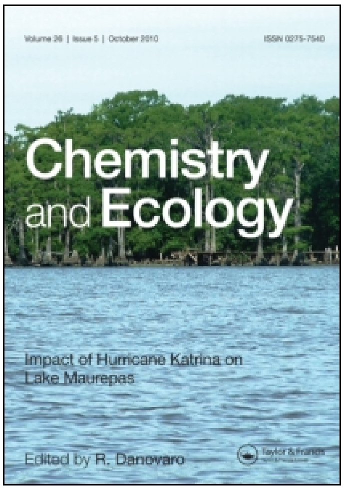

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Decline in metallophytes in tertiary polluted floodplain grasslands in the Netherlands: experimental evidence for metal and nutritional changes in soil as driver factors

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Decline in metallophytes in tertiary polluted floodplain grasslands in the Netherlands: experimental evidence for metal and nutritional changes in soil as driver factors

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Metallophyte vegetation along the River Geul has been almost completely replaced by grasses during the last decades. Field investigations indicated that this was accompanied by higher alkalinity and phosphate availability in the soil, related to the closure of the metal industry and intensification of agricultural practices. An experiment with a full factorial design for phosphate and zinc availability indicated that the metallophytes *Silene vulgaris* and *Thlaspi caerulescens* did not grow on zinc-poor soils, irrespective of phosphate availability. The grass *Holcus lanatus* performed well on phosphate-rich soil, irrespective of zinc availability. An experiment with zinc-poor and zinc-rich floodplain soils confirmed the high zinc demand of the metallophytes *T. caerulescens* and *Armeria maritima* and the zinc independence of *H. lanatus*. A third experiment indicated that a reduced zinc availability due to liming affected only the metallophyte *T. caerulescens*; it had no effect on the growth of the grass *Festuca rubra*. This means that increasing alkalinity leads to a decrease in zinc availability, limiting the growth of at least some metallophyte species. An increase in phosphate availability stimulates growth in more competitive fast-growing grasses under zinc-rich as well as zinc-poor conditions.

Keywords: alkalinisation; floodplain grasslands; metallophytes; metal availability; phosphate; restoration

1. Introduction

Soils enriched or polluted with metals, and their characteristic vegetation, can be found in many parts of the world [1]. In general, three types of metalliferous soils are distinguished: (1) primary polluted soils, including naturally enriched soils in the vicinity of mineral veins; (2) secondary polluted soils on metalliferous mine spoils; and (3) tertiary polluted soils indirectly polluted via streams or the atmosphere, including floodplain grasslands [2]. Metallophytes are plants that have evolved biological mechanisms to survive on these metal-rich soils [3]. They are the result

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of tens, hundreds and often thousands years of strong selective pressure exerted by the soils [4–6]. The restricted geographical area of metallophytes is a key factor in their high rates of population decline and extinction [7,8]. Metallophytes are increasingly under threat and actions towards their conservation are therefore imperative [8,9].

The River Geul area is situated near the borders between Germany, Belgium and the Netherlands. In La Calamine and Plombières (Belgium) over 2.3 million tons of zinc and lead have been produced since pre-Roman times. The most intense period of production was during 1600–1880. In 1880 and 1920, metal extraction stopped but processing of ores continued until 1950. Waste water was added directly to the River Geul, causing widespread dispersal of pyrite- and metal-containing mud in Belgian and Dutch floodplain grasslands [10–12]. As a result of these processes, a very specific zinc-tolerant vegetation type, syntaxonomically belonging to the *Violetum calaminariae* Schwickenrath, developed not only in the secondary polluted Belgian mine spoil areas, but also in the tertiary polluted floodplain grasslands [13–15].

At the time that the Belgian zinc industry became inactive, metallophyte vegetation in the relatively metal-rich secondary polluted mine spoil areas in Belgium remained more or less stable, although it showed a strong decline in the relatively metal-poor tertiary polluted floodplain grasslands of Belgium and the Netherlands [16]. More and more floodplain grasslands (relatively poor in metals compared with mine spoil areas) came into cultivation and were used for agricultural purposes. Since 1933, grasses indicative of fertilised agricultural lands, like *Holcus lanatus* L., *Agrostis capillaris* L. and *Festuca rubra* L., have begun to develop at the cost of the zinc vegetation [17]. By 1960, the overall area of zinc vegetation in the Dutch floodplain grasslands had gradually decreased by 80% [16,18,19]. The habitat of the metallophyte vegetation is now protected on both a national and European scale because the floodplain area forms part of the European Ecological Network ‘Natura 2000’ [20].

Research carried out in the Dutch floodplain of the River Geul, previously and currently vegetated by metallophytes, indicated that metallophytes had been maintained only at relatively acidic locations (pH-$H_2O = 5.0$–5.5) with a relatively high zinc availability (total $Zn > 40\,\mu mol \cdot g^{-1}$ dw; $Zn/Ca > 0.8$; Zn-$H_2O > 59\,\mu mol \cdot kg^{-1}$ dw) in combination with a low phosphate availability (Olsen-P $\ll 1250\,\mu mol \cdot kg^{-1}$ dw). Under all other soil conditions in this area, no metallophytes were present and the grass *H. lanatus* had become the dominant plant species [21]. Total zinc concentrations were high throughout the soil profile (0–50 cm), whereas the top layer contained a relatively high phosphate availability and calcium concentration. Removal of the alkaline and phosphate-rich soil top layer led to an increase in the abundance of metallophytes during at least the first four years, while re-vegetation of *H. lanatus* was nil [21,22]. Field investigations indicated that the decline in metallophytes might be related to changes in land use (increased use of lime-containing fertiliser) and closure of the Belgian metal industry, leading to phosphate enrichment and decreased zinc availability.

In this study, plant growth experiments were performed under laboratory conditions to test the (combined) effects of zinc and phosphate in the soil on the growth of several grasses and local metallophytes. It is hypothesised that the growth of grasses is stimulated at the cost of the original metallophyte vegetation when zinc availability decreases and/or phosphate availability increases. Growth of the grass *H. lanatus* and of two local metallophytes was tested on artificially created soil types with a full factorial design for phosphate and zinc availability. In addition, the effect of alkalinisation and zinc availability on floodplain grasses (*Festuca rubra*) and metallophytes (*Thlaspi caerulescens* (J. & C. Presl) and *Armeria maritima* Willd. subsp. *halleri* (Wallr.) Rothm.) was tested. Plant development was recorded and the elemental composition of the shoot material was compared with plant material collected under field conditions.

