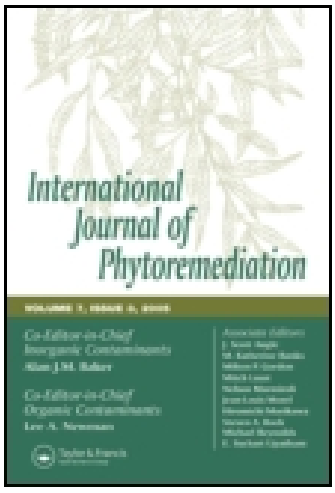

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Phytoextraction of Cadmium and Zinc By *Sedum plumbizincicola* Using Different Nitrogen Fertilizers, a Nitrification Inhibitor and a Urease Inhibitor

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Cadmium (Cd) and zinc (Zn) phytoavailability and their phytoextraction by *Sedum plumbizincicola* using different nitrogen fertilizers, nitrification inhibitor (dicyandiamide, DCD) and urease inhibitor (N-*(n-Butyl)* thiophosphoric triamide, NBPT) were investigated in pot experiments where the soil was contaminated with 0.99 mg kg^{-1} of Cd and 241 mg kg^{-1} Zn. The soil solution pH varied between 7.30 and 8.25 during plant growth which was little affected by the type of N fertilizer. The $(NH_4)_2SO_4$+DCD treatment produced higher NH_4^+−N concentrations in soil solution than the $(NH_4)_2SO_4$ and $NaNO_3$ treatment which indicated that DCD addition inhibited the nitrification process. Shoot Cd and Zn concentrations across all treatments showed ranges of 52.9–88.3 and 2691–4276 mg kg^{-1}, respectively. The $(NH_4)_2SO_4$+DCD treatment produced slightly higher but not significant Cd and Zn concentrations in the xylem sap than the $NaNO_3$ treatment. Plant shoots grown with $NaNO_3$ had higher Cd concentrations than $(NH_4)_2SO_4$+DCD treatment at 24.0 and 15.4 mg kg^{-1}, respectively. N fertilizer application had no significant effect on shoot dry biomass. Total Cd uptake in the urea+DCD treatment was higher than in the control, urea+NBPT, urea+NBPT+DCD, or urea treatments, by about 17.5, 23.3, 10.7, and 25.1%, respectively.

Keywords: cadmium, zinc, nitrogen fertilizers, nitrification inhibitor, *Sedum plumbizincicola*

Introduction

There is widespread contamination of soils by cadmium (Cd) and zinc (Zn) as a result of human activities such as ore mining, industrial processes, and excessive fertilizer applications in intensive agriculture. Soils that are highly contaminated with Zn and Cd can be phytotoxic. Soils polluted with heavy metals such as Zn and Cd pose a risk to human health and many remediation techniques have been used to remove heavy metals from contaminated sites such as soil washing/flushing and electrokinetics (Mulligan *et al.* 2001; Pulford and Watson 2003). However, the most cost effective and promising method for moderately polluted soils is phytoextraction, the use of metal-accumulating plants to remove pollutants from soils by concentrating them in harvestable parts. This is considered to be a cost-efficient and environmentally friendly method to remove metals from contaminated soils *in-situ* (Schwartz *et al.* 2003; Xie *et al.* 2010). *Sedum plumbizincicola*, a new species of the Crassulaceae (Wu *et al.* 2006, 2013), is a remarkable Zn and Cd hyperaccumulator that has high capacity to accumulate, translocate, and tolerate high concentrations of heavy metals. Moreover, it also has a high potential for Zn and Cd phytoextraction in the field (Jiang *et al.* 2010; Liu *et al.* 2011). It is of considerable interest to enhance plant growth and biomass production to increase phytoremediation efficiency. This is because high yields can lead to a large accumulation of metals in the harvestable parts and reduce the time required for remediation.

Nitrogen (N) is an essential element for plant growth and reproduction and N fertilizers, usually in the form of ammonium (NH_4^+), nitrate (NO_3^-) or urea, are commonly used to enhance plant biomass yields and phytoextraction of pollutants from contaminated soils (Loosemore *et al.* 2004; Zaccheo *et al.* 2006; Giansoldati *et al.* 2012). A number of studies have shown that supplying N in the NH_4^+ form to plants can lead to rhizosphere acidification because proton excretion by root cells enhances Zn and Cd uptake by tobacco and sunflower (Loosemore *et al.* 2004; Zaccheo *et al.* 2006).

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Conversely, supplying N as NO_3^- can promote growth and phytoextraction of Cd and Zn by *Noccaea* (*Thlaspi*) *caerulescens* compared with NH_4^+ (Xie *et al.* 2009; Monsant *et al.* 2010, 2011). The results indicate that NO_3^- enhances Cd and Zn uptake and/or translocation from roots to shoots and then increases the bioavailability of these metals in the soil. This is consistent with Hu *et al.* (2013) who found that NO_3^- supplementation enhanced Cd uptake by *S. plumbizincicola* rather than NH_4^+ in hydroponic solution. In addition, Monsant *et al.* (2008) found that Zn phytoextraction efficiency increased after $Ca(NO_3)_2$ addition to a slightly acid soil.

Urea is the predominant form of N fertilizer used because it has a high N content (46% by weight) and a relatively low production cost. However, the fertilizer N efficiency of urea decreases through losses of N as ammonia gas after urea is hydrolyzed by the enzyme urease. Urease inhibitors can block the conversion of urea to ammonia. Similarly, ammonium formed in the soil is rapidly oxidized to nitrate and lost in the nitrification process carried out by specific bacteria. Nevertheless, some reports have shown that nitrification inhibitors can slow down or delay the nitrification process and increase N availability in the soil (O'Connell *et al.* 2004; Zaman and Nguyen 2012).

