMA4* in the root-to-shoot translocation of Cd in *Arabidopsis halleri* and in the detoxification of the cytosol of sensitive cells by acting as an efflux pump. Members of the ABC transporter family that are known to confer Cd tolerance to plants include MRP3 (multidrug-resistance-related protein) (Kolukisaoglu et al., 2002), ATM3 (ABC transporter of the mitochondria) (Kim et al., 2006), and PDR8 (pleiotropic drug resistance) (Kim et al., 2007).

An important role in Cd detoxification is played by phytochelatins (PC) (Howden et al., 1995), which are heavy metal-chelating polypeptides that are synthesized in the cells through a specific non-translational pathway from glutathione. The synthesis of GSH occurs in two ATP-dependent steps, which are catalyzed by γ -glutamylcysteine synthetase (γ -ECS) and glutathione synthetase (May et al., 1998a). These enzymes are encoded by the genes *GSHI* and *GSHII*, respectively (Zhu et al., 1999). An important component of GSH is sulphur (Leustek and Saito, 1999). Sulphur is taken up by plant roots primarily in the form of sulphate (SO_4^{2-}) through high-affinity sulphate transporters, such as the Sultr1 (SULphate TRANSPORTer) subfamily in *A. thaliana* (Saito, 2004). Low-affinity sulphate transporters (LAST) are involved in the translocation of sulphate from roots to shoots (Sun et al., 2005).

Table 6-1: Summary of studies in which Cd effects on the expression of genes coding for enzymes that are involved in Cd uptake and detoxification are reported (↑ = up-regulated, ↓ = down-regulated, → = expression not affected, () = see remarks, n.e. = not expressed, n.d. = not determined).

Gene	Enzyme function ^{a)}	Studies on the effects of Cd on gene expression	System ^{b)}	Plant species	Cd Conc.	Effect		Exposure time / remarks
						Root	Shoot	
<i>HMA4</i>	Heavy-metal detoxification (Mills et al., 2005; Rea, 2007)	(Mills et al., 2003)	H	<i>A. thaliana</i>	100 µM	↓	n.d.	30 hours
						→	→	72 hours
						→	→	72 hours / 7 days
<i>MRP3</i>	Involved in Cd resistance (Tommasini et al., 1998)	(Bovet et al., 2003)	H	<i>A. thaliana</i>	20 µM	↑	→	24 hours
						↑	→	2 weeks
<i>ATM3</i>	Heavy metal detoxification (Kim et al., 2006; Rea, 2007)	(Kim et al., 2006)	A	<i>A. thaliana</i>	50 µM	↑	→	2 weeks
						↑	→	2 weeks
<i>PDR8</i>	Cd resistance by efflux pumping from root epidermal cells (Kim et al., 2007)	(Kim et al., 2007)	A	<i>A. thaliana</i>	50 µM	↑	↑	12 hours
						↑	n.d.	2, 10 and 24 hours
<i>Sultr1</i>	Sulphur uptake by the roots (Smith et al., 1995; Yoshimoto et al., 2002)	(Herbette et al., 2006)	H	<i>A. thaliana</i>	50 µM	→	n.d.	2, 6, and 30 hours/ Sultr1;2
						↑	n.d.	2, 6, and 30 hours/ Sultr1;1
<i>LAST</i>	Uptake, translocation, distribution and reallocation of sulphate in plants (Sun et al., 2005)	(Sun et al., 2005)	H	<i>B. napus</i>	30 µM	↑	→	2 and 3 days
						↓	→	48 hours
						↓	↑	72 hours
		(Heiss et al., 1999)	H	<i>B. juncea</i>	25 µM	↓	→	48 hours
		(Sun et al., 2007)	H	<i>B. napus</i>	20, 40 µM 80, 120 µM	↓	↓	72 hours

<i>APR</i>	Reduction of APS to sulphite in sulphur assimilation, control function in sulphur pathway (Kopriva and Koprivova, 2004)	(Herbette et al., 2006)	H	<i>A. thaliana</i>	5 μ M 50 μ M	↑ ↑	n.d. n.d.	2 and 6 h, (→) 30 h 2, 6 and 30 hours
		(Weber et al., 2006)	H	<i>A. thaliana</i>	10, 50 μ M	↑	n.d.	2 hours
		(Heiss et al., 1999)	H	<i>B. juncea</i>	25 μ M	↑	↑	48 hours
<i>GSHI</i>	Catalyses the first step in GSH synthesis (May et al., 1998a)	(Xiang and Oliver, 1998)	P	<i>A. thaliana</i>	100 μ M ^{d)}	n.d.	↑	12 hours
			H	<i>A. thaliana</i>	25, 50, 100 μ M 400 μ M	n.d. n.d.	↑ ↓	12 hours
		(Schäfer et al., 1998)	H ^{c)}	<i>B. juncea</i>	25 μ M	↑	↑	10 days
		(Kim et al., 2006)	A	<i>A. thaliana</i>	10 μ M	(↑)	(↑)	2 weeks; () = increased in the "seedlings"
		(Sun et al., 2005)	H	<i>B. napus</i>	30 μ M	↑	↑	2 and 3 days
<i>GSHII</i>	Catalyses the second step in GSH synthesis (May et al., 1998a)	(Herbette et al., 2006)	H	<i>A. thaliana</i>	5, 50 μ M	→	→	2, 6, and 30 hours
		(Xiang and Oliver, 1998)	P	<i>A. thaliana</i>	100 μ M ^{d)}	n.d.	↑	12 hours
			H	<i>A. thaliana</i>	25, 50, 100 μ M 400 μ M	n.d. n.d.	↑ ↓	12 hours
		(Schäfer et al., 1998)	H ^{c)}	<i>B. juncea</i>	25 μ M	→	n.d.	10 days
		(Bovet et al., 2006)	H	<i>N. tabacum</i>	1 μ M	↑	→	7 days
<i>NAS3</i>	Transfer of excess metals from root to shoots (Sharma and Dietz, 2006)		H	<i>N. rustica</i>	1 μ M	↑	→	7 days

a) linked to Cd

b) A = agar, H = hydroponics, P = pot

c) in pots with sand, watered with nutrient solution

d) plants sprayed with Cd solution

In the sulphur assimilatory pathway, sulphate is reduced in several steps to sulphite. The key step in sulphur assimilation is the reduction of adenosine 5'-phosphosulphate (APS). It is catalyzed by the enzyme APS reductase (APR) (Kopriva and Koprivova, 2004). Due to their role in Cd detoxification, it is no surprise that the expression of the genes coding for the respective enzymes, i.e. *Sultr1*, *LAST*, *APR*, *GSHI*, and *GSHII*, is also responsive to Cd exposure (Kopriva and Koprivova, 2004; Sun et al., 2005; Xiang and Oliver, 1998) (see also Table 6-1). Furthermore, although not fully understood, nicotianamines (NA) are involved in Cd chelation, transport and detoxification in plants (Sharma and Dietz, 2006). Nicotianamines are ubiquitously present in plants and synthesized from three molecules of methionine by nicotianamine synthase (NAS) (Sharma and Dietz, 2006).

Most studies investigating the expression of genes involved in Cd uptake and detoxification were carried out using *A. thaliana*, *B. napus* and *B. juncea* in hydroponic or agar systems (Table 6-1). In contrast, there are only few studies on Cd-induced gene expression using *N. tabacum*, which may seem surprising given the health problems for smokers associated with the accumulation of Cd by this plant. The times over which seedlings were exposed to Cd in the experiments investigating Cd effects on gene expression generally ranged between 2 h and 2 weeks. Thus, in such experiments plants were generally harvested at an early vegetative state. Bovet et al. (2003) showed that it is not always possible to extrapolate data on gene expression from seedlings to mature plants. Furthermore, previous experiments were often performed with excessively high Cd concentrations (Ernst et al., 2008). No studies on Cd-related gene expression have so far been performed under field conditions, where plants experience many other stress factors and are grown to maturity.

The aim of this study was to investigate the effects of increased Cd phytoavailability on the expression of genes that are involved in Cd uptake, distribution and detoxification in tobacco plants grown in a phytoextraction experiment on a heavy-metal contaminated soil, in comparison to published results from hydroponic or agar experiments. In this field experiment some plots were treated with elemental sulphur in order to increase the Cd solubility in the soil and thereby the phytoextraction of this soil contaminant.

6.2 Materials and methods

6.2.1 Experimental design

The plants were taken from a field experiment at Witzwil in the Bernese Seeland, Switzerland, on a former wetland soil that had been converted to agricultural use after drainage at the end of the 19th century. The land had been contaminated in the first half of the 20th century through the application of organic but also other wastes from the city of Bern. In the field experiment, which was started in the year 2000, maize (*Zea mays*, cv. Magister), sunflower (*Helianthus annuus*, cv. Sanluca), and tobacco (*Nicotiana tabacum*, cv. Burley 92) were grown on three parallel plots in a 3-year scheme of annual rotation between these crops. Each plot was subdivided into 16 sub-plots (3 m × 12 m) on which four treatments affecting Cd solubility were applied to the soil in four replicates. All fields were fertilized according to the nutritional requirements of the plants. For the study of gene expression reported here, we focus on tobacco plants, which were sampled in 2006 from plots treated with elemental sulphur and from control plots that had not received any chemical treatment apart from fertilization. The Cd-mobilizing effect of sulphur addition results from the decrease in soil pH as elemental sulphur is microbially oxidized to sulphate (Tichý et al., 1997). Previous studies have shown that this effect can be used to enhance phytoextraction of Cd on contaminated soil (Kayser et al., 2000; Wenger et al., 2002). From 2000 to 2006, a total of 1.71 kg sulphur per m² had been added to the top soil (plough layer). Relevant soil properties are listed in Table 6-3.

6.2.2 Cadmium and nutrient concentrations in tobacco

Four randomly selected tobacco plants were sampled at harvest, at the age of 20 weeks, from each of the two treatments (sulphur application, control). The leaves of each plant were grouped into bottom, middle and top leaves, taking the same amount of leaves for each category. Roots were excavated using a garden fork and washed carefully with water to remove soil particles. For gene expression analysis, some fresh material was taken from the root and leaf samples and conserved at -80 °C. The rest was cut into small pieces, oven-dried for three days at 60 °C and ground to a particle size of approximately 0.75 mm using a heavy-duty cutting mill (Retsch, SM1). The root samples were first coarsely ground using a knife mill (Fuchs, 180S), before they were passed through the cutting mill. A subsample of 0.5 g was taken from each sample and microwave-digested in a mixture of 5 ml HNO₃ (65%), 3 ml H₂O₂ (30%), and 2 ml H₂O. The digested samples were diluted by adding Millipore water to a total volume of 25 ml and then filtered (filter paper grade MN 640 d,

average retention capacity 2–4 μm). Cadmium was analyzed by means of Graphite Furnace Atomic Absorption Spectroscopy (GF AAS, Varian GTA120/AA240Z). Concentrations of the micro- and macronutrient elements Cu, Fe, Mg, Mn, S and Zn were determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP OES, Varian Vista-MPX). For quality control certified material of Virginia tobacco leaves (CTA-VTL-2, Dybczynski et al., 1998) was digested and analyzed together with the samples. The maximal relative standard deviation of repeated measurements of the reference samples was 7% and the respective maximal relative bias was 5% for all elements.

6.2.3 Gene expression analysis

Gene expression was analysed by semi-quantitative RT-PCR using frozen plant material (see above). All RT-PCRs were repeated twice on each plant sample. The design of primers was based on tobacco sequences available from the tobacco genome initiative TGI (NC State University, 2008) (Table 6-2 gives a complete list of the primers). The selection of tobacco genes was based on the literature listed in Table 6-1 and on a search using the sequence similarity search program BLAST (Basic Local Alignment Search Tool) (Ye et al., 2006). The BLAST analysis gave a sequence of the tobacco genome that belongs to the *Sultr* family and is close to the sequences of the *AtSultr1;1*, *AtSultr1;2* and *AtSultr1;3* genes. A specific *Sultr* homologue for tobacco could not be assigned. Therefore, the designation *AtSultr1* was chosen. Specific tobacco *APR2* homologues could not be ascertained either. Sequences similar to *AtAPR2* and *AtAPR3* were chosen therefore. Total RNA was purified from the root and leaf samples using the RNeasy Plant Mini Kit (Qiagen, Basel, Switzerland) and stored at $-80\text{ }^{\circ}\text{C}$ after quantification by spectrophotometry. After DNase treatment (RQ1, RNase free, Promega Catalys, Wallisellen, Switzerland), cDNAs were prepared using M-MLV reverse transcriptase (RNase H minus, point mutant, Promega Catalys) as prescribed by the manufacturer and stored at $-20\text{ }^{\circ}\text{C}$. An aliquot of the cDNAs was diluted 1:10 in water and used for PCR. After 2 min denaturation at $95\text{ }^{\circ}\text{C}$, 35 PCR cycles ($95\text{ }^{\circ}\text{C}$ for 30 s, $50\text{--}58\text{ }^{\circ}\text{C}$ (depending primers T_m) for 30 s and $72\text{ }^{\circ}\text{C}$ for 30 s) were run. PCR was performed in a final volume of 15 μL containing the following mixture: PCR buffer 1x, 5 mM MgCl_2 , 0.2 mM dNTPs, 1 μM of both forward and reverse primers, 1 U Go Taq DNA polymerase (Promega Catalys). Thirty-five PCR cycles were performed to ensure transcript detection. For PCR adjustment, we used the housekeeping gene “elongation factor A” (*NtEFA*), the tobacco homologue of the *AtEF1* gene, for the root samples and the *NtTubulin* gene homologue to *At4G14960* for the shoot samples.