2. Materials and methods

2.1. *Background information*

Seeds of *Thlaspi caerulescens*, *Holcus lanatus* and *Festuca rubra* were collected in the zinc-flora nature reserve situated in the tertiary polluted floodplain grasslands in Epen along the River Geul (the Netherlands: 50°45′42″N, 5°55′48″E). Seeds of *Silene vulgaris* (Moench) Garcke var. *humilis* and *Armeria maritima* were collected upstream of the River Geul at a secondary polluted mine spoil in Plombières because both species have become extinct in Epen [16]. Seeds were gradually dried at room temperature for three months. Germination took place (after a cold period of one week at −4 °C) on wet filter paper. After germination, plants were grown up in a mixture of nutrient-poor river sand and metalliferous floodplain soil (2:1) for one week prior to the experiments. Seedlings had the following mean length (±SEM) at the start of the experiments: *T. caerulescens*, 1.0 ± 0.1 cm; *F. rubra*, 1.5 ± 0.2 cm; *S. vulgaris*, 0.9 ± 0.1 cm; *A. maritima*, 0.7 ± 0.1 cm; *H. lanatus*, 2.5 ± 0.3 cm.

Before filling the pots, textile fibre was placed at the bottom to prevent the soil falling out. All pots were inoculated with a soil suspension (25 mL) created by mixing floodplain soil (originating from an area with metallophytes present) with demineralised water ($1\ \mathrm{kg} \cdot \mathrm{L}^{-1}$) at 50 rpm for one day. This was to exclude a possible effect of the absence of area-specific or metal-tolerant micro-organisms in the different soil types. Pots with the same sediment type were put in the same watertight tray to prevent water shortage without causing contamination between the soil types. In the first week, artificial rainwater ($50\ \mathrm{mg\ sea\ salt} \cdot \mathrm{L}^{-1}$, brand: Tropic Marin) was added on top of the pots. After this, seedlings were settled and demineralised water mixed with tap water (9:1) was added to the bottom of the trays weekly ($\sim 3\ \mathrm{L} \cdot \mathrm{week}^{-1}$). The trays were moved each week to minimise any effect of differences in microclimate. The experiments were performed in a climate-controlled room with a temperature of 18 °C, a day/night regime of 15/9 h, a relative humidity of 60% and a light intensity of $250\ \mu\mathrm{mol} \cdot \mathrm{m}^{-2} \cdot \mathrm{s}^{-1}$.

2.2. *Experiment 1: combined effects of phosphate and zinc availability*

Growth of the metallophytes *T. caerulescens* and *S. vulgaris* and the grass *H. lanatus* was tested in a laboratory experiment with a full factorial design for phosphate and zinc availability in the soil. Plants can create various gradients in the availability of phosphate and metals in soil as a consequence of a range of processes induced by the activity of either plant roots or the rhizosphere microflora [23]. Therefore, we decided to carry out our experiments in soil instead of in hydroponics. We decided to create substrates differing in Zn and P availability by mixing soil types. In fact, mixing of soils also happens during flooding events and ploughing of the soil by farmers. The following three soil types were used to create four substrate types differing in Zn and P availability, as indicated in Table 1: river sand from a nature reserve along the River Meuse (poor in phosphate and zinc), agricultural soil from a floodplain along the River Meuse (rich in phosphate and poor in zinc), and metalliferous soil from Plombières (poor in phosphate and rich in zinc). The soils were collected with a shovel at a depth of 0–20 cm. The homogenous river sand and agricultural soil were not sieved. The dry and heterogenous metalliferous soil was sieved ($<2000\ \mu$m). For each substrate type, five individuals per plant species were planted in a black plastic pot (length: 11 cm, width: 11 cm, height: 12 cm). The number of leaves, the length of the longest leaf and the width of the widest leaf were recorded at $t = 0$, 2, 4 and 6 weeks. Estimated shoot size was calculated by multiplication of the number of leaves, the maximum leaf length and width ($W_{\max}$) [24]. Plants were harvested after six weeks. Shoots and roots were separated, dried (24 h at 70 °C) and weighed.

Table 1. Relative contributions (volume %) of river sand, agricultural soil and metalliferous soil in order to create four soil types differing in P and Zn availability.

Treatment	River sand	Agricultural soil	Metalliferous soil
P−Zn−	100	0	0
P−Zn+	75	0	25
P+Zn−	75	25	0
P+Zn+	50	25	25

Note: P− = P-poor; P+ = P-rich; Zn− = Zn-poor; Zn+ = Zn-rich.

Table 2. Parameters of the four soil types ($n = 4$).

	P−Zn−	P−Zn+	P+Zn−	P+Zn+
Organic matter (%)	0.3 (0.03)a	4.4 (0.4)b	0.7 (0.04)a	4.9 (0.3)b
Moisture (%)	18.9 (0.4)a	17.2 (0.2)a	17.6 (0.3)a	17.6 (0.3)a
pH-H_2O	8.42 (0.04)a	7.76 (0.04)a	7.23 (0.15)b	7.07 (0.07)a
Olsen-P	256.6 (4.3)a	420.6 (48.5)a	1596 (82.7)c	1200.4 (61.1)b
Zn-total	0.74 (0.09)b	21.05 (2.40)a	0.75 (0.05)b	32.2 (6.3)a
Ca-total	47.5 (4.3)a	57.1 (6.3)a	48.8 (6.9)a	46.9 (7.0)a
Zn/Ca	0.02 (0.00)a	0.46 (0.06)b	0.02 (0.00)a	0.71 (0.15)b
Zn-DTPA	58.5 (10.9)a	1642.6 (236.5)b	42.6 (2.4)a	1238.7 (118.5)b
NH_4^+–NaCl	8.9 (1.4)a	21.1 (4.3)b	17.3 (0.5)ab	9.1 (1.1)a

Notes: P− = P-poor; P+ = P-rich; Zn− = Zn-poor; Zn+ = Zn-rich. Total concentrations are given in μ mol · g^{-1} dw, extractable concentrations are given in μ mol · kg^{-1} dw. Means and (SEM) are given. Means (within a row) with a same letter do not significantly differ.