Numerous studies have investigated the benefits of the nitrification inhibitor dicyandiamide (DCD) in reducing nitrification. DCD has a significant impact in reducing ammonia oxidizing bacteria (AOB), especially at high nitrogen application rates. Furthermore, total N_2O emissions by DCD were reduced by about 69% with animal urine applied at 600 kg N ha^{-1} (Dai *et al.* 2013). Zaccheo *et al.* (2006) reported that Cd and Zn phytoextraction from contaminated soil by sunflower was increased by NH_4^+ and addition of a nitrification inhibitor. Furthermore, DCD and N-(*n-Butyl*) thiophosphoric triamide (NBPT) applied in the solid form prior to grazing has the most potential to reduce N losses through NH_3 volatilization, N_2O emissions, and NO_3^- leaching in grazed pastoral systems (Zaman and Nguyen 2012). However, DCD application to soil cannot be used for reducing N_2O emissions over the long term because nitrification is limited by saturated soil conditions (Kleina *et al.* 2011). Thus, urease and nitrification inhibitors can decrease the possibility of large losses of nitrate and extend N fertilizer efficiency for plant uptake. It is not presently known whether the N form and urease inhibitor or nitrification inhibitor can influence Cd and Zn phytoextraction by *S. plumbizincicola* under soil conditions.

The aims of the present study were to compare the effects of different N fertilizers on shoot yield and Cd and Zn accumulation by *S. plumbizincicola* under pot experiment conditions. The effects of the nitrification inhibitor DCD and the urease inhibitor NBPT applied alone and in combination in order to extend fertilizer efficiency were investigated, together with the effects of soil properties on phytoextraction during application of different N fertilizers. It was hoped that the results would help us to select the appropriate N fertilizer form and inhibitors to enhance Cd phytoextraction from soils by *S. plumbizincicola*.

Materials and Methods

Reagents

Dipotassium hydrogen phosphate (K_2HPO_4), ammonium sulphate $(NH_4)_2SO_4$ (AR grade, XLHG, China), sodium nitrate ($NaNO_3$), urea ($CO(NH_2)_2$), dicyandiamide (DCD, $C_2H_4N_4$), N-(*n-Butyl*) thiophosphoric triamide (NBPT), nitric acid (HNO_3) (GR grade, Nanjing Chemical Reagent, Co., Ltd., China), calcium chloride ($CaCl_2$), and 30% hydrogen peroxide (H_2O_2) (GR grade, Nanjing Chemical Reagent, Co., Ltd., China), were used in this experiment.

Soil Characteristics and Sample Preparation

Soil was collected from the top 20 cm of a field contaminated with heavy metals from a smelter at Fuyang city, Zhejiang province, east China. The soil was air dried, ground with a mortar and pestle, and sieved through a 2 mm nylon mesh for physical and chemical fraction analysis (Bao 2000). Total heavy metal concentrations in the soil were determined using an atomic absorption spectrophotometry (AAS) (Varian SpectrAA 220FS, 220Z, Varian, Palo Alto, CA) after digestion of 0.20–0.25 g sub-samples with 10 ml of HCl-HNO_3 (5: 5, v/v). Selected properties of the soil are listed in Table 1.

Pot Experiment

Air-dried and homogenized soil equivalent to 1.0 kg (oven dry basis) was placed in each plastic pot (16 cm upper diameter, 14 cm basal diameter and 12 cm high). Pots containing soil were allocated to one of eight treatments as follows: (1) control with no amendment (CK); (2) 250 mg kg^{-1} N as $NaNO_3$;

Table 1. Properties of the soil from Fuyang city, Zhejiang Province, east China

Soil Property	Value
Cd (mg kg^{-1})	0.99
Cu (mg kg^{-1})	58.5
Pb (mg kg^{-1})	85.3
Zn (mg kg^{-1})	241
Al (g kg^{-1})	17.5
Ca (g kg^{-1})	31.2
Cr (mg kg^{-1})	42.4
Fe (g kg^{-1})	13.2
K (mg kg^{-1})	1561
Mg (mg kg^{-1})	1636
Mn (mg kg^{-1})	323
Na (mg kg^{-1})	149
Ni (mg kg^{-1})	15.2
Total N (mg kg^{-1})	1940
Available N (mg kg^{-1})	162
Available P (mg kg^{-1})	0.63
Available K (mg kg^{-1})	34.0
Organic matter (g kg^{-1})	35.5
pH (soil: water 1: 2.5)	7.99

(3) 250 mg kg^{-1} N as $(NH_4)_2SO_4$; (4) 250 mg kg^{-1} N as $(NH_4)_2SO_4$, plus 2.5 mg kg^{-1} DCD; (5) 250 mg kg^{-1} N as urea; (6) 250 mg kg^{-1} N as urea, plus 2.5 mg kg^{-1} DCD; (7) 250 mg kg^{-1} N as urea, plus 2.5 mg kg^{-1} NBPT; and (8) 250 mg kg^{-1} N as urea, plus 2.5 mg kg^{-1} NBPT and 2.5 mg kg^{-1} DCD. There were 8 replicate pots for treatments (2) and (4), and 4 replicate pots for other treatments. K_2HPO_4 was applied as a basal fertilizer at a rate of 350 mg kg^{-1}.