Table 6-2: List of primers used for the RT-PCRs

Gene	primer		<i>Arabidopsis thaliana</i> homologue
<i>NtHMA_a</i>	forward	CACCCAGCTTCTTTGGCAATTG TTC	<i>AtHMA4</i>
	reverse	GAGACTTGA ACTCGGTCACCA	
<i>NtMRP_a</i>	forward	GAGGATGTTCTCAGCTTCA	<i>AtMRP3</i>
	reverse	AGGTACGGGCCAACAAAAG	
<i>NtATM_a</i>	forward	AATCAGAGATGCAAACGATGC	<i>AtATM3</i>
	reverse	TTCCAGAATGGGTGTCAAAG	
<i>NtPDR_a</i>	forward	GGGATCAATGTATGCTGCTG	<i>AtPDR8</i>
	reverse	TTCAAATCCAATCATAGCATAGACA	
<i>NtSultr_a</i>	forward	CCCCAAAACAGGACATATTCA	<i>AtSultr1</i>
	reverse	CAATGGTGAGACCAGCAATAA	
<i>NtLAST</i>	forward	TATTCTGGCTACCGGCTATTG	<i>BnLAST**</i>
	reverse	GATAATGACCCACAATGTTCA	
<i>NtAPR_a</i>	forward	AAGGAGTGTGGATTGCACAAG	<i>AtAPR2</i>
	reverse	TTAACACCTGAACCAGCCAAC	
<i>NtAPR_b</i>	forward	GCTAATTGGTGGT GATTTGGA	<i>AtAPR3</i>
	reverse	TGTGTAACCTCCCAGAGGATG	
<i>NtGSHI*</i>	forward	TGCAGCCTATTGCTACAGCTC	<i>AtGSHI</i>
	reverse	ATGCACACAACCTTCTCCAAG	
<i>NtGSHII*</i>	forward	GAACTGTTCTGGAGTTGGA	<i>AtGSHII</i>
	reverse	AATTACCAACCTAGACAGTGATTGC	
<i>NtNAS</i>	forward	GGCATCTGACCCTGACTTGTA	<i>AtNAS3</i>
	reverse	CGTCTGTTGGATGGTGGATAG	
<i>NtTubulin</i>	forward	ATTTGTTGACTGGTGCCCAAC	<i>AtTubulin</i>
	reverse	TCTTCATCGTCAACTTCAGCA	
<i>NtEF_a</i>	forward	TTGGAAATGGATATGCTCCAG	<i>AtEF1</i>
	reverse	CACCAACAGCAACAGTTTGAC	

* in the tobacco genome only 1 copy for each gene is apparently present

** *Brassica napus* homologue

6.2.4 Statistical analysis

Treatment effects and variations between plant parts were analysed by means of one-way analysis of variance and tested for significances between group means by the Bonferroni post hoc test using SPSS version 15. Differences were considered significant if $p < 0.05$.

6.3 Results

6.3.1 Treatment effects on soil properties

The pertinent changes in soil parameters that were found in response to the sulphur treatment are shown in Table 6-3. The acidity generated by the oxidation of the applied sulphur markedly decreased the soil pH from 7.7 to 6.5. Buffering of the acidity by dissolved carbonate led to a reduction of the calcium carbonate content from 2.7 to 1.4%. The acidification of the soil caused soluble Zn to increase by an order of magnitude from 81 to 614 $\mu\text{g kg}^{-1}$, while soluble Cd was doubled from 1.67 to 3.49 $\mu\text{g kg}^{-1}$. In addition, soluble Fe

Table 6-3: Total and soluble concentrations of selected metals, total S concentration, pH and CaCO₃ content of treated and control top soil samples (0–20 cm). Standard errors are in parentheses. Asterisks indicate significant differences between the treatments.

Property	Method ¹⁾	Unit	Control	Sulphur treatment
Cd conc.	HNO ₃	mg kg ⁻¹	1.444 (0.095)	1.366 (0.121)
	NaNO ₃	$\mu\text{g kg}^{-1}$	1.673 (0.439)	3.493 (0.516) *
Cu conc.	HNO ₃	mg kg ⁻¹	588 (27)	593 (30)
	EDTA	mg kg ⁻¹	284 (13)	280 (14)
Fe conc.	HNO ₃	g kg ⁻¹	20.42 (0.11)	20.19 (0.15)
	EDTA	g kg ⁻¹	0.74 (0.00)	1.00 (0.00) *
Mg conc.	HNO ₃	g kg ⁻¹	2.95 (0.15)	2.68 (0.20)
	EDTA	g kg ⁻¹	0.28 (0.01)	0.29 (0.01)
Mn conc.	HNO ₃	mg kg ⁻¹	999 (57)	953 (70)
	EDTA	mg kg ⁻¹	131 (5)	195 (5) *
S conc.	XRF	g kg ⁻¹	3.13 (0.08)	7.13 (0.34) *
Zn conc.	HNO ₃	mg kg ⁻¹	683 (41)	645 (55)
	NaNO ₃	$\mu\text{g kg}^{-1}$	81 (14)	614 (88) *
pH	H ₂ O		7.71 (0.03)	6.48 (0.09) *
CaCO ₃ content ²⁾		%	2.72 (0.32)	1.38 (0.14) *
C _{org} content ³⁾		%	11.1 (0.3)	11.0 (1.1)

¹⁾ HNO₃: extraction with boiling 2 M HNO₃

NaNO₃: extraction with 0.1 M NaNO₃

EDTA: extraction with 0.5 M ammonium acetate and 0.03 M EDTA

metal analysis of the extractions: Cd by means of GF AAS (Graphite Furnace Atomic Absorption Spectroscopy), analysis of all other elements by means of ICP OES (Inductively Coupled Plasma Optical Emission Spectrometry)

XRF: determination by means of X-Ray Fluorescence

H₂O: measurement of pH in H₂O at a soil:solution ratio of 1:2.5

²⁾ Determination by means of calcimeter

³⁾ Determination with potassium dichromate method

and Mn slightly increased, whereas there were no significant effects on soluble Cu and Mg. The total S concentration was approximately doubled in the sulphur treated plots.

6.3.2 Treatment effects on element uptake by the plants

The sulphur treatment had no visible effect on plant phenotypes (Figure 6-1) and did not affect the biomass production (data not shown). The plants showed no signs of toxicity and grew well in both treatments.



Figure 6-1: Phenotypes of tobacco plants in the control treatment (a), and in the sulphur treatment (b).

The mean leaf Cd concentration increased from $1.42 \pm 0.09 \text{ mg kg}^{-1}$ in the controls to $2.30 \pm 0.13 \text{ mg kg}^{-1}$ in the sulphur treatments. These values are in the concentration range of tobacco plants grown on uncontaminated land and used for cigarette production (Lugon-Moulin et al., 2004). The sulphur treatment significantly increased leaf Cd concentrations along the entire length of the stem (Figure 6-2a). The concentration was the highest in the bottom leaves but the differences were not significant between the positions. In contrast, Lugon-Moulin et al. (2004) reported clearly higher concentrations in bottom than in top leaves. Root Cd concentrations on the other hand, did not respond to the sulphur treatment in

our field experiment. As a result, the leaf/root Cd concentration ratio increased from 5.5 in the control to 7.2 in the sulphur treatment. This suggests that the sulphur treatment stimulated the xylem loading of Cd. Cellular detoxification in the roots generally includes both compartmentalization of Cd complexes and xylem loading (Clemens et al., 2002).

The plant S concentrations were significantly increased by the increased sulphur supply (Figure 6-2b). However, the ratio between leaf and root S concentrations was not affected. In each treatment, the root S concentration was less than half that of the leaf concentration,

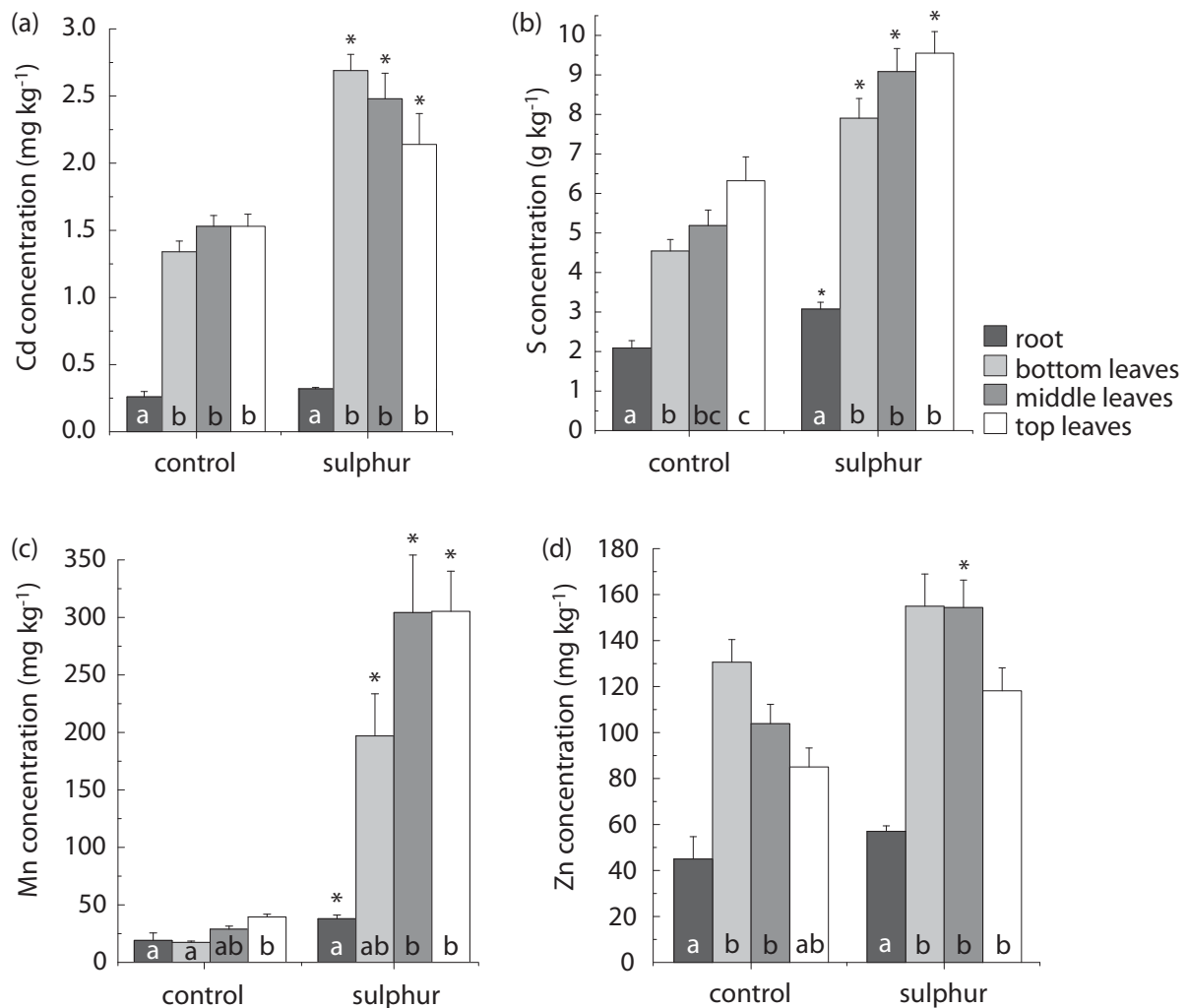


Figure 6-2: Mean concentrations of Cd (a), S (b), Mn (c) and Zn (d) in field grown tobacco leaves and roots in control and sulphur treatments. Letters indicate significant differences between plant parts within a treatment; asterisks indicate significant differences between treatments for the respective plant parts.

indicating that the S taken up by the roots was effectively translocated into the shoots. As in the case of Cd, the increased concentration of EDTA-soluble Mn and NaNO₃-soluble Zn in the S-treated soil (Table 6-3) were associated with enhanced accumulation of these elements in the shoots (Figure 6-2c,d). The increase in Zn accumulation was marginal and significant only in younger leaves. However, leaf Mn concentrations increased by an order of magnitude. In the roots the concentration of this element was doubled by the sulphur treatment, whereas the concentration of Zn was not significantly increased. The sulphur treatment also increased the concentration of Mg from a mean leaf concentration of $4.34 \pm 0.16 \text{ g kg}^{-1}$ to $5.18 \pm 0.08 \text{ g kg}^{-1}$, although no change was observed in the EDTA-extractable Mg concentration of the soil (Table 6-3). The root Mg concentration was 1.25 g kg^{-1} , with and without sulphur treatment. On the other hand, there was no change in Fe accumulation, although EDTA extractable soil Fe concentrations were significantly increased by the S-treatment. For Fe we measured $140 \pm 34 \text{ mg kg}^{-1}$ in the roots and $81 \pm 6 \text{ mg kg}^{-1}$ in the shoots, pooled over both treatments (i.e. with and without sulphur application). In addition, no treatment effects on Cu accumulation were observed. The concentration in roots and shoots were $20.1 \pm 2.6 \text{ mg kg}^{-1}$ and $26.0 \pm 1.3 \text{ mg kg}^{-1}$, respectively, in both treatments.