Soil mixing may lead to alterations in NH_4^+ versus NO_3^- availability, which may in turn lead to considerable changes in rhizosphere pH and thus Zn availability [25]. However, this effect can be excluded in our experiment because the NaCl-extractable NH_4^+ concentration was very low and pH was high in all soil types used (Table 2). Previous attempts to create these substrates by adding fertiliser and metals failed. As under natural conditions, quick addition of large amounts of fertiliser and metals leads to a shock effect resulting in die back of plants. Only after ageing will a new soil equilibrium establish which is suitable for plant growth (Lucassen, unpublished results).

2.3. *Experiment 2: effect of alkalinisation*

Growth of the metallophyte *T. caerulescens* and the grass *F. rubra* was tested on floodplain soil collected with a shovel (0–40 cm) from a site previously vegetated by metallophytes. The soil was thoroughly mixed and distributed into 10 plastic pots (diameter: 18 cm, height: 10 cm). Half of the pots were spiked with 20 g of $CaCO_3$ which was mixed to decrease the Zn/Ca ratio of the soil to below the critical value of 0.66 characteristic of floodplain soils with zinc-flora growth [21]. Five individuals per plant species were subsequently planted in each pot ($n = 5$). Plant material was collected after three months.

2.4. *Experiment 3: effect of zinc availability in floodplain soils with a moderate phosphate availability*

Growth of the metallophytes *T. caerulescens* and *A. maritima* and the grass *H. lanatus* was tested on floodplain soil collected at a zinc-rich location currently vegetated by metallophytes and at a zinc-poor location vegetated by *H. lanatus*. Previous research has shown that phosphate was mainly accumulated in the first 20 cm of the soil [21]. Soil was collected with a shovel at a depth of

20–30 cm to prevent an interacting effect of differences in phosphate availability on plant growth between the two soil types. For each substrate type, 12 plastic pots (length: 20 cm, width: 11 cm, height 5 cm) were filled with unsieved material. For each plant species, four pots were planted with 10 specimens per sediment type ($n = 4$). The five largest plants were harvested per pot after three months.

2.5. *Field measurements*

In order to determine a realistic range for the elemental composition of metallophytes and grasses, shoot material of several plant species was collected at metalliferous sites in Plombières (Belgium), La Calamine (Belgium) and Epen (the Netherlands) in August 2004.

2.6. *Processing of soil and plant material*

An elemental analysis was carried out to determine the total amount of elements (metals, total-P and total Ca) in the various soil types as well as in the plant material. An amount of 200 mg of dried material (70 °C for 24 h) was digested for 17 min with 4 mL of concentrated nitric acid and 1 mL 30% hydrogen peroxide using a Milestone type mls 1200 Mega microwave. An Olsen-P extraction was carried out to determine the concentration of plant-available phosphorus in the soil [26]. A H_2O-extraction (17.5 g of fresh soil and 50 mL of demineralised water) was carried out to determine the pH of the soil. A diethylene triamine pentaacetic acid (DTPA)-extraction was carried out to determine the concentration of extractable Zn in the soil [27]. A NaCl-extraction (17.5 g of fresh soil and 50 mL 0.01 M NaCl at 100 rpm for 2 h) was carried out to determine the amount of soil-extractable ions in the soil. All extracts were sampled with Teflon rhizon soil samplers (Eijkelkamp Agrisearch, the Netherlands) connected to vacuum nitrogen pre-flushed serum bottles (30 mL). Organic matter content was determined by loss on ignition (550 °C for 4 h).

2.7. *Chemical analysis*

Total concentrations of elements (Zn, Pb, Cd, Ca, Mg, S, Mn, K, Fe, Al, P) in digests, Olsen-P and DTPA-extracts were analysed using an inductively coupled plasma mass spectrophotometer (ICP-MS). Quality assurance measures included blanks, replicate analyses and matrix spikes (standard certified reference chemicals). Recoveries from matrix spikes ranged from 95 to 107%. Repeated analyses did not reveal differences $>5\%$. NH_4^+ and NO_3^- concentrations in the NaCl extracts were analysed with Technicon AA II systems using salicylate [28]. pH-H_2O was measured with a combination pH electrode with an Ag/AgCl internal reference (Orion Research).

2.8. *Data analysis*

Overall effects on plant dry weight and plant composition at the end of the experimental period were analysed using general linear model (GLM) multivariate analyses with design (species and soil type) as the fixed factor. Differences within soil type or treatments were analysed using GLM univariate analyses with soil type or species as the fixed factor. Data on estimated shoot size were analysed using GLM repeated measures. Statistical differences between groups were analysed using Tukey's HSD post-hoc tests. Kolmogorov–Smirnov tests were used in all cases to investigate whether the responses met the parametric assumptions of normality. Homogenity of variance was checked with Levene's test. Parameters on plant composition of experiment 1 and on plant material from the field were subjected to a natural log transformation. SPSS 16.0 was used as the statistical package.

3. Results

3.1. *Experiment 1: combined effects of phosphate and zinc availability*

Results are shown in Table 2 and Figures 1–3. The concentration of plant-available phosphate (Olsen-P) was, as expected, significantly higher in soil types enriched with agricultural soil