Healthy and uniform shoots of *S. plumbizincicola* were collected from an old Pb/Zn mine area in the suburbs of Hangzhou city, Zhejiang province and grown for two weeks in nutrient solution containing (in mM) calcium nitrate [$Ca(NO_3)_2$ $4H_2O$] 1.00, magnesium sulfate [$MgSO_4$ $7H_2O$] 0.5, monopotassium phosphate [K_2HPO_4] 0.5, KCl 0.1, 2-(N-morpholino) ethanesulfonic acid [MES] 1.00, potassium hydroxide [KOH] 0.5, boric acid [H_3BO_3] 10, sodium molybdate dihydrate [Na_2MoO_4 $2H_2O$] 0.2, manganese sulfate [$MnSO_4$ $4H_2O$] 1.8, copper sulfate [$CuSO_4$ $5H_2O$] 0.31, nickel sulfate [$NiSO_4$ $6H_2O$] 0.5, iron ethylenediamine-N, N'-bis (2-hydroxyphenylacetic acid) [Fe-EDDHA] 100, and zinc sulfate [$ZnSO_4$ $7H_2O$] 5.00. *S. plumbizincicola* was then transplanted in plastic pots with four replicates and each pot contained four plants. A soil moisture sampler was inserted in each pot to allow sampling of the soil solution. The pots were arranged randomly in a glasshouse with natural daylight, no supplementary illumination, and a day/night temperature range of 20–30°C. The pots were watered with deionized water when required to maintain soil moisture at approximately 70% water holding capacity (WHC).

Xylem Sap Extraction and Plant Analysis

On day 34, four pots of plants in treatments 2 ($NaNO_3$) and 4 ($[NH_4]_2SO_4$ plus DCD) were harvested for xylem sap analysis. The xylem sap was extracted following the methods of Hu *et al.* (2013). Shoots were cut with a razor blade at their base and the stem stubbles at 2–3 cm height were retained. Sap exuding from the cut surfaces was collected with 1.5-ml ultrafiltration centrifuge tubes filled with cotton held in a serum cap of a small vial. Xylem sap absorbed in the cotton was collected by centrifuging at 3000 rpm for 30 sec. Samples were kept on ice until they were transferred to a freezer (–18°C) for storage. Cd and Zn concentrations in the xylem sap were determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (VI 1 STA-PRO, Varian, Palo Alto, CA). The shoots were thoroughly washed with distilled water and oven-dried in paper bags at 75°C for 5 days. Dried tissues were then weighed immediately upon removal from a desiccator. They were then ground to less than 0.25 mm for analysis of heavy metals and other elements using AAS after digestion with concentrated HNO_3: 30% H_2O_2 (3: 1, v/v).

Soil Solution Analysis

Soil solution samples were collected with soil moisture samplers (Rhizon SMS, Rhizosphere Research Products, the Netherlands) after 3, 7, 14, 28, 49, 73, and 95 days to determine pH, heavy metal concentrations, NH_4^+−N and NO_3^-−N. NH_4^+−N and NO_3^-−N concentrations were determined using a continuous flow analyzer (CFA: SAN++ System, The Netherlands).

Plant Analysis

Plants were harvested after 80 and 100 days when the soil water content decreased to 40–50% WHC. Shoots and roots were cut at their junction, washed thoroughly with tap water, rinsed with deionized water, and oven dried at 50°C for 5 days in paper bags. The fresh weights (FW) and dry weights (DW) were determined. Sub-samples of the ground shoots (0.2–0.3 g) were digested with concentrated HNO_3: 30% H_2O_2 (3: 1, v/v) mixture in an electro-thermostatic blast oven and the Cd and Zn concentrations were determined using AAS. A Certified Reference Material (GBW10014, provided by the Institute of Geophysical and Geochemical Exploration, Langfang, Hebei province, China), replicate samples, and blanks were included in all element analyses for quantitative verification of the results.

Soil Analysis

Soil was collected from all treatments after harvesting the plants to determine soil water content, extractable N, and heavy metal concentrations. Soil samples were air dried, homogenized in an agate mortar and passed through a 100-mesh nylon sieve. Soil extractable Cd and Zn and NH_4^+−N and NO_3^-−N concentrations were extracted with $CaCl_2$. Soil was shaken at 25°C for 2 hours with 0.01 M $CaCl_2$ (soil to extractant ratio 1: 5) and then filtered. The extractable fractions of Cd and Zn and NH_4^+−N and NO_3^-−N concentrations were then determined by AAS and CFA, respectively. The total shoot Cd and Zn uptake (dry matter basis) was calculated by multiplying the shoot dry weight biomass by the shoot Cd and Zn concentrations.

Statistical Analysis

The data were statistically analyzed using the SPSS version 19 software package to perform one way analysis of variance (ANOVA). Mean values were compared using Duncan's multiple range test at the 5% level.

Results

Changes in Soil Solution Properties

Soil Solution pH

The original pH value of the tested soil was 7.99 and the soil solution pH varied between 7.30 and 8.25 during plant growth and a similar trend was observed among the treatments (Fig. 1). When the soil solution samples were first examined after 14 days the pH values were only slightly different among treatments. After 49 days the urea+NBPT+DCD treatment had the lowest soil solution pH, 0.16 units lower than the urea treatment, 0.30 lower than $(NH_4)_2SO_4$+DCD, 0.32 lower than

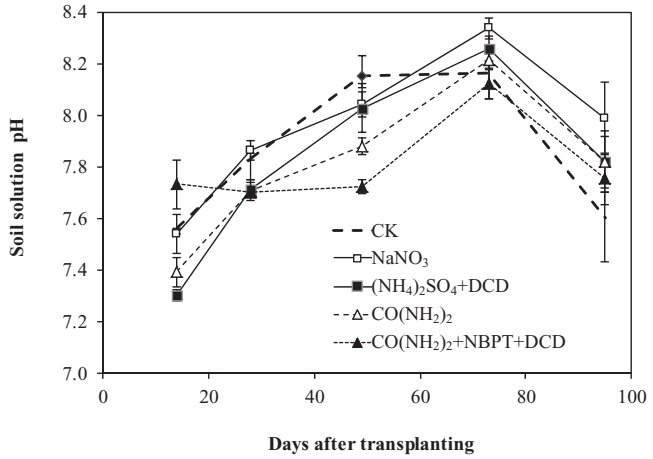

Fig. 1. Effects of N form, nitrification inhibitor (DCD), and urease inhibitor (NBPT) on soil solution pH.