6.3.3 Gene expression

In the control plants none of the genes involved in Cd transport and detoxification was expressed in the roots (Figure 6-3a). However, most of them were constitutively expressed in the shoots (Figure 6-3b). The sulphur treatment induced the expression of *NtHMA_a*, *NtMRP_a*, *NtATM_a*, and *NtPDR_a* also in the roots. The activation of the ABC transporters and HMA in the roots was likely linked to heavy metal transport and may explain the increased root-to-shoot translocation of Cd that was indicated by the increased shoot/root Cd concentration ratio.

In the leaves, *NtMRP_a* was constitutively expressed in all positions with and without sulphur treatment. Also Bovet et al. (2003) found no changes in shoot *AtMRP3* expression upon addition of Cd to the nutrient solution. In contrast to results obtained with other plants reported in the literature (Table 6-1), *NtATM_a* was down-regulated in the bottom leaves of the field-grown plants in the sulphur treatment. *NtPDR_a* was randomly expressed in the leaves, independently of treatment and leaf age (or position along the stem), suggesting that *NtPDR_a* responded to local stress conditions in the field, e.g. pathogens. Stein et al. (2006) reported that *AtPDR8* transporter in the leaves of *A. thaliana* also play a role in pathogen infection.

regulated in the bottom leaves and partly also in the middle leaves. The observed increases in root gene expression caused by the sulphur treatment agree with findings reported by Herbette et al. (2006) and Weber et al. (2006). As *NtSultr_a* was up-regulated in the roots of our tobacco plants, and Herbette et al. (2006) found a strong up-regulation of *Sultr1;1* expression in *Arabidopsis* exposed to elevated Cd concentrations, while *Sultr1;2* remained unaffected by Cd, *NtSultr_a* seems to be more closely related to *AtSultr1;1* than to *AtSultr1;2*.

The sulphur treatment only marginally induced *NtGSHI* expression in the roots, while there was no expression of root *NtGSHII*, neither in the control, nor in the sulphur treatment. It is possible that the base level of glutathione was either sufficient for Cd detoxification, or glutathione production was stimulated otherwise. May et al. (1998a) and Herbette et al. (2006) related increased *GSHI* activity in response to Cd exposure to post-transcriptional up-regulation of *GSHI*. Both genes were more strongly expressed in younger (top) than in the older (bottom) leaves independent of the treatment. As in the case of most other genes investigated here, the sulphur treatment appears to further down-regulate *NtGSHI* and *NtGSHII* in the older leaves. The *NtNAS_a* gene was not expressed in both root and shoot (data not shown).

6.4 Discussion

In Table 6-4 we compared the expression of Cd-induced genes between tobacco cultivated in sulphur amended soil to expectations based on the published data listed in Table 6-1. The expression of the genes in the roots of our plants mostly agreed with the reported results. Exceptions were *NtLAST*, *NtGSHII* and *NtNAS_a*, which were not expressed at all, and *NtHMA_a* expression which was increased in contrast to findings reported in the literature (Courbot et al., 2007; Mills et al., 2003). The effect of Cd on *HMA4* expression appears to vary in *Arabidopsis* species. Mills et al. (2003) found a decreased expression of *AtHMA4* in *A. thaliana* roots exposed to Cd. They considered the down-regulation of the gene as a positive effect, as it leads to a reduction in the amount of Cd transferred from the roots to the shoot. In contrast, Courbot (2007) found no change in the *AhHMA4* expression in *A. halleri* upon Cd exposure. Verret et al. (2004) showed that *AtHMA4* is located in the plasma membrane and that the *AtHMA4* gene is expressed in tissues surrounding the root vascular vessels. Therefore, they suggested that the *HMA4* transporter may be involved in xylem loading of Cd in *A. thaliana*. A direct relationship

between the increased expression of *NtHMA_a* in the roots and the increased leaf/root Cd concentration ratio and the increased Cd concentration in the leaves is conceivable.

In addition to the higher exposure of the roots to Cd in the sulphur treatment, the increased solubility of other metals, in particular Mn and Zn, may have affected the expression of some genes, for example the *NtHMA_a* gene. The HMA4 transporter belongs to a group of ATPase transporters involved not only in Cd, but also in Co, Pb and Zn detoxification (Mills et al., 2003). Mills et al. (2003) tested the effect of a range of elements

Table 6-4: Summary of the effects of sulphur application on gene expression in roots and shoots of field grown tobacco plants in comparison to expectation based on the literature discussed in the text.

gene	field		expected Cd effect	
	root	shoot	root	shoot
<i>NtHMA_a</i> (<i>AtHMA4</i>)	↑	↓ a	○	→
<i>NtMRP_a</i> (<i>AtMRP3</i>)	↑	→	↑	→
<i>NtATM_a</i> (<i>AtATM3</i>)	↑	↓ a	↑	→
<i>NtPDR_a</i> (<i>AtPDR8</i>)	↑	→	↑	↑
<i>NtSultr_a</i> (<i>AtSultr1</i>)	↑	↓ a	○	○
<i>NtLAST</i> (<i>BnLAST</i>)	■	■	○	○
<i>NtAPR_a</i> (<i>AtAPR2</i>)	↑	↓ a	↑	↑
<i>NtAPR_b</i> (<i>AtAPR3</i>)	↑	↓ a	↑	↑
<i>NtGSHI</i> (<i>AtGSHI</i>)	↑	↓ a	↑	○
<i>NtGSHII</i> (<i>AtGSHII</i>)	■	↓ a	→	○
<i>NtNAS_a</i> (<i>AtNAS3</i>)	■	■	↑	→

legend:

↑ up-regulated

↓ down-regulated

→ unchanged

○ ambiguous

■ not expressed

a: in the older leaves

on the expression of *AtHMA4* and showed an up-regulation in the roots of *A. thaliana* after treatment with Mn and Zn, whereas Cd led to a down-regulation. The increased Mn concentration in the roots as well as the higher solubility of Zn and Mn in the soil after sulphur application may explain the up-regulation of *NtHMA_a* in our field-grown tobacco in spite of the increase in soluble Cd.

The strong up-regulation effects in the roots on genes that are putatively involved in Cd detoxification suggest that the ATPase and the ABC transporters might have been activated by both the increased Cd concentration and the increased availability of sulphur. The influence of sulphur supply on the expression of these genes in plants has received scant attention. Kolukisaoglu et al. (2002) and Glombitza et al. (2004) found that leaf *AtMRP3* was not only inducible by Cd, but also by other stressors such as xenobiotics and oxidative stress. Considering that an increased S supply would enhance glutathione production and that glutathione is involved in the reduction of oxidative stress in plants (May et al., 1998b), the Cd effect on *NtMRP_a* may have been partially enhanced by the increase in soil S concentration in the sulphur treatments.

In the leaves, however, most of the genes studied here were either not affected or then down-regulated, as in the case of the bottom leaves, while up-regulation or no effects were expected according to the literature. The down-regulation of the genes in the bottom leaves in our plants may have been due to a senescence effect. In other studies the experimental plants were harvested well before maturity and not divided into bottom, middle and top leaves. The bottom leaves of our plants showed first signs of senescence at harvest. Down-regulation has been reported for many genes in senescent leaves (Quirino et al., 2000). Down-regulation of genes involved in sulphur uptake and assimilation might also result from changes in sulphur supply. Many genes involved in the acquisition and assimilation of sulphate from soil are known to respond to the S status of a plant (Hawkesford, 2000; Leustek and Saito, 1999; Saito, 2000). Sulphur starvation has been found to activate sulphur acquisition and assimilation mechanisms in the same way as the exposure of plants to Cd (Herbette et al., 2006; Yoshimoto et al., 2002). *AtSultr1;1* is strongly up-regulated in roots and shoots when the availability of sulphate is insufficient. In addition, the expression of *AtAPR* was found to be up-regulated in the roots and shoots of *A. thaliana* under sulphur deficiency (Hirai et al., 2003; Takahashi et al., 1997). Sun et al. (2007) found that *BnLAST* was up-regulated in the shoots of *B. napus* under sulphur deficiency. Little is known about the effects of excessive sulphur supply, a condition that was more likely the case in our field experiment than sulphur deficiency, on gene expression. An excess of SO_4^{2-} in nutrient solution was found to decrease the activity of APS sulphurylase (Brunold et al., 1987). This

enzyme was recently found to be identical with APR (Suter et al., 2000). Thus, it is plausible that the expression of *NtSultr_a*, *NtAPR_a* and *NtAPR_b* was down-regulated in the bottom leaves of the field-grown tobacco plants in response to an over-supply of sulphate.

The expression of the genes that were under study here (along with *NtHMA4*, see above) may also have been affected by the increase in the solubility of Fe, Mg, Mn, and Zn. While e.g. Xiang and Oliver (1998) and Kim et al. (2006) found no effects of Zn on the expression of *AtGSHI*, *AtGSHII* and *AtATM3* in *A. thaliana*, Bovet et al. (2003) found a slight up-regulation effect of Zn on *AtMPR3* expression. Similar to Cd, Zn is known to up-regulate the expression of *Sultr1;1* in roots (Ernst et al., 2008). Zinc may thus also have been involved in the up-regulation of these genes in the S-treatment of our field experiment. Magnesium and Fe effects on the expression of the investigated genes have not been reported. The detoxification of cells through ABC transporters is mediated by MgATP-energized GS-conjugate transport (Rea, 1999, 2007) but this does not necessarily indicate that there are changes in the gene expression when the soluble Mg concentration in the soil changes. However, the increase in plant Fe was not significant, and the increases in plant Zn and Mg were not significant in all leave fractions, either. There are no reports of the effects of Mn on the expression of the studied genes other than *HMA4*. Manganese was the element of which the accumulation was increased most by the sulphur treatment.

The decrease in soil pH itself might have an effect. Changes in rhizosphere pH can affect a wide range of proteins such as ABC-transporters (Reeve et al., 2004) or enzymes of bacteria and plants involved in sulphur assimilation (APR) (Setya et al., 1996). Sulphate transporters are pH-dependent (Saito, 2004), and glutathione production in *Escherichia coli* bacteria was found to decrease with external pH (Smirnova et al., 2003). The impacts of changes in soil pH on gene expression have so far not been reported for the genes that were studied here.

6.5 Conclusion

The up-regulation observed in the expression of most of the investigated genes in the roots agreed well with findings reported from hydroponic or agar experiments in the literature. However, there were also a few notable disagreements, in particular the up-regulation of the *NtHMA_a* in the roots or the down-regulation of most of the investigated genes in the older leaves. These findings indicate that factors other than increased exposure to Cd may have played an important role, in particular the higher concentrations of S and

soluble Mn, Fe and Zn in the soil, and the lower soil pH. The leaves investigated here were obtained from mature plants, while hydroponically or agar grown plants are usually harvested at a very young stage. Nevertheless, our results indicate that hydroponic or agar experiments are useful predictors of gene expression effects that may be expected in field conditions. In order to become more comparable to field situations, future laboratory studies on the expression of genes expected to respond to environmental stresses should also look into the effects of combinations of different stress factors and investigate such effects also at later developmental stages.