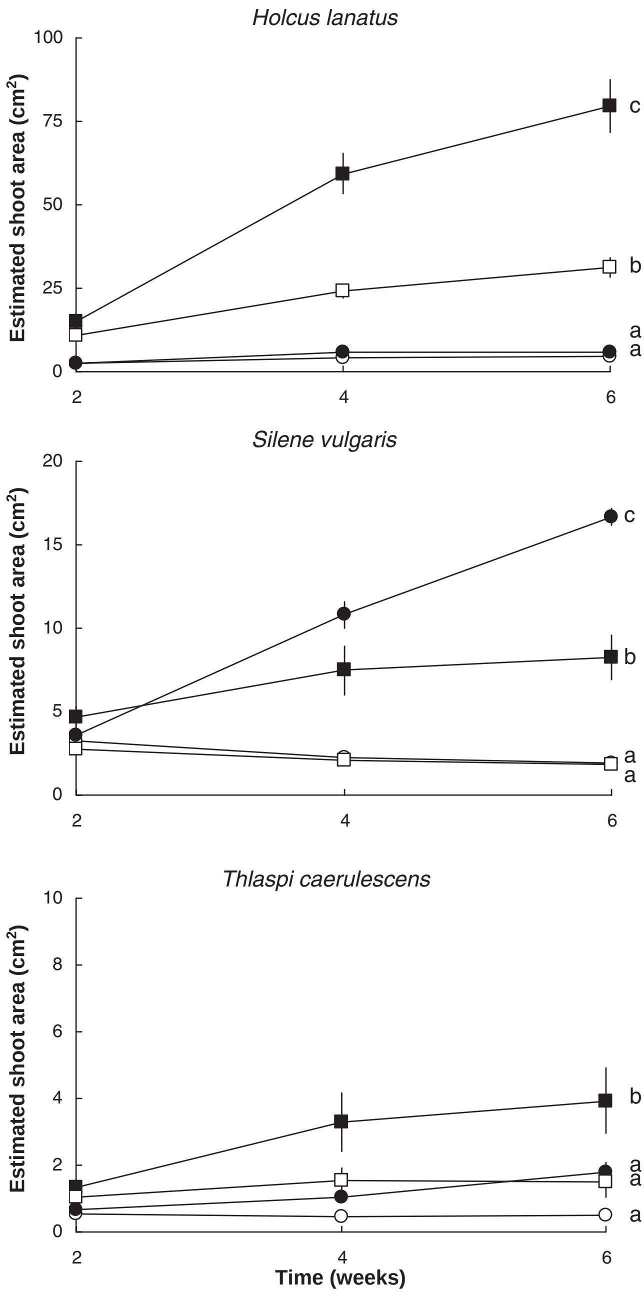

Figure 1. Mean (+SE) estimated shoot area of the grass *H. lanatus* and the metallophytes *S. vulgaris* and *T. caerulescens* growing on soil with a full factorial design for zinc and phosphate availability for 1.5 months. (○) Phosphate- and zinc-poor ($n = 4$). (●) Phosphate-poor and zinc-rich ($n = 4$). (□) Phosphate-rich and zinc-poor ($n = 4$). (■) Phosphate- and zinc-rich ($n = 4$). Means with the same letter do not differ significantly.

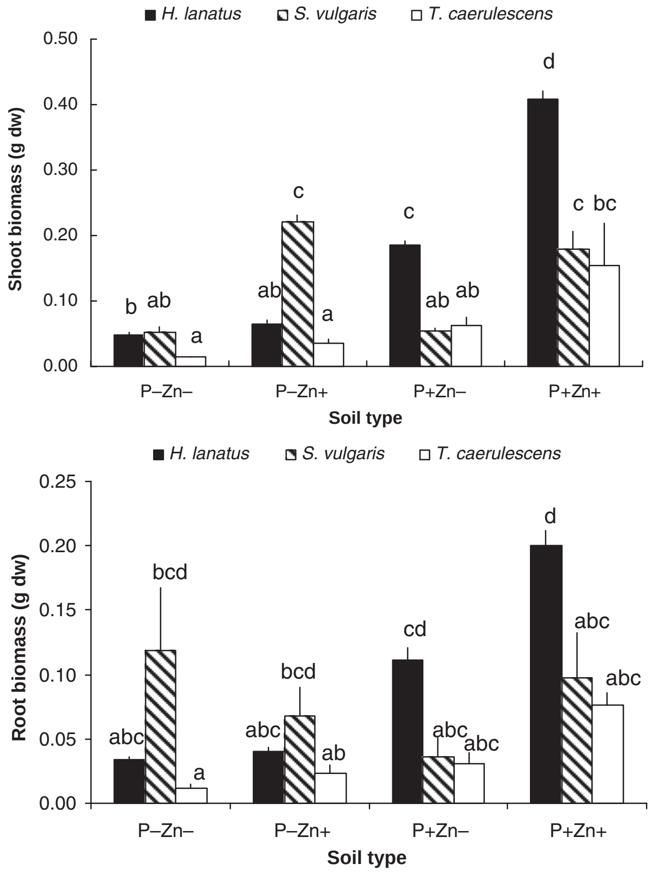

Figure 2. Mean (+SE) shoot and root biomass of the grass *H. lanatus* and the metallophytes *S. vulgaris* and *T. caerulescens* after 6 weeks of growth on soils with a full factorial design for phosphate (P) and zinc (Zn) availability. −, Poor; +, rich. Means with the same letter do not differ significantly.

(P+Zn− and P+Zn+) than in those without agricultural soil (P−Zn− and P−Zn+). The concentrations of total (Zn-total) and extractable zinc (Zn-DTPA) were, as expected, significantly higher in soil types enriched with metalliferous soil (P−Zn+ and P+Zn+) than in those without metalliferous soil (P−Zn− and P+Zn−) (Table 2).

Root and shoot biomass production were significantly affected by soil type ($p < 0.001$), plant species ($p < 0.001$) and their interaction ($p < 0.001$). Shoot biomass of *S. vulgaris* remained low if growing on zinc-poor soil under phosphate-poor as well as phosphate-rich conditions (P−Zn−, P+Zn−). Shoot biomass production increased significantly on zinc-rich soil under phosphate-poor as well as phosphate-rich conditions (P−Zn+, P+Zn+). In this case, the maximum length and width of the leaves was lower and the total length of the plants higher, explaining the lower estimated shoot size of the plants on zinc-rich soil under P-rich conditions compared with P-poor conditions. Shoot biomass production of *T. caerulescens* increased significantly only if growing on soil rich in both zinc and phosphate (P+Zn+). Growth of *H. lanatus* increased significantly if growing on the phosphate-rich soil type, especially in combination with a high zinc availability (P+Zn−, P+Zn−). *H. lanatus* growing on phosphate-rich soil contained the highest estimated shoot sizes of the investigated plant species.