$NaNO_3$, and 0.43 lower than the control ($p < 0.05$). The soil solutions showed a significant ($p < 0.05$) increase in pH after 73 days of growth of *S. plumbizincicola* and the pH declined in all treatments by 0.37–0.57 units after 95 days. In all N treatments the soil pH showed a small increase compared to the control soil. The soil solution pH was approximately 0.17 units higher in the $NaNO_3$ treatment than in the $(NH_4)_2SO_4$+DCD treatment.

Ammonium and Nitrate Concentrations in the Soil Solution (NH_4^+−N, NO_3^-−N)

The dynamics of soil solution NH_4^+−N showed a similar trend in all treatments. The NH_4^+−N concentration decreased continuously in all treatments during plant growth. The lowest soil solution NH_4^+−N content after 3 days was found in the control, $NaNO_3$ and urea+NBPT+DCD treatments. The concentration of NH_4^+−N ($p < 0.05$) was significantly higher in the $(NH_4)_2SO_4$+DCD treatment than in $NaNO_3$ between day 3 and 14 (Fig. 2a). When $NaNO_3$ was added soil NH_4^+−N changed with time in a similar fashion to the control. Furthermore, NH_4^+−N concentrations in the soil solution of $NaNO_3$ treatment were below the detection limit after 28 days. Thus, DCD inhibited the nitrification process in the soil and then led to higher NH_4^+−N concentrations than in the $NaNO_3$ treatment.

Moreover, the concentration of NH_4^+−N was significantly higher in the urea treatment without inhibitors than in the urea treatment with the two inhibitors combined between day 3 and 14. When urea was added the hydrolysis of urea in soil led to increased NH_4^+−N on day 3 and then nitrification led to decreased NH_4^+−N and increased NO_3^-−N. This result confirms that the urease inhibitor (NBPT) was blocking the conversion of urea to ammonia over a period of 14 days.

The results in Fig. 2B show that soil solution NO_3^-−N concentrations decreased significantly after DCD application compared with no DCD application. The combination of the two inhibitors gave lower NO_3^-−N concentrations than DCD only. $NaNO_3$ application had a significant effect ($p < 0.05$)

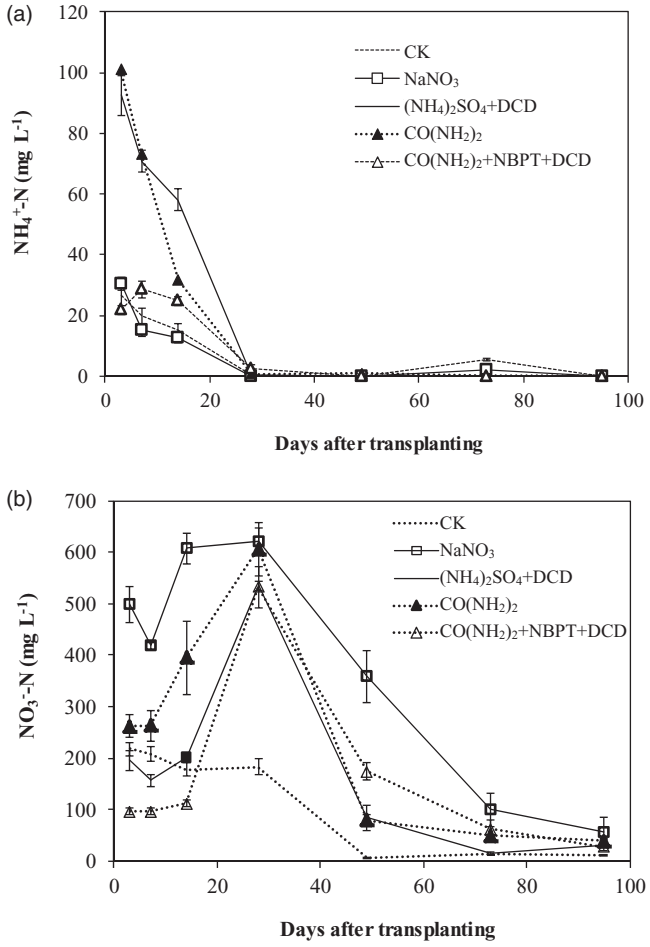

Fig. 2. Effects of N form, nitrification inhibitor (DCD), and urease inhibitor (NBPT) on soil solution (a) ammonium and (b) nitrate concentrations.

on soil solution NO_3^-−N concentrations compared to the control. Soil NO_3^-−N content in the $NaNO_3$ treatment increased from 499 to 621 mg L^{-1} after 28 days and then decreased subsequently. The soil solution NO_3^-−N concentrations were significantly affected by the treatments; the addition of the $(NH_4)_2SO_4$+DCD treatment reduced NO_3^-−N concentrations by 12–84% compared with the $NaNO_3$ treatment and the addition of the urea+NBPT+DCD treatment reduced NO_3^-−N concentrations by 63–72% compared with urea without inhibitors.