6.6 Acknowledgements

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6.7 References

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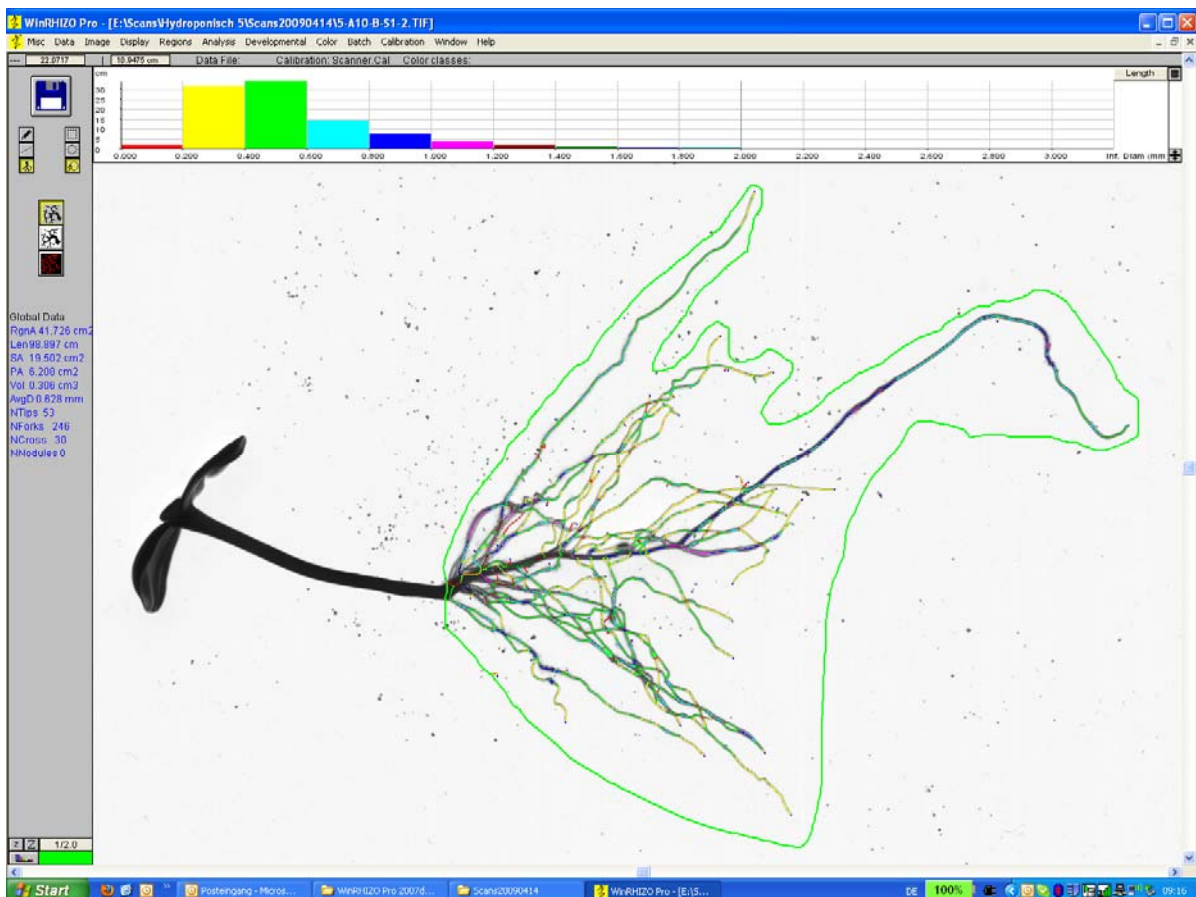
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7

Effect of auxin and EDDS on sunflower growth and heavy metal uptake

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Abstract

The aim of this study was to increase metal phytoextraction by addition of the biodegradable chelating agent EDDS and to alleviate toxic metal effects by the growth-promoting phytohormone IAA. We investigated the effects of combined auxin (indole-3-acetic acid, IAA) and EDDS (ethylene diamine disuccinic acid) applications on growth, mineral nutrition and metal accumulation of heavy-metal stressed sunflowers (*Helianthus annuus*, cv. Sanluca) grown in nutrient solution and pot experiments.

Three sets of experiments were performed. At first we tested five different auxin concentrations (0, 10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} M) applied in combination with Pb (2.5 μ M) or Zn (15 μ M) to evaluate the most efficient auxin concentration on root and shoot growth of metal stressed sunflowers grown in nutrient solutions. Root and shoot growth was most effectively increased in both metal treatments by auxin application at a concentration of 10^{-10} M. Auxin also significantly increased Zn extraction at all application rates, whereas it had no effect on Pb extraction. In the second set of experiments we applied combinations of auxin (10^{-10} M vs. 0 M) and EDDS (500 μ M vs. 0 μ M) treatments (i.e. 8 combinations) to sunflowers grown in nutrient solutions with either Pb or Zn. Auxin increased growth in Pb, but not in Zn treatments. EDDS significantly decreased metal uptake by the plants. The lead concentrations of the EDDS-treated plants were even below the detection limit. Again, auxin significantly increased Zn uptake in EDDS treatments. In a third experiment we grew sunflowers in pots filled with metal-contaminated soil (1.37 mg kg⁻¹ Cd, 685 mg kg⁻¹ Zn, 535 mg kg⁻¹ Cu, 630 mg kg⁻¹ Pb). The soil was treated again with various combinations of EDDS (0, 0.25, 0.5 and 1 mmol kg⁻¹), and auxin (0, 10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} and 10^{-8} mol kg⁻¹). EDDS increased the solubility of Zn and Cu in the soil and also the Cu concentration of the shoots, whereas Zn and Cd accumulation by the shoots was decreased. Auxin only caused minor effects.

The results of the hydroponic experiments show that auxin indeed may alleviate toxic effects of Pb and Zn on plant root and shoot growth and in the same time increase the phytoextraction potential, also in combination with chelants such as EDDS. Effects obtained in the hydroponics, however, are not easily translated into conditions encountered by plants in pot experiments with soil.

7.1 Introduction

Phytoextraction potentially is an attractive strategy to clean up metal-polluted agricultural and other soils, provided that sufficiently high extraction rates can be achieved. In order to enhance phytoextraction it has been proposed to solubilise the polluting metals in soils by adding chelating agents to the soil and thus to increase their availability for uptake by plants. Tandy et al. (2006a) showed that the application of the biodegradable chelant EDDS (ethylene diamine disuccinic acid) to soil increased the uptake of essential and non-essential metals by sunflowers in pot experiments. The addition of EDDS, however, decreased plant dry weight. The addition of the growth promoting phytohormone auxin has been proposed to counteract negative effects from chelating agents as well as from heavy metal stress (Israr and Sahi, 2008; Liphadzi et al., 2006; Liu et al., 2007).

The uptake of water and solutes by plant roots strongly depends on total root surface, length and branching pattern. There is a gradient in the uptake of water along the root axis, declining from the apex to the basal zones, which also affects nutrient uptake. This decline in water uptake in the basal zones is caused by the formation of suberin in the rhizodermis, the exodermis and the secondary and tertiary endodermis. Suberin acts as an efficient barrier against the uptake of unessential elements via the apoplast. These barriers are temporarily disrupted in the basal zones where lateral roots penetrate the cortex. Water and element uptake may increase again in these zones (Marschner, 1995). Auxin is known to induce root growth by enhancing cell division, cell extension and inducing lateral root growth (Taiz and Zeiger, 2000), and may thus increase the uptake of water and solutes over the increased root surface. Rhizosphere bacteria, such as some strains of *Pseudomonas* and *Acinetobacter*, were found to produce indole-3-acetic acid (IAA), the most common auxin in plants, and thereby to stimulate root elongation, lateral root production and increase nutrient (P, Fe, Zn) uptake into roots of crop plants (Lippmann et al., 1995). Root inoculation with rhizosphere bacteria strains also had similar effects on root morphology as exogenous IAA application (Lippmann et al., 1995).

Healthy plants in general maintain the endogenous auxin concentration close to a level that maximizes growth. Exogenously applied auxin to healthy plants thus only causes marginal growth-promoting effects. Optimum auxin concentrations of shoots vary between 10^{-6} and 10^{-5} M (Taiz and Zeiger, 2000). Auxin was found to increase root growth when applied at very low concentrations, i.e. between 10^{-12} and 10^{-10} M, whereas higher concentrations inhibit growth (Gaspar et al., 2002; Jagnow et al., 1991). Salisbury and Ross (1992), however, gave a range between 10^{-7} and 10^{-13} M for positive growth effects of auxin.

These endogenous auxin concentrations, however, might be suboptimal for stressed plants. Both, inoculation of plant roots with auxin producing bacteria and the application of IAA to plant roots were found to allow better root and sometimes also shoot growth of plants that were stressed with drought or metals (Israr and Sahi, 2008; Leinhos and Bergmann, 1995; Liu et al., 2007; Sheng and Xia, 2006). The effects of phytohormones depend on many factors such as plant species, affected plant part, developmental stage of the plant, hormone concentration, interaction between the diverse phytohormones as well as environmental conditions (Salisbury and Ross, 1992). It is therefore necessary to determine the effects of a given auxin concentration case by case.

The aim of this study was to increase metal phytoextraction by addition of the biodegradable chelating agent EDDS and to alleviate toxic metal effects by the growth-promoting phytohormone IAA. We therefore carried out hydroponic and pot experiments to determine the auxin concentrations giving the best root and shoot growth of Pb and Zn stressed sunflowers. We furthermore tested the effects of combined auxin and EDDS applications to soil or nutrient solution on plant growth and heavy metal uptake.

7.2 Materials and methods

7.2.1 Hydroponic experiments

In a first step, we carried out screening experiments with different concentrations of Pb (125, 62.5, 31, 15.5, 7.8, 3.9, 2.5 and 1 μM) and Zn (122, 61, 30.5, 15 μM) in nutrient solution to determine at which concentrations the metals stunted plant growth without killing them. The highest concentrations (125 μM P, 122 μM Zn) were chosen on the basis of the experiments of Tandy et al. (2006b), who investigated the effect of EDDS on heavy metal uptake by sunflowers. The screening experiments revealed that our sunflower variety appeared to be more sensitive to Zn and Pb in solution than those used by Tandy et al. (2006b), which may also have been due to the fact that we performed our experiments at an earlier stage of growth. Treatments with 2.5 μM Pb and 15 μM Zn (single element contamination) were found to give the desired results of a light metal stress.

In a next step, we performed two sets of screening experiments (Table 7-1). In the first set we applied auxin concentrations between 10^{-9} and 10^{-12} M or no auxin, with and without metal stress (either 15 μM Zn, 2.5 μM Pb). The experiments were carried out in four replicates each. In the second set we grew seedlings in solutions with Zn, Pb or no metal,

and applied auxin and EDDS in various combinations as specified in Table 7-1. These experiments were carried out in five replicates each.

The experiments were carried out in a climate chamber applying a 16 h (22° C) / 7 h (15° C) day/night cycle with 0.5 h transition time between day and night phases. Seeds of *H. annuus* (cv. Sanluca) were germinated in silica sand. Six days old seedlings were transferred into aerated brown 1 l bottles containing nutrient solution (Figure 7-1). Two different nutrient solutions were prepared. The first was a modified 10% Hoagland nutrient solution in which NaFe(II)EDTA was replaced by FeSO₄·7 H₂O to avoid interference between EDTA and EDDS. The other solution was the treatment solution (Table 7-1). Here, all micronutrients and KH₂PO₄ were omitted to avoid competition between metals and precipitation of Pb phosphate. 2000 μM MES (pH buffer) was added to maintain a solution pH of 6. The two solutions were freshly prepared and exchanged alternately every three days, starting with the Hoagland solution and ending after 18 days of treatment, so that every solution was applied three times during the experiment. This alternation allowed maximizing the treatment time while avoiding P and micronutrient deficiency stress.

Table 7-1: Treatment solutions of the hydroponic experiments with different auxin concentrations, with and without Zn, Pb and EDDS.

Set	Treatment	Auxin (M)	Metal (μM)	EDDS (μM)	Combinations #
1	Auxin/Zn	0; 10 ⁻¹² ; 10 ⁻¹¹ ; 10 ⁻¹⁰ ; 10 ⁻⁹	0; 15	0	10
	Auxin/Pb	0; 10 ⁻¹² ; 10 ⁻¹¹ ; 10 ⁻¹⁰ ; 10 ⁻⁹	0; 2.5	0	10
2	Auxin/Zn/EDDS	0; 10 ⁻¹⁰	0; 15	0; 500	8
	Auxin/Pb/EDDS	0; 10 ⁻¹⁰	0; 2.5	0; 500	8

At harvest, plants were separated into root and shoot and weighed to determine wet masses. After examination of root growth using Winrhizo (see chapter 7.2.3), the samples were dried at 60 °C for 3 days and weighed again to determine dry mass.