Patterns in shoot biomass development between the various soil types were related to the total concentration of metals (Zn, Pb and Cd) and phosphorus in the shoot material. *T. caerulescens*

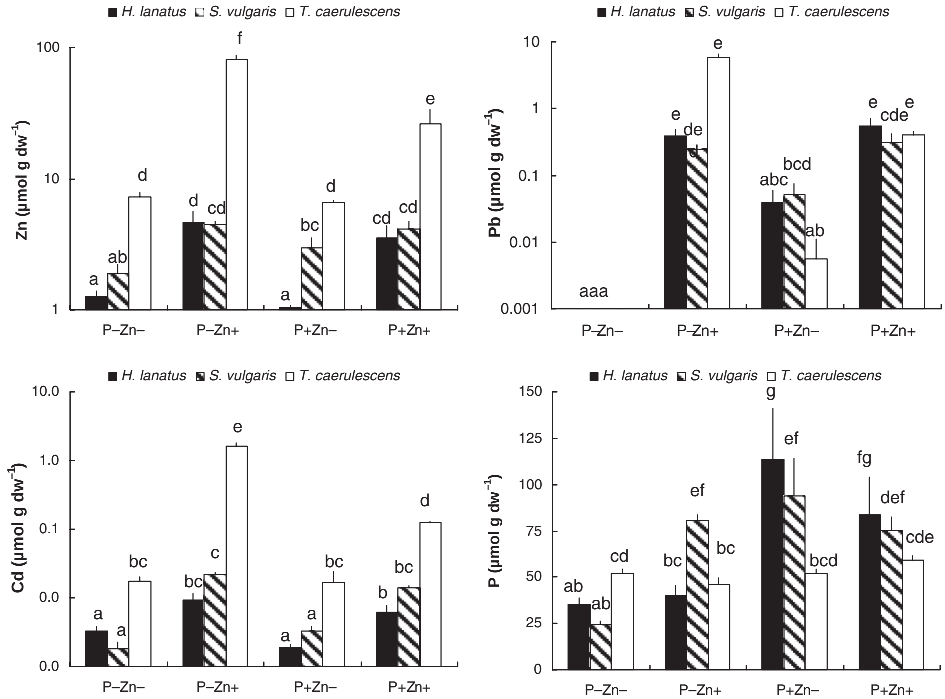

Figure 3. Mean (+SE) concentrations of Zn, Pb, Cd and P in the shoot of the grass *H. lanatus*, and the metallophytes *S. vulgaris* and *T. caerulescens* growing on soil with a full factorial design for phosphate (P) and zinc (Zn) availability for 1.5 months. −, Poor; +, rich. Means with the same letter do not differ significantly.

grew best on zinc-rich soil rich in phosphate (P+Zn+). Under that condition, its shoots contained combined high concentrations of zinc and phosphorus. *S. vulgaris* grew best on zinc-rich soil independent of the phosphorus content. Under both conditions, its shoots contained combined high concentrations of zinc and phosphorus. The grass *H. lanatus* grew best on both phosphate-rich soil types. Under both conditions its shoots contained high concentrations of phosphorus, whereas, by contrast to the two metallophyte species, zinc concentrations in the shoots were variable.

3.2. *Experiment 2: effect of alkalinisation*

The results of experiment 2 are shown in Table 3 and Figure 4. The total Ca concentration in the floodplain soil increased significantly from 42 to 245 μmol · g^{-1} dw due to liming. As a result, the

Table 3. Soil characteristics of the floodplain soil (previously dominated by metallophytes) with and without $CaCO_3$ addition.

	$-CaCO_3$ ($n = 5$)	$+CaCO_3$ ($n = 5$)
Zn-total	53.4 (0.3)	52.1 (0.5)
Ca-total	41.9 (0.6)	244.7 (9.9)**
Zn/Ca	1.27 (0.03)	0.21 (0.01)**
pH-H_2O	6.55 (0.10)	7.29 (0.17)*
Olsen-P	247.7 (15.2)	240.2 (10.2)
Zn-DTPA	6111 (282)	4202 (71)**

Notes: Total concentrations are given in μmol · g^{-1} dw, extractable concentrations are given in μmol · kg^{-1} dw. Mean and (SEM) are given. *$p < 0.05$; **$p < 0.001$.

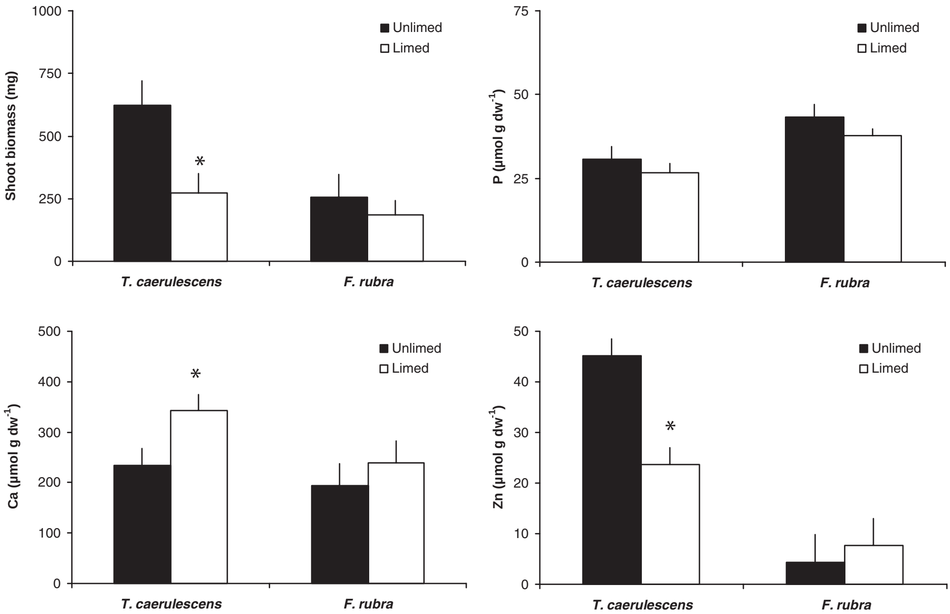

Figure 4. Mean (+SE) shoot biomass development and concentrations of P, Ca and Zn in the shoot of the metallophyte *T. caerulescens* and the grass *F. rubra* growing on limed (Zn/Ca $= 0.21$, $n = 5$) and unlimed (Zn/Ca $= 1.27$, $n = 5$) floodplain soil with a low P availability (Olsen-P = 240–250 $\mu mol \cdot kg^{-1}$ dw) for 3 months. The soil (total Zn = 52–53 $\mu mol \cdot g^{-1}$ dw) originated from a location previously dominated by metallophytes. $^{*}p < 0.05$.