Cd and Zn Concentrations in the Soil Solution

The Cd and Zn concentrations in the soil solution are shown in Fig. 3. The concentrations of Cd ranged from 0.19 to 0.77 μg L^{-1} within 95 days. Moreover, the soil had higher solubility of Zn compared with Cd in all treatments on day 28. The presence of DCD, NBPT, or both inhibitors combined did not significantly decrease or increase the Cd concentration in the solution compared with urea with or without inhibitors. Within four weeks of fertilization, Cd had increased by approximately 49 and 70% in the $(NH_4)_2SO_4$+DCD and urea

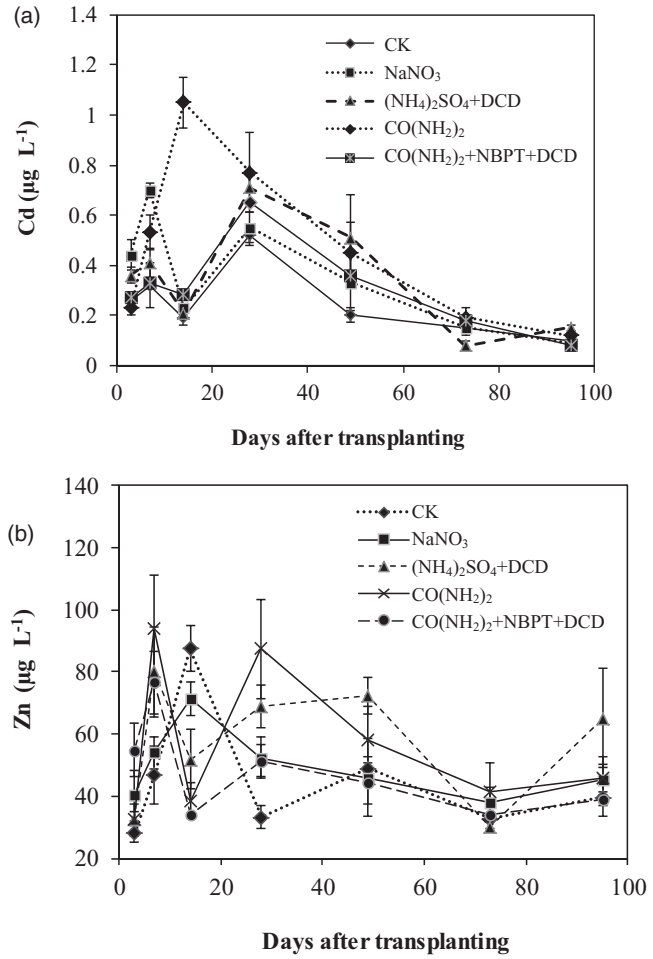

Fig. 3. Effects of N form, nitrification inhibitor (DCD), and urease inhibitor (NBPT) on (a) Cd and (b) Zn concentrations in the soil solution.

treatments, respectively. The highest soil solution Cd concentration, 0.77 μg L^{-1}, was found on day 28 in the soil receiving urea. However, there were no significant differences among treatments on day 28 (Fig. 3A).

The concentration of Zn in the soil solution ranged from 28.0 to 87.5 μg L^{-1} (Fig. 3B). Zn concentrations in the control treatments were similar to those observed in the fertilizer treatments on day 3 but urea+NBPT+DCD gave a high Zn concentration compared with the other treatments. The addition of the nitrification inhibitor (DCD) and urease inhibitor (NBPT) slightly affected the solubility of Zn in the soil. There was no difference in the soil solution pH among treatments. Urea+NBPT+DCD gave a high Zn concentration and the nitrification and urease inhibitors impacted slightly the solubility of Zn in soil.

Soil Extractable Nitrogen and Heavy Metals

Soil Extractable NH_4^+−N and NO_3^-−N Concentrations

Soil was collected after harvesting plants on day 100 and extracting with $CaCl_2$ to determine NH_4^+−N and NO_3^-−N concentrations. Ammonium and nitrate extractable with $CaCl_2$ were used to estimate potential NH_4^+ and NO_3^- release from soil to the solution. This may be widely applicable as an index for plant-available nitrogen in fertilized soils. Table 2 shows that the highest extractable NH_4^+−N contents were found in treatments at the last period of plant growth with the following order: $NaNO_3$ > $(NH_4)_2SO_4$ > urea+NBPT+DCD > $(NH_4)_2SO_4$+DCD > urea+DCD (44.8, 36.7, 36.3, 24.2, and 15.2 mg kg^{-1} soil, respectively). The highest NO_3^-−N contents were found in treatments in which $NaNO_3$ was supplied to the soil and there was no significant difference from the urea+NBPT+DCD treatment. Moreover, with the addition of urea the soil NO_3^-−N concentrations significantly increased compared with the control. Application of DCD reduced the NO_3^-−N concentrations in the urea treatment. In contrast, application of NBPT with urea increased the NO_3^-−N concentrations when compared with urea alone.

Table 2. Effects of N form, nitrification inhibitor (DCD), and urease inhibitor (NBPT) on extractable soil solution ammonium and nitrate concentrations on day 100 (mg kg^{-1})

Treatment	Extractable NH_4^+−N	Extractable NO_3^-−N
Control	N.D.	46.5 ± 10.5b
$NaNO_3$	44.8 ± 4.5a	804 ± 255a
$(NH_4)_2SO_4$	36.7 ± 0.0ab	244 ± 165ab
$(NH_4)_2SO_4$+DCD	24.2 ± 12.1ab	324 ± 15ab
$CO(NH_2)_2$	N.D.	484 ± 45ab
$CO(NH_2)_2$+DCD	15.2 ± 7.8b	294 ± 67ab
$CO(NH_2)_2$+NBPT	N.D.	605 ± 306ab
$CO(NH_2)_2$+NBPT+DCD	36.3 ± 10.0ab	792±116a

Values are means ± SE from four individual replicates. Within each column means followed by the same letter are not significantly different by Duncan's multiple range test at the 5% level. N.D., not detected.