Figure 7-1: Sunflowers growing in hydroponic solution.

7.2.2 Pot experiments

Pots were filled with heavy metal contaminated soil ($1.37 \text{ mg kg}^{-1} \text{ Cd}$, $685 \text{ mg kg}^{-1} \text{ Zn}$, $535 \text{ mg kg}^{-1} \text{ Cu}$, $630 \text{ mg kg}^{-1} \text{ Pb}$) from the field experiment at Witzwil (described in Chapters 3 and 5). After field sampling, the soil was oven-dried at $40 \text{ }^\circ\text{C}$ until constant weight was reached, and then sieved to 1 cm grain size. Portions of 500 g soil were filled into 1 l pots and moistened with 330 g deionised water (this water content gave the best growth according to a preliminary experiment). The watered pots were kept in the climate chamber mentioned above for about 5 days. Thereafter the soil was loosened and the water content adjusted to 280 g per pot. Three seeds were planted per pot, and $0.13 \text{ g kg}^{-1} \text{ N}$, $0.164 \text{ g kg}^{-1} \text{ P}$, $0.13 \text{ g kg}^{-1} \text{ K}$ and $0.042 \text{ g kg}^{-1} \text{ Mg}$ were added to the soil of each pot in the form of a 50 ml nutrient solution.

The water content was re-adjusted to 280 g per pot every third day, to reach the required amount of 330 g after addition of the 50 ml treatment solution (Table 7-2). Six days after sowing the seedling density was thinned to one plant per pot and applications of auxin were started. Auxin was applied at six different solution concentrations every third day from day 6 on. EDDS was given only once (on day 9). The treatment solutions were added in portions

of 50 ml per pot. The applied auxin concentrations were 0, 10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} and 10^{-8} mol kg⁻¹ dry soil and EDDS was applied at 0, 0.25, 0.5 and 1 mmol kg⁻¹. This led to a total of 24 different treatment combinations (no. of replicates see Table 7-3) which were once analysed separately and once pooled, considering only the different auxin or EDDS concentrations, respectively.

Table 7-2: Treatment of the pots after sowing.

Day	Treatment
0	Water content adjustment to 280 g water pot ⁻¹ , sowing, 50 ml fertilizer solution
3	Water content adjustment to 330 g water pot ⁻¹
6	Plant thinning, adjustment to 280 g water pot ⁻¹ , 50 ml auxin treatment
9	Water content adjustment (280 g), 50 ml EDDS and auxin treatment
12	Water content adjustment (280 g), 50 ml auxin treatment
15	Water content adjustment (280 g), 50 ml auxin treatment
18	Water content adjustment (280 g), 50 ml auxin treatment
21	Water content adjustment (280 g), 50 ml auxin treatment
24	Harvest

In order to distribute the time-consuming harvest work over time, plants were sown with a shift of three days between subsequent runs. In each run, 12 different treatments with one pot each were performed (Table 7-3), leading to a total of 8 runs with 12 pots. All plants that were treated in the same way (in different runs) were considered as replicates (3 to 5 replicates, see Table 7-3). The application of 1 mmol EDDS kg⁻¹ soil were stopped after performing three experimental runs because leaves showed signs of toxicity at the highest EDDS treatment level (Figure 7-2).

After 24 days of growth the plants were harvested. The soil was gently washed from the roots, while the plants were kept in water for the analysis of root parameters using Winrhizo (see chapter 7.2.3). The root washing water was collected together with the entire soil of each pot and used for the extraction of soluble soil metals after adjusting the concentration of NaNO₃ to 0.1 M (ART and ACW, 2007). The > 2 mm fraction of each pot was removed by sieving and the remaining fine earth shaken for 2 hours after addition of extractant solution at a ratio of 2.5 l kg⁻¹ dry soil. Sampling the entire amount of soil of each pot allowed retrieving the complete root system of each plant and in the same time obtaining

representative soil samples for the extraction of soluble metals and for the measurement of soil pH. Roots and shoots were separated and washed with deionised water. After weighing to obtain the wet weight the plant samples were oven dried at 60 °C for 3 days and then weighed again to obtain the dry weight.

Table 7-3: Treatments applied in the 8 experimental runs. Light grey fields represent one pot (control (C) and EDDS treatments without auxin); dark grey fields represent one pot per auxin treatment (10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} and 10^{-8} mol kg⁻¹ = 5 pots), leading to a total of 12 pots per run.

Treatment	C	Auxin	EDDS			Auxin + EDDS		
Auxin (mol kg ⁻¹)	0	10^{-12} to 10^{-8}	0	0	0	10^{-12} to 10^{-8}	10^{-12} to 10^{-8}	10^{-12} to 10^{-8}
EDDS (mmol kg ⁻¹)	0	0	0.25	0.5	1.0	0.25	0.5	1.0
Run 1								
Run 2								
Run 3								
Run 4								
Run 5								
Run 6								
Run 7								
Run 8								
*	5	5	4	4	3	4	4	3

* Number of replicates per treatment and auxin concentration.



Figure 7-2: Sunflowers with signs of toxicity after soil treatment with 1 mmol EDDS kg⁻¹ soil.

7.2.3 Root growth

All roots were scanned with a desktop scanner (Epson expression 10000XL, Epson, Japan) that was equipped with water trays receiving the roots and a positioning system for the trays. In the hydroponics experiment plant roots were scanned before they were transferred into the bottles and again at harvest. The scans at the beginning of the experiment were used to check if the plants used in the different treatments were comparable. Root morphology parameters (length, volume and surface area) were determined by means of the image analysis software WinRhizo (version Pro 2007d, Régents Instruments, Canada). As the root systems were quite large, they were divided into several portions that were scanned separately. Total root length, volume and surface area were determined by summing up the individual scans for a given plant. The available version of WinRhizo was not able to distinguish between root branching and root crossing. Therefore, root branching was not analyzed.

7.2.4 Metal analysis of plant and soil samples

Samples of about 0.25 g of each plant (or less in cases where this amount of material was not available) were microwave-digested in 6 ml HNO₃ (65%), 2 ml H₂O₂ (30%), and 2 ml H₂O for metal analysis. The digested samples were filtered and diluted by adding Millipore water until an extract volume of 25 ml was obtained. The elements Ca, Cu, Fe, K, Mg, P and Zn were analysed by means of ICP OES (Varian, Vista MPX). Lead was analyzed by means of ICP OES or, if concentrations were below the ICP OES detection limits, by means of stripping voltametry using a mercury anode (Metrohm 797 VA Computrace). Cadmium was analysed by means of GF AAS (Varian GTA120/AA240Z).

7.2.5 Statistics

Treatment differences were determined by analysis of variance followed by Fisher's least significant difference post hoc analysis. The data were log-transformed in order to normalize frequency distributions. Differences were judged significant if the error probability was 5% or less ($p < 0.05$).

7.3 Results

7.3.1 Hydroponics experiments (set 1)

Figure 7-3 shows the results of the experiments “set 1” (see Table 7-1). None of the auxin concentrations had a significant effect on plant dry weight when added alone (data not shown). Without addition of auxin, the application of 15 μM Zn decreased plant dry weight almost by half in comparison to the control without additional Zn (Figure 7-3a). When auxin was added in combination with Zn, shoot growth was still significantly decreased but much less than in the treatments with Zn alone, while the effect on root growth was no longer significant. The application of 2.5 μM Pb led to similar but smaller effects as Zn. Auxin again reduced or at least tended to reduce the toxic metal effect. The addition of 10^{-10} M auxin was so effective in compensating the toxic Pb effect that it became insignificant in the shoots (Figure 7-3b), whereas the other auxin concentrations had no significant growth-promoting effect. The concentration of 10^{-10} M auxin was thus chosen for the experiments with EDDS (set 2, see below) because it was effective in alleviating toxicity for both metals.

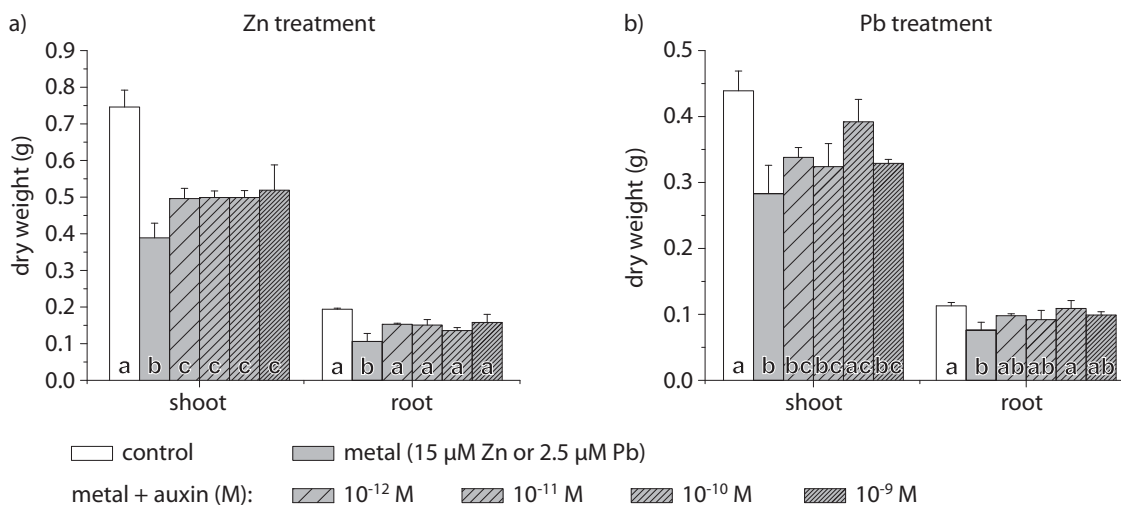


Figure 7-3: Shoot and root dry weight of sunflowers grown in nutrient solution to which Zn (a) or Pb (b) were added with and without auxin. “ 10^{-12} M” to “ 10^{-9} M” represent the applied auxin concentrations in combination with the respective metal. Error bars give the standard errors of the means. Different letters indicate significant differences between treatments ($p < 0.05$).

We expected that auxin would increase the ratio between root surface area (or root volume) and root dry matter, as it is known to cause cell elongation and cell wall loosening.

Root volume closely correlated ($R \approx 1$) with root surface area for both treatments (data not shown). Therefore we chose root surface area as parameter. In the experiment where plants were treated with different auxin concentrations in presence and absence of Zn, the treatments with 10^{-10} and 10^{-11} M auxin exhibited a significantly higher ratio between root surface area and root dry weight than in the controls without auxin (Figure 7-4a). In the respective experiment with Pb the increase was only significant for 10^{-10} M auxin (Figure 7-4b). The highest auxin concentration (10^{-9} M) showed the smallest effect with both metals.

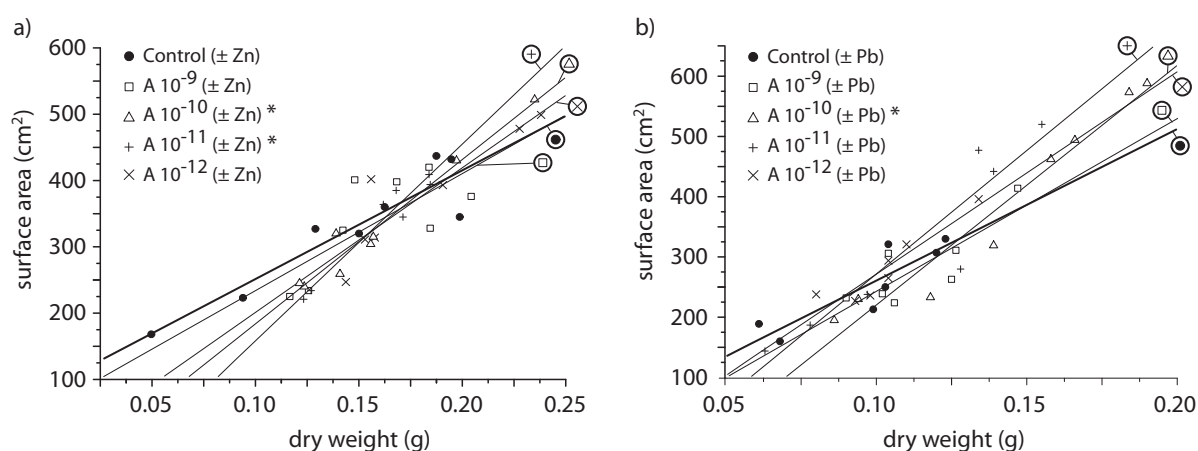


Figure 7-4: Regression lines between root surface area and root dry weight of hydroponically grown sunflowers treated with different auxin concentrations in presence and absence of Zn (a) or Pb (b). The bold lines represent the regression line for the control plants. Asterisks in the legend indicate that the difference between auxin-treated and control plants was significant (different slopes of the regression lines).

