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1 Does the stress tolerance of mixed grassland communities change in a
2 future climate? A test with heavy metal stress (zinc pollution)

3
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1 **Abstract**

2 Will species that are sensitive/tolerant to Zn pollution still have the same sensitivity/tolerance in
3 a future climate? To answer this question we analysed the response of constructed grassland
4 communities to five levels of zinc (Zn) supply, ranging from 0 to 354 mg Zn kg⁻¹ dry soil, under
5 a current climate and a future climate (elevated CO₂ and warming). Zn concentrations increased
6 in roots and shoots with Zn addition but this increase did not differ between climates. Light-
7 saturated net CO₂ assimilation rate (A_{sat}) of the species, on the other hand, responded differently
8 to Zn addition depending on climate. Still, current and future climate communities have
9 comparable biomass responses to Zn, i.e. no change in root biomass and a 13% decrease of
10 above-ground biomass. Provided that the different response of A_{sat} in a future climate will not
11 compromise productivity and survival on the long term, sensitivity is not altered by climate
12 change.

13

14

15 **Capsule**

16 This study is the first to examine plant responses to a heavy metal (Zn) in a changing climate,
17 and shows that the tolerance of plants to Zn stress will not be altered in a future climate.

18

1 **Introduction**

2 Zinc contamination of soils creates an important environmental problem. Zinc enters the soil for
3 example through mining and smelting activities, land application of sewage sludge or traffic.
4 Since heavy metals are very persistent in the soil and the contamination appears to be quite
5 permanent (half life of Zn 1000-3000 years) (Kabata-Pendias and Pendias, 2001), contaminated
6 soils will continue to be a problem in the coming decades when the global climate is likely to
7 change drastically (IPCC, 2007). If not stabilized or removed, metals can be dispersed in the
8 environment through water and wind erosion and through percolation. Different remedial
9 techniques such as in situ extraction, leaching or ex situ stabilization exist, but these techniques
10 are very costly and some of them disturb the structure and functioning of the soil (Mulligan et
11 al., 2001). Phytostabilisation or revegetation can offer an inexpensive, environmentally friendly
12 solution. This method aims to prevent the soil with the present pollutant from dispersing through
13 wind or water and, to prevent the pollutant from leaching to groundwater. The success of
14 revegetation depends totally on the metal tolerance of the selected plant species (Gardea-
15 Torresdey et al., 2005; van der Lelie et al., 2001). The question arises whether species that are
16 successful today will still have the same tolerance in a future climate?
17 In the current climate, *Lolium perenne* L. has been found a suitable species (Arienzo et al., 2004;
18 Bidar et al., 2007; Pichtel and Salt, 1998) except for the fact that it has a high nitrogen (N)
19 demand (Soussana et al., 2005). The amount of N in the polluted soil determines largely how
20 long plants can sustain on these unfertilized soils. Therefore, adding N₂-fixing species to the
21 plant community could benefit the establishment of a vegetation in the long term. As suggested
22 by Remon *et al.* (2005) the use of indigenous species, combined into a self sustainable vegetation
23 cover, would be the most effective for site restoration, provided that the species thrive on metal

1 contaminated soils. In addition, Olson and Fletcher (2000) put forward that after screening and
2 field testing, it is unlikely that the same species will be found equally useful under different
3 climatic conditions.

4 In the near future, plant communities are likely to grow in a substantially different environment
5 with higher temperatures and CO₂ concentrations (IPCC, 2007). So far, these factors have never
6 been studied in combination with Zn pollution, while they can change aspects of basic plant
7 functioning that might alter the plant's response to Zn pollution.