Zn/Ca ratio decreased significantly from 1.27 to 0.21 and the concentration of extractable zinc (Zn-DTPA) decreased significantly from 6100 to 4200 $\mu mol \cdot kg^{-1}$ dw. Shoot biomass development was significantly affected by soil type ($p = 0.026$) and plant species ($p = 0.018$), but not by their interactive effect ($p = 0.118$). Shoot biomass for *T. caerulescens* decreased significantly due to liming, whereas shoot biomass for the grass *F. rubra* did not. Decreased growth in *T. caerulescens* was accompanied by a significant increase in the concentration of Ca and a significant decrease in the concentration of Zn in the shoot. There were no significant differences in the concentration of other elements measured in the shoot including Fe (data not shown).

3.3. *Experiment 3: effect of Zn availability in floodplain soils with a moderate P availability*

The results of experiment 3 are shown in Table 4 and Figure 5. The Zn-poor floodplain soil showed a significantly lower Zn/Ca ratio although the total concentration of Ca was significantly higher in this soil type. The availability of nutrients did not differ significantly between soil types. The amount of extractable Zn (Zn-DTPA) was, as expected, significantly higher in the Zn-rich soil than in the Zn-poor soil. Shoot biomass development was significantly affected by soil type ($p = 0.004$), plant species ($p = 0.042$) and their interactive effect ($p = 0.032$). On the Zn-poor soil, *T. caerulescens* developed a significantly lower shoot biomass compared with the Zn-rich soil. The biomass development of *A. maritima* showed a similar trend but the effect was not significant between the two soil types ($p = 0.167$). Growth of the grass *H. lanatus* was not affected by the Zn concentration of the soil ($p = 0.672$). The concentration of Zn, Mg, P and S in the shoots differed significantly between the two soil types for most of the plant species. There were no such significant differences in the concentrations of other elements measured. The concentration of Zn in shoots was significantly lower in *A. maritima* and *H. lanatus* if growing on Zn-poor soil. *T. caerulescens* did not develop significant differences in Zn concentration in its shoots between

Table 4. Soil characteristics of two floodplain soil types with a low P availability (collected at a depth of 20–30 cm) differing in Zn concentration.

	High Zn ($n = 12$)	Low Zn ($n = 12$)
Organic matter (%)	8.3 (0.3)	8.0 (0.2)
Moisture (%)	21.1 (1.5)	20.3 (0.5)
Zn-total	50.0 (0.6)	2.3 (0.05)**
Ca-total	44.4 (1.1)	89.8 (0.5)*
Zn/Ca	1.14 (0.04)	0.03 (0.00)**
pH-H_2O	5.95 (0.10)	6.34 (0.14)
Olsen-P	401.2 (22.3)	421.2 (12.6)
Zn-DTPA	2567 (35)	101 (5)**

Notes: Total concentrations are given in μmol g^{-1} dw, extractable concentrations are given in μmol kg^{-1} dw. Mean and (SEM) are given. *$p < 0.05$; **$p < 0.001$.

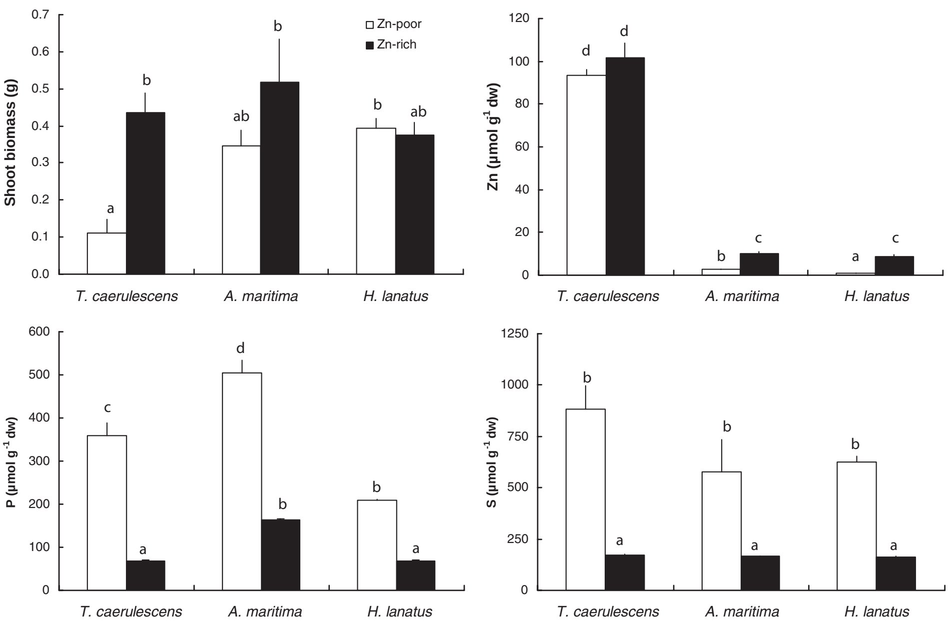

Figure 5. Mean (+SE) shoot biomass development and concentration of Zn, P and S in the shoot of the grass *H. lanatus* ($n = 4$) and the metallophytes *T. caerulescens* ($n = 4$) and *A. maritima* ($n = 4$) growing on Zn-poor (white bars: 2 μmol · g^{-1} dw) and Zn-rich (black bars: 50 μmol · g^{-1} dw) floodplain soil with a moderately high P availability (Olsen-P = 400–420 μmol · kg^{-1} dw) for 3 months. Means with a same letter do not significantly differ.

both soil types. For all plant species, the concentration of Mg in their shoots was higher if growing on the Zn-rich soil (results not shown). For all plant species, the concentration of P and S was significantly higher if growing on the Zn-poor soil.

Concentrations of elements in the shoot material of the plant species under field conditions are given in Figure 6. *T. caerulescens* showed a much wider range in the concentration of metals (Zn, Pb and Cd) as well as in the concentrations of Ca and S compared with the other plant species. The mean concentrations of Zn, Pb and Cd in the shoots were significantly higher in *T. caerulescens* compared with the other plant species. There were no significant differences in metal concentrations in the shoots between *S. vulgaris*, *A. maritima* and *H. lanatus*. The Ca concentrations in the shoots were lower in *H. lanatus* compared with metallophyte plant species. There were no significant differences in the concentration of phosphorus in the shoots of the four plant species.