In contrast to the treatment effects on growth, there was no difference between the Zn concentration of the plants exposed to Zn alone and those exposed to auxin and Zn in combination (Figure 7-5a). There was also no difference between the control and auxin only (without metals) treated plants (data not shown). The shoot Pb concentration of plants exposed to Pb in combination with 10^{-12} M auxin was lower than for the respective Pb treatment without auxin (Figure 7-5b). All other auxin concentrations had no significant effect on Pb accumulation in the shoots, although there was a trend for the lower auxin concentrations to decrease Pb accumulation. Auxin also decreased root Pb concentrations. This effect was strongest at the two highest auxin concentrations, while it was not significant at 10^{-11} M auxin. This pattern in shoot and root Pb concentrations led to an increased root-to-shoot translocation factor of Pb with increasing auxin concentrations. The highest value of

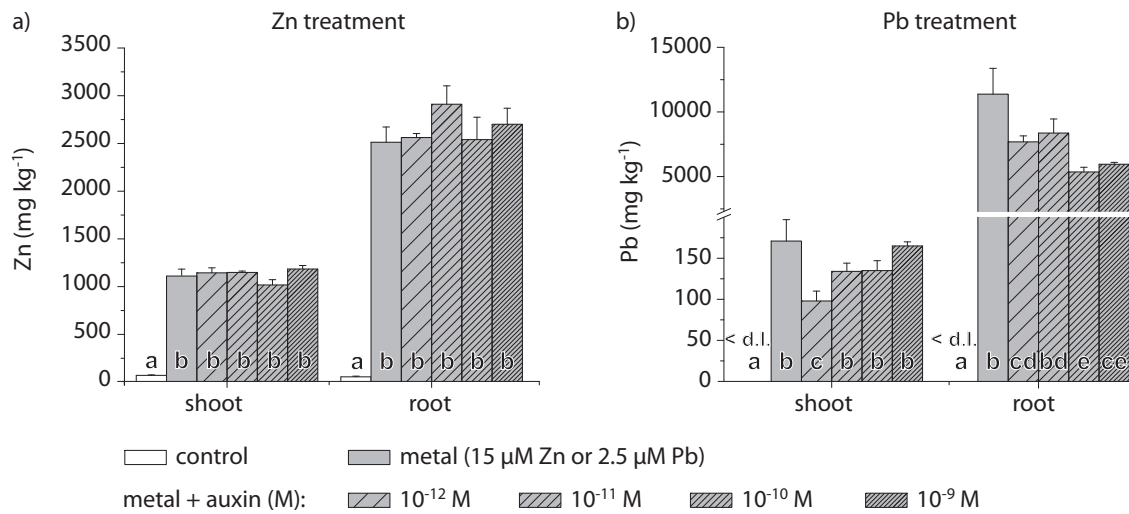


Figure 7-5: Zn and Pb concentrations of sunflower plants grown in nutrient solutions to which (a) Zn and (b) Pb were added with and without auxin. “10⁻¹² M” to “10⁻⁹ M” represent the applied auxin concentrations in combination with the respective metal. Error bars give standard errors of the means. Different letters indicate significant differences between treatments ($p < 0.05$).

this factor (0.028) was found for plants treated with 10⁻⁹ M auxin. It decreased to 0.025 (10⁻¹⁰ M), 0.016 (10⁻¹¹ M) and finally to 0.013 for plants treated with 10⁻¹² M auxin. For plants exposed to Pb without auxin addition the factor was 0.015.

We found no beneficial effects of auxin on the uptake of nutrients into the above-ground biomass (shoots of Zn and Pb treated plants as well as shoots treated without heavy metal application, data not shown). In the Pb experiment we even measured decreasing concentrations of several nutrients (Ca, Cu, K, Mg, P and Zn) in the roots with increasing auxin concentrations in the nutrient solution. The same tendency also was found for Fe (Table 7-4).

Not only auxin, but also the metals themselves were found to affect nutrient concentrations. The application of Pb reduced shoot Fe and Zn concentrations, while it increased the concentrations of Fe and Zn in the roots (data not shown). This means that the application of Pb reduced the root-to-shoot translocation of these two metals. The root-to-shoot translocation factor decreased from 0.016 to 0.008 for Fe and from 1.245 to 0.468 for Zn. Also the Zn treatment (experiment Auxin/Zn) reduced the root-to-shoot translocation factor for Fe (from 0.036 to 0.011).

Table 7-4: Mean nutrient concentrations (Ca, Cu, Fe, K, Mg, P and Zn) in the roots of sunflower plants grown in nutrient solutions to which Pb and different concentrations of auxin (10^{-9} to 10^{-12} M) were added. Standard errors of the means are in parentheses. Different letters indicate significant differences between treatments ($p < 0.05$).

Nutrient	Pb + 10^{-9} M	Pb + 10^{-10} M	Pb + 10^{-11} M	Pb + 10^{-12} M	Pb only
Ca (g kg ⁻¹)	3.9 (0.1) ^a	4.3 (0.3) ^a	4.3 (0.1) ^{ab}	4.8 (0.2) ^{bc}	5.5 (0.6) ^c
Cu (mg kg ⁻¹)	91 (12) ^a	132 (22) ^a	133 (23) ^a	149 (8) ^a	157 (23) ^a
Fe (g kg ⁻¹)	4.4 (0.4) ^a	5.0 (0.9) ^a	6.3 (1.0) ^a	5.7 (0.7) ^a	10.0 (0.6) ^b
K (g kg ⁻¹)	80 (5) ^a	84 (2) ^{ab}	88 (2) ^{ab}	98 (2) ^{bc}	105 (4) ^c
Mg (g kg ⁻¹)	1.6 (0.1) ^a	1.7 (0.0) ^{ab}	1.7 (0.0) ^{ab}	1.8 (0.0) ^b	1.9 (0.0) ^b
P (g kg ⁻¹)	5.0 (0.1) ^a	6.0 (0.3) ^{ab}	5.8 (0.2) ^{ab}	6.7 (0.5) ^{ab}	7.4 (0.6) ^b
Zn (mg kg ⁻¹)	82 (4) ^a	103 (9) ^{ab}	107 (8) ^{ab}	124 (9) ^{bc}	157 (23) ^b

7.3.2 Hydroponics experiments (set 2)

In the experiments of set 2, in contrast to set 1 (see above), Zn toxicity was not alleviated by the addition of auxin (Figure 7-6a). Not only Zn, but also auxin significantly decreased plant growth compared to the control treatment, added alone as well as in combination with Zn. As this negative auxin effect was not found in the other hydroponics experiments, we suspect that also some other factor may have exerted stress on the plants in this experiment. Excess of auxin is known to decrease root growth, and the range between beneficial and toxic effects is quite narrow. Thus, little additional stress may tip the balance (Salisbury and Ross, 1992).

The application of EDDS had a positive effect on plant growth in both experiments. All EDDS-treated plants looked much healthier, and shoot as well as root weights were significantly larger in the EDDS treated plants than in the plants without EDDS application (Table 7-5). The main reason for that was probably a reduction in Pb and Zn toxicity due to formations of metal complexes with the chelating agent EDDS. Alleviation of metal toxicity through the addition of EDDS was also found in Cu-stressed hydroponically grown *Chrysanthemum coronarium* L. by Wei et al. (2007). The EDDS applications also increased the ratio between root surface area and root dry weight (Figure 7-7), a similar effect as was observed in the treatment with 10^{-10} M auxin in the first set of experiments (Figure 7-4).

EDDS decreased the Zn concentration in plants by about a factor of 9 (Figure 7-8). A similar effect was also found by Tandy et al. (2006b). It can again be attributed to the formation of EDDS complexes with Zn, reducing its uptake by the plants. The addition of

auxin to EDDS treated plants significantly increased the Zn concentration (from $81 \pm 2 \text{ mg kg}^{-1}$ to $117 \pm 4 \text{ mg kg}^{-1}$). In other words, the combination of auxin with EDDS was more effective for Zn extraction than the application of EDDS alone.

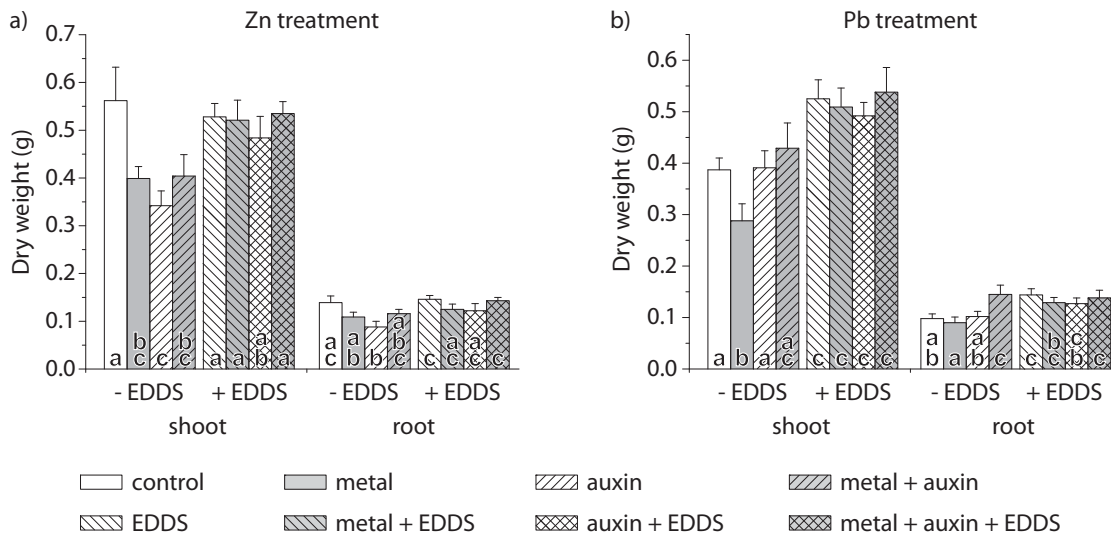


Figure 7-6: Dry weight of sunflower plants grown in nutrient solutions to which (a) Zn and (b) Pb was added, with and without auxin (10^{-10} M) and EDDS. Error bars show the standard errors of the means. Different letters indicate significant differences between the treatments ($p < 0.05$).

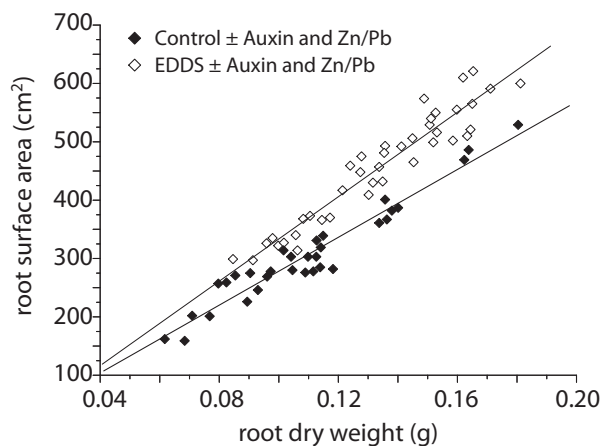


Figure 7-7: Regression lines between root surface area and root dry weight of sunflower plants grown in nutrient solutions to which EDDS was applied or not, with and without auxin, Zn and Pb (pooled samples). The slopes of the two regression lines are significantly different.

In contrast to the screening experiment of the first treatment set (Figure 7-5), auxin increased the Pb concentration of the shoots from 117 ± 5 to 147 ± 5 in the second treatment set (data not shown). In the roots, however, in agreement with the results of set 1, Pb concentrations were around 40% lower in the Pb+auxin treatment (5.0 g kg^{-1}) than in the treatment with Pb alone (8.2 g kg^{-1}). This suggests that plant growth was limited by Pb toxicity as Pb and auxin treated plants grew better than Pb only treated (Figure 7-6). EDDS significantly decreased root and shoot Pb concentrations, in the shoots even to values below the detection limit. The roots of EDDS treated plants contained 8.4 ± 0.7 and $8.3 \pm 0.1 \text{ mg kg}^{-1}$ Pb when treated with and without auxin, respectively (data not shown).