8 The influx of Zn into plants could change under climate change for several reasons. Organic
9 matter exerts a major control on the availability of metals to plants (McBride et al., 1997). If the
10 soil contains a large amount of soil organic matter (SOM), a stimulation of mineralization under
11 warming might increase the release of Zn (Antoniadis and Alloway, 2001). On the other hand,
12 Zn uptake could change when the magnitude of the root surface is modified; elevated CO₂
13 increases root biomass (Cotrufo and Gorissen, 1997; Hebeisen et al., 1997) while warming
14 reduces it (De Boeck et al., 2008; Soussana et al., 1996); or when the intrinsic root Zn uptake
15 rate, which is characterized by Michaelis-Menten kinetics (Broadley et al., 2007), increases due
16 to warming. Whether or not more Zn enters plants in a future climate, their Zn tolerance will still
17 depend on the relation between Zn tissue concentration and plant functioning. Zinc that has
18 entered the shoot can disturb plant functioning through negative effects on several aspects of
19 photosynthesis, e.g. chlorophyll content, ribulose-1,5-bisphosphate carboxylase/oxygenase
20 (RubisCO) activity or photochemical processes (Monnet et al., 2001; Vanassche and Clijsters,
21 1986a, b). Then again, in a future climate, higher CO₂ concentrations generally stimulate
22 photosynthesis (Drake et al., 1997) which could overcome any down regulation by Zn.

1 To study Zn tolerance in a future climate, grassland communities were subjected to different
2 levels of Zn addition either under current conditions or under simultaneous elevated CO₂ and
3 warming. We exposed plant communities with multiple species, such that responses arose both
4 from the species-specific sensitivities and interspecific interactions. The two advantages of this
5 choice: (i) the complexity of real communities is approximated, and (ii) changes in species
6 composition of the communities can be studied, outweigh the shortcoming that the intrinsic
7 responses of the species in the absence of competitors or facilitators cannot be determined. The
8 current study focuses particularly on the following hypotheses: (i) climate change exacerbates
9 the response of grassland communities to Zn addition by stimulating Zn uptake; (ii) climate
10 change mitigates the stress response of grassland communities to Zn addition by reducing
11 negative effects of Zn on photosynthesis.

12

13

1 **Material and Methods**

2 *Experimental set-up*

3 This research is part of a larger experiment in which synthesized grassland communities are
4 subjected to different stressors in a future climate. The current paper will focus on Zn addition to
5 the soil. The experiment is located at the Drie Eiken Campus, University of Antwerp, Wilrijk,
6 Belgium (51° 09' N, 04° 24' E), where average annual precipitation is 776 mm (evenly
7 distributed throughout the year) and average annual air temperature 10.8 °C. The grassland
8 communities were grown in six sunlit, climate-controlled chambers, facing south. The distances
9 between the chambers were maximized to avoid mutual shading. The interior surface area was
10 1.5 x1.5 m, the height at the north side 1.5 m and at the south side 1.2 m. The top of the
11 chambers consisted of a colourless polycarbonate plate (4 mm thick), whereas the sides were
12 made of polyethylene film (200 µm thick), both UV transparent. Three of the six chambers
13 tracked the current climate with current air temperature (T_{air}) and CO₂ concentration, while the
14 other three chambers were exposed to a future climate scenario with 3 °C warming and a target
15 CO₂ concentration of 620 ppm (further referred to as 'current' and 'future climate', respectively).
16 Every half hour, T_{air} was monitored with a combined humidity–temperature sensor (Siemens,
17 type QFA66, Erlangen, Germany). During the experiment (7 May – 4 October 2007), monthly
18 average outside T_{air} was 14.5, 17.6, 17.4, 17.4 and 14.6 °C in May, June, July, August and
19 September, respectively. In the current climate chambers T_{air} was on average 0.3 ± 1.6 °C (SD)
20 higher than outside, while the future climate chambers were 3.3 ± 2.1 °C (SD) warmer than
21 outside. The CO₂ concentration was measured and regulated with a CO₂ control group with a
22 CO₂ analyser (WMA-4, PPSystems, Hitchin, UK). In the current climate chambers the