Urea hydrolysis occurred after urea application as evidenced from the lower extractable soil solution NH_4^+ concentrations in the urea treatment compared to the control treatment on day 100, and was significantly ($p < 0.05$) increased by DCD or the combined addition of both inhibitors. On day 100 the urease inhibitor had low efficiency in inhibiting urea hydrolysis because extractable NH_4^+−N was undetectable but high extractable NO_3^-−N was present in the soil.

Soil Extractable Cd and Zn

$CaCl_2$-extractable Cd and Zn were sampled on day 80 and 100 for determination of heavy metal concentrations. On day 80 the extractable Cd concentrations appeared to be highest in the $(NH_4)_2SO_4$ treatment and lowest in the $NaNO_3$ treatment (Table 3) but the difference was not significant. The extractable Zn concentration was not significantly ($p > 0.05$) affected by treatment.

The N fertilizers had no significant effect on extractable Cd concentrations on day 100. The concentration of Cd and Zn ranged from 0.50−1.00 μg Cd kg^{-1} and 0.18−0.38 mg Zn kg^{-1}. Soil extractable Cd in the $(NH_4)_2SO_4$ treatment was twice as likely to occur in the soil in the control treatment. The

Table 3. Effects of N form and nitrification inhibitor (DCD) on the extractable Cd and Zn in soil on day 80 and 100

	Day 80		Day 100	
Treatment	Cd (μg kg^{-1})	Zn (mg kg^{-1})	Cd (μg kg^{-1})	Zn (mg kg^{-1})
Control	2.98±0.23a	0.23±0.02a	0.50 ± 0.02c	0.22 ± 0.03b
$NaNO_3$	2.80±0.11a	0.31±0.08a	0.69 ± 0.26abc	0.28 ± 0.01ab
$(NH_4)_2SO_4$	5.31±1.43a	0.34±0.14a	1.00 ± 0.06a	0.24 ± 0.03b
$(NH_4)_2SO_4$+DCD	3.84±0.64a	0.29±0.06a	0.61 ± 0.01b	0.18 ± 0.04b
$CO(NH_2)_2$	4.69±1.24a	0.28±0.08a	0.62 ± 0.04b	0.18 ± 0.04b
$CO(NH_2)_2$+DCD	3.13±0.35a	0.23±0.04a	0.61 ± 0.08bc	0.26 ± 0.06ab
$CO(NH_2)_2$+NBPT	3.82±0.14a	0.24±0.02a	0.54 ± 0.19bc	0.24 ± 0.04b
$CO(NH_2)_2$+NBPT+DCD	3.32±0.29a	0.27±0.02a	0.94 ± 0.13a	0.38 ± 0.06a

Values are means ± SE from four individual replicates. Within each column means followed by the same letter are not significant by Duncan's multiple range test at the 5% level.

urea mixed with the combined inhibitors gave high extractable Zn concentrations compared with the control at about 72.7% on day 100. The effects of the treatments on $CaCl_2$-extractable Cd and Zn concentrations were similar to those on heavy metals in the soil solution.

Heavy Metal Concentrations in Xylem Sap

Table 4 shows the effects of $NaNo_3$ and $(NH_4)_2SO_4$+DCD on Cd and Zn concentrations in xylem sap of 34-day-old *S. plumbizincicola*. The ability of $(NH_4)_2SO_4$+DCD to enhance Cd and Zn uptake was demonstrated by slightly higher Cd and Zn concentrations in xylem sap and higher plant dry weights compared to the $NaNO_3$ treatment. The average shoot dry weights ranged from 1.00 and 1.24 g pot^{-1} in the $NaNO_3$ and $(NH_4)_2SO_4$+DCD treatments, respectively.

Shoots of plants grown with $NaNO_3$ were found to have higher Cd concentrations at 24.0 mg kg^{-1} than plants supplied with the $(NH_4)_2SO_4$+DCD treatment at 15.4 mg kg^{-1}. Zn concentrations in the plant shoots supplied with $(NH_4)_2SO_4$+DCD and $NaNO_3$ were not significantly different, with 1193 and 1044 mg kg^{-1}, respectively (data not shown).

Plant Biomass and Metal Concentrations

The *Sedum* plants of all treatments were harvested 100 days after transplanting. In the present study *S. plumbizincicola* showed no toxicity symptoms. Fertilizer N application had no significant effect on *S. plumbizincicola* shoot dry biomass. The average shoot dry weights of plants ranged from 3.56 to 5.40 g pot^{-1} but the differences were not significant (Table 5).

Furthermore, no significant differences occurred in the Cd and Zn shoot concentrations between treatments; this lack of difference may be attributed to the lack of difference in the pH of the soil solution and average dry biomass. Shoots of plants grown in the $(NH_4)_2SO_4$+DCD treatment were found to have higher Cd concentrations at 83.3 mg kg^{-1} compared with plants supplied with the $NaNO_3$ treatment at 52.9 mg kg^{-1} of approximately 57.5%. According to the xylem sap analysis, soil supplied with $(NH_4)_2SO_4$+DCD led to slightly higher Cd concentrations but slightly lower Zn concentrations in the shoots than the $NaNO_3$ treatment.

There was no effect of N fertilizer type or inhibitor on Cd and Zn concentrations in shoots or dry biomass. Shoot Cd and Zn concentrations ranged from 52.9 to 83.3 mg kg^{-1} and 2691 to 4276 mg kg^{-1}, respectively. However, total Cd metal uptake by *S. plumbizincicola* shoots varied from 211 to 339 μg pot^{-1}. Total Cd uptake followed the order $CO(NH_2)_2$+DCD > $(NH_4)_2SO_4$+DCD > $CO(NH_2)_2$+NBPT+DCD > $(NH_4)_2SO_4$ > CK > $CO(NH_2)_2$+NBPT > $CO(NH_2)_2$ > $NaNO_3$. The total Cd uptake in the urea+DCD treatment was higher than in the control, $CO(NH_2)_2$+NBPT, $CO(NH_2)_2$+NBPT+DCD, and $CO(NH_2)_2$ by about 17.5, 23.3, 10.7, and 25.1%, respectively.