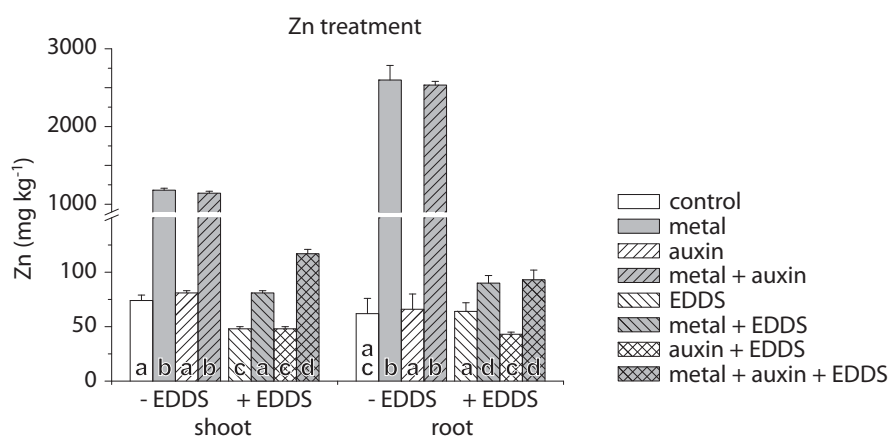


Figure 7-8: Shoot and root Zn concentrations of sunflower plants grown in nutrient solutions to which Zn was added, with and without auxin (10^{-10} M) and EDDS. Error bars show the standard errors of the means. Different letters indicate significant differences between the treatments ($p < 0.05$).

The application of EDDS significantly reduced the concentrations of Cu and Fe in the plant roots in both experiments of “set 2”. In experiment “Auxin/Pb/EDDS”, also Zn was significantly decreased by EDDS (Table 7-5). For Cu and Zn these effects were associated also with reduced accumulation in the shoots, whereas the concentration of Fe in the shoots was highly increased. As shoot Fe concentration positively correlated with dry weight, Fe deficiency would be an additional explanation for the lower shoot dry weight of the plants treated without EDDS. The modification of the Hoagland nutrient solution ($\text{FeSO}_4 \cdot 7 \text{ H}_2\text{O}$ instead of NaFe(II)EDTA) may have had a negative impact on the growth of the sunflower variety used here (Sanluca). Tandy et al. (2006b) did not find such effects in their EDDS experiments, although they used the same nutrient solutions. One difference was that they used another variety of sunflowers (Iregi).

Table 7-5: Comparison of mean shoot and root dry weights, and Cu, Fe and Zn concentrations of sunflower plants grown in nutrient solutions to which EDDS was added or not, with and without Zn, Pb and auxin (mean values of the pooled data of all plants from the experiments of “set 2” and only the Pb experiment of “set 2”, respectively). Standard errors of the means are in parentheses. Different letters indicate significant differences between the treatments ($p < 0.05$).

Parameter	Experiments	-EDDS		+EDDS	
Root dry weight (g)	set 2 (both)	0.110 (0.005)	^a	0.134 (0.004)	^b
Shoot dry weight (g)	set 2 (both)	0.396 (0.017)	^a	0.516 (0.012)	^b
Root Cu (mg kg ⁻¹)	set 2 (both)	174 (6)	^a	35.4 (2.1)	^b
Shoot Cu (mg kg ⁻¹)	set 2 (both)	21.4 (0.5)	^a	12.6 (0.3)	^b
Root Fe (mg kg ⁻¹)	set 2 (both)	6609 (308)	^a	143 (8)	^b
Shoot Fe (mg kg ⁻¹)	set 2 (both)	75 (8)	^a	119 (6)	^b
Root Zn (mg kg ⁻¹)	set 2 (Auxin/Pb/EDDS)	78.2 (5.2)	^a	37.3 (1)	^b
Shoot Zn (mg kg ⁻¹)	set 2 (Auxin/Pb/EDDS)	68.5 (2.6)	^a	43.6 (1)	^b

7.3.3 Pot experiment

The auxin treatment had no influence on the solubility of Cu and Zn in the soil, regardless of the applied auxin concentration (data not shown). EDDS, on the other hand, significantly increased soluble soil Cu and Zn concentrations, and the solubilisation effect increased with the concentrations of EDDS (Figure 7-9). The concentrations of soluble Cd and Pb were below our detection limits (i.e. < 0.005 mg kg⁻¹ for Cd and < 0.02 mg kg⁻¹ for Pb). The soil pH of 7.1 was not affected by the auxin or EDDS treatments.

None of the 24 different auxin-EDDS treatments had a significant effect on plant growth (data not shown). Irrespective of additional EDDS application, the application of auxin did not affect plant growth, except for the treatment with 10^{-10} mol kg⁻¹ auxin at which shoot but not root dry weight was slightly, but significantly, reduced (Figure 7-10a). Addition of 10^{-9} or 10^{-11} mol kg⁻¹ auxin significantly increased shoot dry weight in comparison to the addition of 10^{-10} mol kg⁻¹ auxin, but not to the control. Thus a toxic effect of the auxin is rather unlikely. A positive growth effect of auxin as found in our hydroponics experiments and in previous pot experiments by Reiner (2008) could not be confirmed.

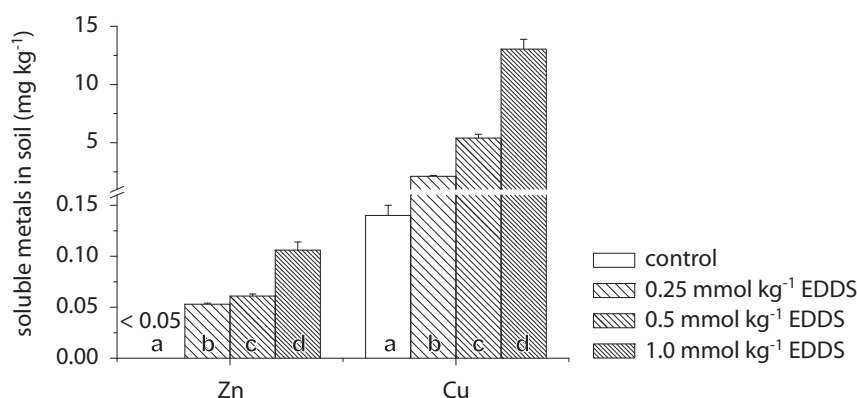


Figure 7-9: Soluble soil Zn and Cu concentrations in pots with sunflowers after treatment with various EDDS concentrations (EDDS alone or in combination with auxin). Error bars show the standard errors of the means. Different letters indicate significant differences between the treatments ($p < 0.05$).

Also EDDS produced only minor effects. While EDDS had increased the growth of metal-exposed plants in the hydroponics experiments, shoot growth was slightly decreased at the two highest EDDS concentrations in the pot experiments (Figure 7-10b). Also root growth was slightly reduced in the treatment with the second-highest EDDS application rate, and there was also tendency of root growth reduction at the highest application rate. Growth reductions due to EDDS applications were also observed by Luo et al. (2005) and Tandy et al. (2006a).

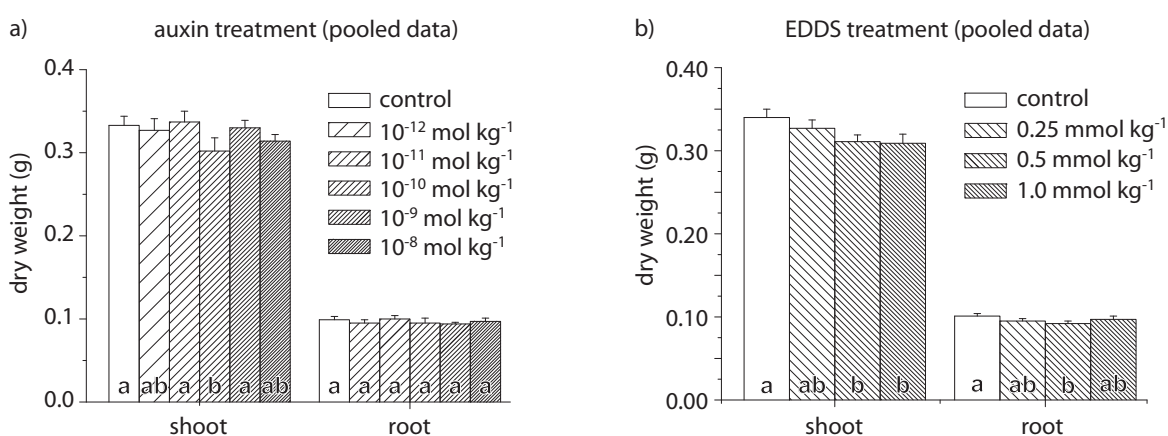


Figure 7-10: Shoot and root dry weight of pooled auxin (a) and EDDS (b) treated sunflowers grown in pots. Error bars show the standard errors of the means. Different letters indicate significant differences between the treatments ($p < 0.05$).

There was also hardly any effect of the 24 treatments on metal uptake by the plants (data not shown). Auxin applications regardless of the EDDS concentrations had only minor effects on metal concentrations in roots and shoots (Table 7-6). The only effects found were a slight increase in root Zn concentration after exposure to 10^{-12} mol kg⁻¹ auxin and a decrease in shoot Cd concentration in plants exposed to 10^{-11} mol kg⁻¹ auxin. The shoot Pb concentrations were all below the detection limit.

Table 7-6: Mean concentrations of Zn, Pb, Cu and Cd in root and shoot of pooled auxin treated sunflowers grown in pots. Standard errors of the means are in parentheses. Asterisks indicate a significant difference ($p < 0.05$) between auxin treated and control (0 mol kg⁻¹ auxin) plants.

Auxin	Zn (mg kg ⁻¹)		Pb (mg kg ⁻¹)		Cu (mg kg ⁻¹)		Cd (mg kg ⁻¹)	
	root	shoot	root	shoot	root	shoot	root	shoot
0 mol kg ⁻¹	166 (7)	109 (2)	74 (5)	< 2	150 (6)	23 (3)	1.5 (0.1)	0.4 (0.0)
10^{-12} mol kg ⁻¹	192 (9)*	106 (4)	80 (5)	< 2	163 (6)	24 (4)	1.8 (0.1)	0.4 (0.0)
10^{-11} mol kg ⁻¹	183 (7)	101 (3)	83 (9)	< 2	154 (6)	26 (4)	1.4 (0.1)	0.3 (0.0)*
10^{-10} mol kg ⁻¹	172 (9)	107	89 (10)	< 2	148 (7)	24 (3)	1.6 (0.1)	0.4 (0.0)
10^{-9} mol kg ⁻¹	164 (9)	109	69 (5)	< 2	148 (7)	26 (5)	1.6 (0.1)	0.4 (0.0)
10^{-8} mol kg ⁻¹	182 (8)	107	73 (4)	< 2	157 (4)	27 (5)	1.5 (0.1)	0.4 (0.0)

Although EDDS increased the solubility of Zn in the soil (Figure 7-9), root and shoot Zn concentrations were either not affected by EDDS or rather slightly reduced (Table 7-7). Similarly, EDDS also showed a tendency to decrease plant Pb and Cd concentrations. Copper accumulation, in contrast, strongly increased with increasing EDDS application rates. The application of 1 mmol kg⁻¹ EDDS increased the accumulation of Cu by the shoots four-fold. Also Tandy et al. (2006a) found that EDDS affected the accumulation of Cu from soil more strongly than the accumulation of Zn, Pb and Cd. However, they found a strong increase in accumulation for all four metals. Similarly, Liphadzi et al. (2006) found that EDTA increased the accumulation of Cu, Zn, Pb and Cd by sunflower, and combined application of auxin further increased this effect. Luo et al. (2005) observed that EDTA was more effective than EDDS in solubilising and increasing plant uptake of Pb and Cd, but still observed a significant treatment effect of EDDS.

Table 7-7: Mean concentrations of Zn, Pb, Cu and Cd in root and shoot of pooled EDDS treated sunflowers grown in pots. Standard errors of the means are in parentheses. Different letters indicate significant differences between the treatments ($p < 0.05$).