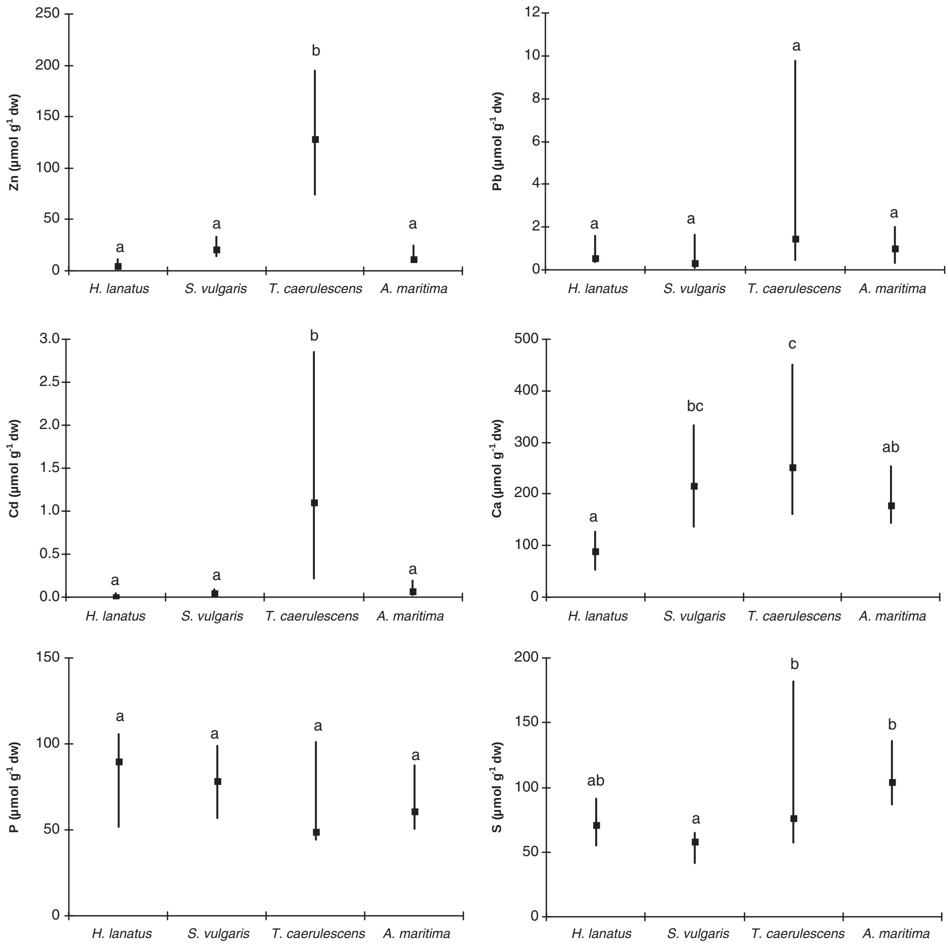

Figure 6. Shoot elemental composition of the grass *H. lanatus* ($n = 6$) and the metallophytes *S. vulgaris* ($n = 6$), *T. caerulescens* ($n = 6$) and *A. maritima* ($n = 8$) under field conditions. Total range and median level are given. Means with the same symbol do not differ significantly.

4. Discussion

The laboratory experiments suggest that growth of metallophytes in tertiary polluted floodplain grasslands of the River Geul may be affected by the availability of zinc and phosphate in the soil. At a relatively low zinc availability (due to decreased zinc application or alkalinisation) biomass development in metallophytes decreased strongly. At a relatively high phosphate availability, growth of the more competitive grass *H. lanatus* was strongly enhanced, irrespective of the zinc concentration in the soil.

The experiment with a full factorial design for phosphate and zinc availability in the soil indicated that metallophytes, such as *S. vulgaris* and *T. caerulescens* which are dominant at metal-rich habitats, only performed well if growing on metal-rich soils, as indicated by several ecological field studies [29–31]. The better performance of *T. caerulescens*, originally from a metal-rich habitat, with high Zn supply has also been shown in hydroponics [32]. However, nutritional requirements of metallophytes can differ and thus responses to soil fertilisation due to agricultural practices may differ. We showed that *S. vulgaris* only increased in shoot biomass if growing on zinc-rich soil, irrespective of phosphate availability (Figures 1 and 2). Under these conditions, shoots of *S. vulgaris* contained high concentrations of metals (Zn, Pb and Cd) combined with high concentrations of phosphorus (Figure 3). *T. caerulescens* only showed an increase in biomass on zinc-rich soil if combined with a high phosphate availability (Figure 1). Under this condition, shoots of *T. caerulescens* also contained high concentrations of metals (Zn, Pb and Cd) combined with high concentrations of phosphorus (Figure 3). However, the grass *H. lanatus* did not grow on soils poor in phosphate and strongly increased in biomass in phosphate-rich soil, irrespective of zinc availability (Figures 1 and 2). The chemical composition of the shoots confirmed that, in contrast to the investigated metallophytes, growth of *H. lanatus* did not depend upon the combination of high phosphorus and high zinc availability. *H. lanatus* plants growing on soil rich in phosphate but poor in zinc performed well and developed high concentrations of phosphorus in combination with low concentrations of metals (Zn, Pb and Cd) in its shoots. These results indicate that *H. lanatus* will easily outcompete the slow-growing metallophyte vegetation when phosphate availability increases.