The addition of fertilizer N and the inhibitors did not significantly decrease shoot Zn concentrations. The highest total Zn uptake by *S. plumbizincicola* was found in plants grown in soil without fertilizer N. The total Zn metal uptake in the $NaNO_3$ treatment was higher than in the $(NH_4)_2SO_4$ treatment by about 11.8%.

Table 4. Cd and Zn concentrations in the xylem sap of 34-day-old *S. plumbizincicola*

Treatment	Cd (μg L^{-1})	Zn (mg L^{-1})
$NaNO_3$	420±25a	17.6±1.1a
$(NH_4)_2SO_4$+DCD	547±100a	22.4±7.1a

Values are means ± SE from four individual replicates. Within each column means followed by the same letter are not significantly different by *t* test at the 5% level.

Discussion

It is known that fertilizer N applied to soils will increase the biomass of most plant species and accelerate the rate of nitrification by soil microorganisms. The urease inhibitor and nitrification inhibitor can decrease the risk of large losses of nitrate and extend N fertilizer uptake by plants. In contrast with previous reports the N fertilizers did not significantly increase plant biomass and phytoextraction efficiency of *S.*

Table 5. Effects of N form, nitrification inhibitor (DCD), and urease inhibitor (NBPT) on the biomass, Zn, and Cd concentrations and total metal uptake by shoots of 100-day-old *S. plumbizincicola*

		Metal concentrations		Total metal uptake	
Treatment	Dry biomass (g pot^{-1})	Cd (mg kg^{-1})	Zn (mg kg^{-1})	Cd (μg pot^{-1})	Zn (mg pot^{-1})
Control	4.29±0.84a	68.4 ± 2.4a	4276 ± 54a	293	18.3
$NaNO_3$	3.99±0.81a	52.9 ± 7.2a	3804 ± 305a	211	15.2
$(NH_4)_2SO_4$	3.56±0.86a	83.3 ± 4.9a	3763 ± 416a	298	13.4
$(NH_4)_2SO_4$+DCD	4.36±0.20a	77.9 ± 7.8a	3081 ± 416a	339	13.4
$CO(NH_2)_2$	4.04±0.65a	65.9 ± 1.3a	3279 ± 112a	266	13.2
$CO(NH_2)_2$+DCD	5.40±0.39a	65.8 ± 9.7a	2691 ± 374a	355	14.5
$CO(NH_2)_2$+NBPT	3.66±0.35a	74.5 ± 4.0a	4037 ± 222a	272	14.8
$CO(NH_2)_2$+NBPT+DCD	4.57±0.80a	69.5 ± 20.5a	3189 ± 209a	317	14.6

Values are means ± SE from four individual replicates. Within each column means followed by the same letter are not significantly different by Duncan's multiple range test at the 5% level.

plumbizincicola. However, the results do not support the hypothesis that the addition of DCD in combination with NBPT and urea fertilizer will increase N uptake by reducing losses of N by nitrate leaching and denitrification. Comparing our data with the results of Xie *et al.* (2009), the total soil nitrogen can be considered to be about 0.14% and N fertilizer was added at a rate of 150 mg kg^{-1} and mixed with 10% of DCD. However, in our study we used soil that contained approximately 0.19% total N and mixed it with 250 mg kg^{-1} N fertilizer and 1% of DCD or/and NBPT. Thus the DCD and NBPT rates may not have been sufficient to ameliorate soil acidification and enhance metal uptake.

After application to the soil urea is usually hydrolyzed by urease and ammonium carbonate is produced. The ammonium carbonate then reacts with hydrogen ions to produce NH_4^+. NH_4^+ may then change to NH_3 which can be lost through gaseous volatilization. This process involves *Nitrosomonas* and *Nitrobacter* bacteria. Nitrification of ammonium can occur in two to three weeks. The optimal pH for nitrification is 7.0 to 8.2. In some cases it has been reported that ammonia, especially under alkaline conditions (> pH 8.5), may preferentially increase free ammonia and this may lead to inhibition of nitrite oxidizers (Smith *et al.* 1997).

The soil solution pH value did not decrease enough to become acidic between days 14 and 95 after transplanting. The soil solution pH varied between 7.30 and 8.25 during plant growth. When $(NH_4)_2SO_4$ or urea is added to the soil H^+ ions are released into the rhizosphere resulting in a decrease in rhizosphere pH that is likely a result of nitrification. Conversely, $NaNO_3$ addition to the soil increases the pH in the rhizosphere (Zaccheo *et al.* 2006; Monsant *et al.* 2008; Xie *et al.* 2009).

The nitrification inhibitor DCD can maintain N in the ammonium form in the soil and remains effective for three to six weeks depending upon environmental conditions. After the application of DCD to the soil NO_3^-—N concentrations were significantly decreased. This result confirms that DCD inhibited ammonium oxidation by deactivating the ammonia monooxygenase (AMO) enzyme of the soil ammonia-oxidizers for the nitrification process (Amberger 1989). The combined application of NBPT and DCD produced low NO_3^-—N contents when compared with urea without any inhibitor. This confirms that the inhibitors reduced the transform of urea to ammonium and nitrate.