1 concentration was 375 ± 17 ppm (SD) while in the future climate chambers it was within 10%
2 and 20% of the target of 620 ppm during 84.4% and 95.6% of the time, respectively.
3 Each chamber contained 30 grassland communities with the same species composition in PVC
4 containers (24 cm inner diameter, 40 cm height), filled with sandy soil (93.2% sand, 4.6% silt,
5 2.2% clay; field capacity $0.13 \text{ m}^3 \text{ m}^{-3}$; pH 7.6; total Kjeldahl-N 0.42 g kg^{-1} ; 1% C in humus;
6 background Zn concentration 21.4 mg kg^{-1} dry soil; cation exchange capacity (CEC) 3.9 meq
7 100 g^{-1} fresh weight). The containers were placed side-by-side so that a close canopy could form,
8 and were buried in the soil to avoid unnatural soil temperatures. Irrigation was calculated from
9 the monthly rainfall over the period 1995-2005 and corrected for the differences in
10 evapotranspiration (ET) inside and outside the chambers. To this end, De Boeck *et al.* (2006)
11 calculated ET inside current climate chambers from changes in soil water content (SWC) and the
12 amount of administered water, and the outside ET with Hamon's equation (Haith and
13 Shoemaker, 1987) based on day length, vapour pressure and T_{air} . The containers were watered
14 every two days according to the 10 year average of 14 to 15 raining days per month during the
15 growing season. Total monthly irrigation matched 61.5, 64.4, 85.1, 80.2, 80.9 and 69.7 mm in
16 May, June, July, August, September and October, respectively. The future climate chambers
17 received the same amount of water as the current climate chambers, so that any enhanced
18 consumption would result in aggravated soil drought. Water could freely drain from the
19 containers while capillary rise was prevented by a drainage system placed below the chambers.
20 Profile probe tubes for the PR2 soil moisture sensor (Delta-T Devices Ltd., UK) were installed in
21 each community and SWC was measured every 10 days between 22 June and 29 September
22 2007. Soil water content was not altered by the climate scenario ($F_{1,4} = 1.95$, $p = 0.235$;
23 ANCOVA; see *Data analysis*), did not change during the growing season ($F_{1,5} = 0.03$, $p = 0.880$)

1 and was on average 12.0%, which was well above wilting point (6.9%) and below field capacity
2 (13%).

3 In each chamber five containers were randomly chosen to develop five Zn treatments, yielding
4 three replicates (chambers) per treatment. The lowest Zn level (no Zn added) had two additional
5 containers per chamber available from other experiments. Zinc was mixed under the soil 40 days
6 before the seedlings were transplanted to the containers, so that the soil could stabilize. Per
7 container 240 ml with different doses of a ZnCl₂ stock solution was administered, resulting in
8 five Zn concentrations added; 0, 12.5, 20.8, 29.2 and 41.7 g Zn L⁻¹ (further referred to as level 0,
9 1, 2, 3 and 4, respectively). In addition, all communities were fertilized with 15 g m⁻² NH₄NO₃,
10 7.5 g m⁻² P₂O₅, 15 g m⁻² K₂O and micro-elements (19 mg m⁻² Fe, 10 mg m⁻² Mn, 3 mg m⁻² Zn, 2
11 mg m⁻² Cu, 7 mg m⁻² B, 1 mg m⁻² Mo). The fertilizer was given dissolved in water (assuring no
12 percolation), in four equal amounts evenly spread over the growing season.

13

14 *Grassland communities*

15 The plant communities were established between 7 and 10 May 2007 by transplanting five-
16 week-old seedlings. Each community contained 18 plants and was composed of six equally
17 represented species from three functional groups (two species per group): grasses (*Lolium*
18 *perenne* L., *Poa pratensis* L.), non-N₂-fixing dicots (*Plantago lanceolata* L., *Rumex acetosa* L.)
19 and N₂-fixing dicots (*Lotus corniculatus* L., *Medicago lupulina* L.). All these species have a
20 perennial life cycle, co-occur in temperate European grasslands and have comparable heights.
21 The 18 plants (three per species) were placed in a hexagonal grid with a 4.5 cm interspace.
22 Interspecific interactions were maximized by avoiding clumping and by using five different
23 designs. The five designs were randomly distributed over the treatments.

1

2 *Biomass and metal concentrations*

3 Above-ground (shoot and stubble) and below-ground (root) biomass was harvested at the end of
4 the growing season (1 to 4 October 2007). Above-ground biomass was subdivided by species.
5 Total below-ground biomass per community was estimated from 12 soil cores (2 cm diameter)
6 per container. To adequately represent the total root biomass in the soil, six cores were taken
7 directly below the plants (one per species) and six cores in the middle of a triangle between three
8 plant positions. Root samples were washed until they were free of soil. All plant material was
9 dried at 70 °C for 48 hours and then weighed.