It is well known that metallophytes of *Violetum calaminariae* Schwickenrath have a high zinc demand and can suffer Zn-deficiency where Zn availability decreases due to the rapid physiological inactivation of zinc [29,33]. Ecotypes of *H. lanatus* tolerant to zinc toxicity also tolerate zinc deficiency [34]. This is in agreement with our findings. Ecotypes of *H. lanatus* are Zn tolerant because of a strong accumulation of Zn in the roots. By means of this tolerance mechanism, Zn-tolerant ecotypes of *H. lanatus* can even perform better on soils with low Zn availability compared with Zn-sensitive ecotypes [34]. The relatively high zinc demand of metallophytes was also shown by the significantly lower biomass development of *T. caerulescens* growing on floodplain soil poor in Zn ($2\,\mu mol \cdot g^{-1}dw$) compared with growing on floodplain soil rich in Zn ($50\,\mu mol \cdot g^{-1}$ dw). The biomass of *A. maritima* showed this trend although it was not significantly different. By contrast, biomass development in *H. lanatus* was obviously unaffected by the Zn content of the soil (Figure 5). In *A. maritima* and *H. lanatus*, the Zn concentration in the shoot was significantly lower if grown on the Zn-poor soil. This was not evident for *T. caerulescens* which is an hyperaccumulator [35]. In all investigated plant species, the very low Zn availability in the soil was accompanied by the development of extremely high concentrations of P and S in the shoots (Figure 5). The availability of P (Olsen-P) and the total S concentration in the two soil types were, however, equal (Table 4). The P concentration in the shoots of plant species growing on Zn-rich floodplain soil were comparably high compared with concentrations in plants growing under field conditions (Figure 6). However, if growing on the Zn-poor soil, P concentrations in the shoots of *T. caerulescens* and *A. maritima* were 5–10 times higher than in plants growing under field conditions. For *H. lanatus* growing on Zn-poor soil, the P concentration in the shoots was only two times higher than for plants growing under field conditions. From solution culture experiments carried out with a high phosphorus but low Zn supply, it appeared that zinc deficiency is often associated with an exceptionally high phosphorus content and even symptoms of phosphorus toxicity in mature leaves [36–40]. The much higher phosphorus content in shoot dry matter of zinc-deficient plants supplied with high phosphorus concentrations can, to some extent, be attributed to a 'concentration effect', particularly in older leaves. Comparable effects have been found in experiments carried out with barley (*Hordeum vulgare* L. cv Weeah) in soil and it was concluded that Zn plays a specific role in the signal-transduction pathway responsible for the regulation of genes encoding high-affinity transporters in plant roots [41]. The effect of zinc deficiency on sulfur uptake is probably comparable with phosphorus. Phosphorus toxicity levels might vary between plant species. P toxicity levels of 1% of its dry weight for tomato (*Solanum lycopersicum* L.) plants were found [42]. It was stated that P contents $>2\%$ of its dry weight can be generally considered toxic to plants [43]. The P concentrations were 1.55 and 1.11% for *A. maritima* and *T. caerulescens*, respectively, whereas the concentration was 0.62% in *H. lanatus*. This might indicate that the metallophytes have suffered P toxicity under laboratory conditions. Under natural conditions, plants will very likely not survive on these soil types as they are slow growing, small-sized plants and thus weak competitors.

Liming can decrease the plant availability of Zn much more than of any other mineral nutrient, including P, and may enhance the risk of Zn deficiency in plants [44,45]. The results of the liming experiment indicated that liming led to decreased Zn availability, as reflected by the lower DTPA-extractable Zn fraction, although P-availability (Olsen-P) in the soil remained unaffected (Table 3). The addition of lime results in a reduced DTPA-extractable Zn fraction by increasing the Zn fraction bound to iron hydroxides at the cost of the exchangeable-Zn fraction and the Zn fraction bound to organic matter [46]. At high pH, Zn availability is limited by the low solubility of carbonates and hydroxides [47]. The floodplains along the River Geul are very rich in iron (400 μmol $\cdot$ g^{-1} dw) and it is therefore likely that adsorption to iron hydroxides, next to the formation of Zn carbonates, plays an important role in the solubility of Zn in the soil [21]. At low pH (5.0) no Zn is bound to hydrous ferric iron, whereas at high pH (7.0) all Zn is bound and insoluble [48]. In addition, it was shown that Ca inhibits Zn uptake in metallophytes by competition for the uptake of these elements at the roots [49]. Several authors concluded that the Zn/Ca ratio in soil is a better measure for expressing Zn availability than the total Zn concentration in the soil [50–52]. Overall, liming of floodplain soil resulted in the development of significantly higher Ca and a significantly lower Zn concentrations in the shoots of *T. caerulescens*, accompanied by a lower biomass development. By contrast, growth of the grass *F. rubra* remained unaffected. This is in agreement with the findings of the first experiment showing the zinc demand of metallophytes and zinc independence of grasses. Liming can also reduce the availability of iron. In our experiment, the concentration of Fe^{2+} in the pore water decreased although not significantly (from 225 ± 35 to 127 ± 19 μmol $\cdot$ L^{-1}; $p = 0.085$). We also did not find a significant decrease in the concentration of iron in the shoot material for any of the three investigated plant species following liming (results not shown).

The laboratory experiments suggest that the decline in metallophyte vegetation in tertiary polluted floodplain grasslands of the River Geul may have been caused by the decreasing zinc availability and/or increasing P availability in the grasslands occurring since the closure of the metal industry and intensification of agricultural activities in the area in the previous century. Pyrite-bearing mine wastes at neutral or slightly alkaline pH can oxidise within months or a few years to produce extreme acidity [53] and in the past will have led to acidification of the grasslands after flooding events. Because of the closure of the mining industry, potentially acidifying pyrite particles were no longer present in the river water or had become inactive after some years [54]. A higher alkalinity in the soil, leading to a lower Zn availability, will have been additionally stimulated by the increased use and application of artificial lime-containing fertiliser in agriculture [19]. As a result of these processes, growth of metal-demanding metallophyte vegetation may have been hampered. Owing to the increased application of fertiliser leading to increased P availability, more competitive grasses including *H. lanatus* and *F. rubra* could have become dominant irrespective of the zinc concentration in the soil. A study carried out at 23 sites in the Harz Mountains in Germany indicated that the specific metallophyte vegetation of relatively metal-rich secondary polluted mine spoils was not only controlled by metal availability but also by low soil fertility. It was shown that metallophyte vegetation types with some *H. lanatus* cover were positively correlated with nitrogen availability in the soil [30]. This indicates that increasing nutrient inputs might be a threat to metallophyte vegetation in general. It is likely that metallophytes may be more exposed to threat in low-metal polluted soils like tertiary polluted floodplain grasslands than at high metal-polluted soils (like secondary polluted mine spoil areas) as the first may be more easily reused for agricultural purposes.

Earlier field investigations and the current laboratory investigations indicated that removal of the P- and Ca-enriched top soil layer might be an option to restore metallophyte vegetation in floodplain grasslands with a high Zn content in the soil profile (>40 μmol $\cdot$ g^{-1} dw and Zn/Ca > 0.8).

In fact, small-scale restoration experiments carried out in floodplains of the River Geul have shown that removal of the alkaline and phosphate-rich soil top layer enables recolonisation of introduced metallophytes and suppression of grasses at least during the first four years after soil removal [21,22].

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