The most important factor controlling Cd solubility in soil is the soil pH (Impellitteri *et al.* 2001). Normally, Cd solubility increases strongly when the soil pH is lower than 6 (Brümmer and Herms 1983) but solubility was low at pH 7 to 8 in our study. The tested soil showed low Cd solubility in the soil solution because the soil pH was 7.99. Furthermore, the N fertilizers did not significantly affect soil Zn and Cd concentrations. The addition of DCD or NBPT or both inhibitors combined produced no significant change in Cd concentration in the solution compared with urea either with or without inhibitors.

The extractable NH_4^+—N concentrations in the $(NH_4)_2SO_4$ treatments appeared to be higher than in the $(NH_4)_2SO_4$+DCD treatment but there was no significant difference. In addition, the urea+NBPT+DCD treatment was higher in extractable NH_4^+—N concentrations than the urea+DCD and urea treatments. The urea+NBPT treatment did not show any detectable NH_4^+—N. The extractable NO_3^-—N concentrations increased significantly with the addition of urea compared with the control. Application of DCD reduced the NO_3^-—N concentrations when compared to the urea treatment without DCD. However, application of NBPT with urea increased the NO_3^-—N concentrations when compared to urea without NBPT. Urea hydrolysis occurred after urea application, as evident from the lower extractable soil solution NH_4^+ concentrations in the urea treatment compared to the control treatment on day 100, and was significantly ($p > 0.05$) increased by DCD or the two inhibitors combined. Pushenreiter *et al.* (2001) found that $(NH_4)_2SO_4$+DCD treatment had little effect on Ni uptake by *Thlaspi goesingense* because the DCD was degraded and did not reduce acidification in the rhizosphere.

The soil extractable Cd and Zn concentrations were not significantly ($p > 0.05$) affected by the treatments. Soil extractable Cd in the $(NH_4)_2SO_4$ treatment was twice as likely to be found in the control treatment soil. Urea combined with both inhibitors produced a 73% higher extractable Zn concentration than the control.

Shoot Zn concentrations of plants supplied with $(NH_4)_2SO_4$+DCD and $NaNO_3$ were not significantly increased at 1193 mg kg^{-1} and 1044 mg kg^{-1}, respectively. This result is in contrast with Monsant *et al.* (2010) who reported that NO_3^- led to higher Zn accumulation than NH_4^+. However, some studies have found that N fertilizers of unknown form of N increased the shoot biomass of the Zn hyperaccumulator *N. caerulescens* but reduced the shoot Zn concentration (Bennett *et al.* 1998; Sirguey *et al.* 2006).

Some studies have reported that the application of N fertilizers will result in the acidification of soils by nitrification (Black 1992; Juo *et al.* 1995, 1996) and reduce soil pH and increase Cd uptake by crops (Eriksson 1990). In the present study the *S. plumbizincicola* plants were healthy and grew well in all treatments. However, the fertilizer N application had no significant effect on shoot dry biomass or shoot Cd and Zn concentrations. One possible explanation is that the low application rate of the N fertilizers of about 250 mg N kg^{-1} was not adequate to significantly increase shoot biomass and phytoextraction efficiency.

The highest total Zn uptake by *S. plumbizincicola* was found in plants that were grown in soil without N fertilizers. However, the total Zn metal uptake in the $NaNO_3$ treatment was about 13% higher than in the $(NH_4)_2SO_4$ treatment. The addition of $(NH_4)_2SO_4$ to the soil showed a high efficiency for increasing Cd uptake by *S. plumbizincicola* when compared with $NaNO_3$ and urea. Zhu *et al.* (2011) found that $(NH_4)_2SO_4$ showed a high efficiency to increase Cd uptake of *Sedum alfredii* when compared with urea and $Ca(NO_3)_2$ $4H_2O$.

The addition of DCD in combination with NBPT to soil decreased metal concentrations compared to treatments without DCD. Kawakami *et al.* (2012) found that DCD addition to the soil decreased plant N uptake, N use efficiency, leaf chlorophyll content, plant growth, and yields of cotton. They concluded that addition of NBPT to urea improved N fertilization in cotton. However, DCD addition to urea with NBPT reduced the efficiency of NBPT by inhibiting nitrification, accumulating NH_4^+ after DCD degradation in soil, and led to phytotoxicity of DCD to cotton plants. It also increased ammonia N losses.

The $CaCl_2$ extraction method was adopted to study Cd and Zn in contaminated soil after *S. plumbizincicola* growth. In the pot experiment four consecutive crops of plants resulted in a significant reduction in bioavailable Cd in the soils after a long period. Fitz and Wenzel (2002) proposed that hyperaccumulators enhance heavy metal solubility in rhizosphere soils, thereby increasing plant metal uptake. However, bioavailable Zn in our soil did not decrease when compared with day 80 and 100.

The current study has demonstrated that the yield of *S. plumbizincicola* was higher when N was supplied as NH_4^++DCD rather than as NO_3^-. The urea+DCD treatment gave a high dry biomass and high total metal uptake when compared among the treatments. However, without the fertilizer N treatment a high rate of Zn phytoextraction was found when both inhibitors were included.

Conclusions

The present study investigated the effects of different nitrogen fertilizers, the nitrification inhibitor DCD and the urease inhibitor NBPT on the soil Cd and Zn phytoavailability and their phytoextraction by the hyperaccumulator *S. plumbizincicola*. The addition of DCD inhibited the nitrification process in the soil and then led to higher NH_4^+−N concentrations than in the $NaNO_3$ treatment. The doses of DCD and NBPT used in this study may not have been sufficient to reduce soil pH and enhance metal phytoavailability. The N fertilizer and inhibitor application had no significant effect on shoot dry biomass or shoot Cd and Zn concentrations. Further research is therefore needed to study the appropriate application rates of N fertilizers and nitrification inhibitors in order to substantially enhance the biomass and phytoextraction efficiency of *S. plumbizincicola* under field conditions.

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