EDDS	Zn (mg kg ⁻¹)		Pb (mg kg ⁻¹)		Cu (mg kg ⁻¹)		Cd (mg kg ⁻¹)	
	root	shoot	root	shoot	root	shoot	root	shoot
0	186 (7) ^a	112 (2) ^a	92 (6) ^a	< 2	149 (5) ^a	12 (0) ^a	1.6 (0.1) ^a	0.4 (0.0) ^a
0.25 mmol kg ⁻¹	164 (6) ^b	102 (2) ^b	72 (4) ^b	< 2	146 (4) ^a	18 (0) ^b	1.5 (0.1) ^{ab}	0.4 (0.0) ^a
0.5 mmol kg ⁻¹	175 (7) ^{ab}	102 (2) ^b	68 (3) ^b	< 2	152 (4) ^a	29 (2) ^c	1.7 (0.1) ^a	0.4 (0.0) ^a
1.0 mmol kg ⁻¹	180 (7) ^{ab}	110 (3) ^a	76 (7) ^b	< 2	172 (5) ^b	52 (3) ^d	1.4 (0.1) ^b	0.3 (0.0) ^b

7.4 Discussion

The application of auxin to the nutrient solution, particularly at a concentration of 10^{-10} M, alleviated Pb and Zn toxicity in hydroponically grown sunflowers (Figure 7-3). Growth promoting effects of auxin on Pb-stressed plants was also observed in other studies. Liu et al. (2007) found that 100 μ M IAA in nutrient solution significantly increased root dry matter in *Sedum alfredii* grown in a solution with 200 μ M Pb. Our findings show that auxin can also reduce stress by other metals such as Zn. This is in line with the general role of auxin in stress alleviation. Leinhos and Bergmann (1995) found that auxin also alleviated drought stress and suggested that exogenously applied IAA may serve in mediating morphological reactions of plants in response to stresses, in particular by increasing root growth.

Auxin not only increased shoot and root dry weight of our plants (Figure 7-3), but also root length, volume and surface area, and with that also the ratio between surface area and dry weight (Figure 7-4). Also Liu et al. (2007) found increased root length, surface area and volume of auxin treated Pb stressed *S. alfredii* (see above). According to the “acid growth theory” auxin promotes root growth by enhancing cell enlargement (Taiz and Zeiger, 2000). Lippmann et al. (1995) found that the main effect of auxin on root growth in *Zea mays* was to enlarge the lateral root system by increased branching and less by increasing the size of lateral roots. Also Chaudry and Rasheed (2003) found that 50 mg l⁻¹ IAA increased the number of lateral roots and metaxylem elements in *Pisum sativum* plants exposed to 50 mg l⁻¹ Pb. This agrees with our observations, as the hydroponically grown plants of the present study looked bushier in the auxin treatments than in the controls.

Auxin reduced the concentrations of Pb in the roots of plants exposed to Pb in nutrient solutions (Figure 7-5), while the shoot concentrations remained the same or were slightly

decreased in comparison to the Pb only treated plants. Auxin treated plants were thus probably less stressed and consequently grew better. Also Israr and Sahi (2008) found that the root-to-shoot translocation of Pb increased in hydroponically grown *Sesbania drummondii* treated with increasing IAA concentrations (1, 10, 100 μM). This was not only due to a decrease in root Pb concentration but also to a simultaneous increase in shoot Pb concentration. López et al. (2005), on the other hand, found that root Pb concentrations of hydroponically grown *Medicago sativa* increased with increasing auxin concentrations in the nutrient solution, while there was no trend in the shoots. The effect of auxin on root Pb concentrations may thus be species dependent.

As in some cases the shoot dry matter was increased in the combined treatments with auxin and metals without “diluting” Pb or Zn concentrations (Figure 7-5) also total metal extraction per plant was increased, indicating that auxin has some potential to enhance phytoextraction of polluting metals.

Besides Pb auxin also decreased nutrient concentrations in the roots (Table 7-4). This decrease, however, did not negatively affect root growth. Reiner (2008), on the other hand, found that the concentrations of Ca, Mg and Zn in the roots of metal-stressed agar-grown sunflowers increased when auxin concentrations were increased from 10^{-9} to 10^{-7} M. The plants in the study of Reiner (2008), however, were exposed to multiple metal stresses, whereas the plants in the present study were only stressed with either Pb or Zn alone. López et al. (2007), in contrast, found no effect of auxin concentrations on root Ca, Cu, K and Mg concentrations of *M. sativa* plants exposed to Pb and auxin. It is not evident whether the decreased nutrient concentration in the Pb treated plants of the present study is due to a lower uptake or a lower binding to the root surface. We suspect that some chemical interaction between Pb and auxin hindered the nutrients to bind to the root surface, maybe by building complexes between the positively charged Pb and the negatively charged auxin. Lead in nutrient solution furthermore increased root and decreased shoot Fe and Zn concentrations. Auxin reduced this Pb effect. With Zn no such effects were found.

The application of EDDS substantially decreased Pb and Zn uptake by hydroponically grown plants (Figure 7-8). This is in contrast to the results of Tandy et al. (2006b) who measured a highly increased Pb uptake by plants treated with EDDS and Pb in comparison to plants treated with Pb alone. The combination of EDDS with auxin, on the other hand, slightly increased the Zn concentration in the shoots in comparison to EDDS alone (Figure 7-8), while no such effect was found in the roots. As the Pb concentrations were too low, this effect could not be evaluated in the Pb treated plants. Israr and Sahi (2008), however, found a highly increased Pb uptake into the shoots when EDTA was added in combination with

auxin. This indicates that the combination of chelating agents with auxin may further increase metal uptake by plants.

In contrast to the hydroponics experiments, we found no growth promoting effects of auxin in the pot experiments. Crop yields in the field from which the soil was taken were in the range or even above Swiss average yields for sunflower, maize and tobacco (Fässler et al., 2009), indicating that the elevated metal concentrations had no substantial, if any, negative effect on plant growth. So there was probably not much stress that could have been alleviated by the auxin treatment. Also no combination effect of auxin and EDDS on the uptake of Zn or other metals could be achieved in the pot experiments, even though EDDS increased metal solubility in the soil. This increase was probably still not high enough to cause metal stress to the plants. Liphadzi et al. (2006) grew sunflowers on a moderately and a highly contaminated soil and added IAA. While the biomass of the roots and stems in the moderately contaminated soil was increased, the IAA had no effect on the ones grown in the highly contaminated soil. Too much stress may therefore probably not be alleviated, either.

Another reason for the absence of effect could be that the IAA concentrations applied were too low. Liphadzi et al. (2006) applied IAA solutions with concentrations of 3 and 6 mg l⁻¹ IAA to the soil at a rate of 500 ml per pot (7 kg soil). They furthermore sprayed 500 ml to the upper and lower sides of the leaves. Thus, it is not possible to say anything about the effectiveness of the soil-applied auxin. The bioavailability of phytohormones in soil can be strongly reduced by sorption, in particular to organic matter, and by microbial degradation (Arteca, 1996). Martens and Frankenberger (1993) found that the half-life of IAA in five different soils averaged 37.8 h when soil was treated with of 225 mg IAA kg⁻¹ soil. Thus applications of much higher amounts of IAA to soils are required to produce the same effects as in nutrient solutions.

7.5 Conclusion

The results of the hydroponic experiments show that the addition of auxin (10⁻¹⁰ M) to nutrient solution can alleviate Zn and Pb stress in sunflowers by promoting root growth. Shoot growth was increased without a pronounced, if any, reduction in Pb and Zn accumulation. Phytoextraction efficiency was thus increased, too. The chelant EDDS decreased metal uptake in hydroponically grown plants most likely due to the formation of metal-EDDS complexes, which are less bioavailable than the free metal cations. In the soil the application of EDDS increased the solubility of Zn and Cu. While the Cu concentration

was also increased in the plants, Zn and Cd uptake were decreased. Auxin and/or EDDS applied to soil, on the other hand, showed only minor effects on plant growth and metal uptake. The reason for that may be a higher auxin sorption or microbial degradation in the soil.

The application of auxin to heavy metal stressed plants thus is a suitable method for stress alleviation of plants grown in hydroponic systems. For the applicability to soil systems, however, further investigations are necessary.

7.6 Acknowledgements

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Conclusions

The main goals of this study were (I) to test the applicability of the three metal-solubilising soil amendments ammonium sulphate fertilizer, elemental sulphur and nitrilotriacetic acid (NTA) to field grown maize, sunflower and tobacco and to evaluate their long-term effect on Cd, Cu and Zn uptake by these three plants; (II) to investigate the effect of these three soil amendments on metal allocation within the plants; (III) to investigate the effect of the sulphur treatment on the expression of genes involved in Cd detoxification and sulphur uptake and assimilation in tobacco; and (IV) to test the potential of auxin in alleviating heavy metal stress to plants as well as the effect of the combination of auxin with EDDS on metal uptake by sunflower.

All three treatments were applied in order to increase metal uptake by the cultivated plants. In the case of ammonium sulphate and elemental sulphur it was furthermore expected, that the effect on metal uptake would increase over the years, as soil pH should have decreased over time. However, only the addition of elemental sulphur showed an effect that would make it potentially interesting for phytoextraction purposes. The sulphur treatment decreased soil pH, even more after doubling of the applied amount in the seventh and eighth year, and it increased Cd and Zn uptake by sunflower and tobacco. In the latter cases, metal accumulation was the most increased in the leaves, while the seed metal concentrations remained unchanged or were even decreased. The other two methods failed to enhance phytoextraction. The effects of these methods obtained in previous studies in hydroponics and pot experiments apparently are not easily translated into field situations.

The applied ammonium sulphate treatment was probably not strong enough to decrease the pH of the experimental soil because it was well buffered due to the high contents in CaCO_3 and C_{org} . These two factors were probably also responsible for the slow pH decrease after the application of elemental sulphur in the beginning of the field experiment. The amount of sulphur applied in the elemental sulphur treatment was about 17 times higher than in the ammonium sulphate fertilizer treatment. A higher rate of ammonium sulphate application, however, is not possible for agronomic reasons, because it would lead to N over-fertilization.

The inefficiency of the NTA treatment may have several reasons. It could have been degraded too fast so that the metal-solubilising effect did not last long enough for the plants to accumulate significantly more metals. Another reason may be that metal solubility was increased locally but plant roots avoided the hotspots of high soluble metal concentrations. In pot experiments this possibility usually does not exist because the available volume is confined and the soil is usually homogenized artificially, explaining why pot and field-grown plants often differently respond to soil metal pollution. This is more a problem in the case of NTA than of sulphur, as NTA was applied by local injections when plants had already reached a certain size, because of its fast biodegradability, while sulphur can be ploughed in uniformly before sowing. A third reason may be that NTA is too weak as a chelator for the target metals Cd, Cu and Zn in the Witzwil soil. At least for Cu, EDDS may be better suited to enhance phytoextraction. In the auxin experiments, EDDS increased Cu solubility as well as Cu uptake by sunflowers. But these experiments were performed in pots so that it is not clear if a significant effect would have occurred also in the field at the same application rate.

Corresponding to the increased Cd uptake by tobacco after sulphur application, the sulphur treatment also increased the expression of Cd detoxification related genes in the roots. This increase was expected according to published data from agar and hydroponics experiments, which shows that results from controlled laboratory environments can in some cases be useful predictors of gene expression effects under field conditions. For *NtHMA_a*, however, a decreased expression was expected in the roots, whereas an increase was observed. This may have been due to the increased solubility of Zn and Mn in the soil. These two metals are known to increase *AtHMA4* expression in *Arabidopsis thaliana*. This indicates that not all effects we found were by necessity due to increased Cd solubility. Also other factors such as increased S, Mn, Zn and Fe availability and decreased soil pH may have influenced gene expression. The fact that also gene expression of most of the analyzed genes in the older leaves differed from expectation indicates that there were major differences between laboratory and field conditions influencing gene expression. Another reason for discrepancies between observation and expectation might be the higher age at harvest and the longer exposure of the plants to Cd in the field experiments than in lab experiments.

Auxin was found to alleviate toxic effects of Zn and Pb on the growth of hydroponically grown sunflowers. Auxin furthermore increased Zn uptake in combination with EDDS in comparison to the EDDS treatment alone. Both effects were in agreement with expectation

according to published studies. The effects observed in the pot experiment were not comparable to the ones of the hydroponics experiments.

Our results show that it is not only problematic to translate results from pot experiments to field conditions, but also from hydroponics to pots. They furthermore show that a promising method found in hydroponics experiments needs to be tested for its effectiveness in pot and field experiments. However, it would be premature to say that a method that was unsuccessfully tested at one single field site does not work in general under real-world conditions. The Witzwil soil is quite special due to its high C_{org} content and the high pH. It is therefore not representative for the majority agricultural soils. The proposed amendments should also be tested with other soils and at other locations. To test phytoremediation options proposed for this soil was not unreasonable, however, as such soils are nevertheless quite common in areas where wetland has been drained to gain more agricultural land. Furthermore, the contamination (in particular Cd) of the soil in the study area exists and a solution for its future use still has to be found. For the study site, there are two possible phytomanagement strategies that should be further pursued: (I) the production of crops that do not accumulate elevated amounts of metals in the harvested parts, in particular metal-excluding plants, and (II) the Cd decontamination of the soil with Cd accumulating crops, which would take about 50 years with sulphur addition. In both cases, on-site screening experiments with potential crops should be performed first. Groundwater monitoring for metal leaching is also recommended, in particular in the case of sulphur application.

Curriculum Vitae

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