10 Concentrations of Zn in roots (all levels), Zn in shoots (levels 0, 2 and 4) and Mg in shoots (only
11 *L. perenne* and *M. lupulina*, levels 0, 2, and 4) were measured after microwave digestion with
12 HNO₃ and H₂O₂ (see *Discussion* for importance of Mg). Soil samples were collected at the end
13 of the experiment (5 October 2007), oven dried at 70 °C and analysed for Zn after digestion by
14 warming with HNO₃ and H₂O₂. Metal concentrations were determined using Mass Spectrometry
15 (ICP-MS, Finnigan Element XR, Scientific, Bremen, Germany) for the plant samples and
16 Atomic Emission Spectrophotometry (ICP-AES, iris/CID, Thermo Jarrell Ash, MA, USA) for
17 the soil samples.

18

19 *Gas exchange*

20 Photosynthetic and fluorescence characteristics were measured as universal stress indicators at
21 two moments in the growing season (20-24 August and 17-21 September). Light-saturated net
22 CO₂ assimilation rate (A_{sat}) was determined on the most recently matured leaves of each species
23 in each Zn treatment and chamber (n = 3) with a portable gas exchange system (LI-6400, Li-

1 COR, NE, USA). Leaf chamber conditions were controlled at 380 ppm CO₂ and 23.5 °C (block
2 temperature) in current climate and at 620 ppm and 26.5 °C in future climate, both at saturating
3 photon flux density (1500 μmol m⁻² s⁻¹) and ambient relative humidity.

4

5 *Data analysis*

6 The experiment had a split plot design with climate (future vs. current) as the main plot factor
7 and Zn treatment (further referred to as ‘zinc’) as the subplot factor. Zinc concentrations in
8 shoot, roots and soil, Mg concentrations, biomass and gas exchange measurements were
9 analysed with ANCOVA, translocation factors (see *Results*) with ANOVA. Analyses were
10 performed in SAS (version 9.1, SAS Institute Inc., Cary, NC) using the mixed procedure (Littell
11 et al., 1996) with climate, zinc (co-variable in ANCOVA) and, if appropriate species and
12 measurement period, as fixed factors and chamber as a random factor nested within climate. Soil
13 water content was analysed with repeated measures ANCOVA with time as co-variable (repeated
14 in time). Non-significant terms were excluded from the analyses. The random chamber effect
15 was only significant on SWC and on shoot and root zinc concentration. In case of significant
16 interactions a posteriori analyses were performed: for ANCOVA the parameter estimates were
17 analysed with a t-test and, for ANOVA the means were compared including Tukey corrections
18 for multiple comparisons.

19

1 **Results**

2 *Plant and soil metal concentrations*

3 The achieved Zn concentrations in the soil ($[Zn_{soil}]$) are summarized in Table 1. Addition of Zn
4 increased $[Zn_{soil}]$ (Table 1, 2a), which subsequently increased the root Zn concentration ($[Zn_{root}]$)
5 of the communities (Table 1, 2a). Zn was also translocated to the above-ground plant parts as the
6 shoot Zn concentrations ($[Zn_{shoot}]$) of each species increased as well, with magnitudes depending
7 on species (significant zinc \times species interaction, a posteriori analyses, $p < 0.05$ for each species;
8 Table 2b, Fig. 1). Translocation factors (TF) were calculated for levels 0, 2 and 4 as the ratio of
9 $[Zn_{shoot}]$ (at community level) to $[Zn_{root}]$ (Fig. 2). With Zn addition (Table 2a) TFs were lower
10 than with no Zn added (level 0), indicating that relatively more Zn was restrained in the roots.
11 The future climate neither affected $[Zn_{soil}]$, $[Zn_{root}]$, $[Zn_{shoot}]$ and TF, nor their response to Zn
12 addition (climate and zinc \times climate never significant; $[Zn_{soil}]$, $[Zn_{root}]$, TF (Table 2a); $[Zn_{shoot}]$
13 (Table 2b, Fig. 1)).

14 The Mg concentration increased with increasing zinc addition for *L. perenne* (from level 0 to 4,
15 from 2.34 ± 0.25 to 2.87 ± 0.43 (SE) g Mg kg⁻¹ dry soil; $F_{1,13} = 7.94$, $p = 0.014$) while it did not
16 for *M. lupulina* (3.57 ± 0.37 (SE) g Mg kg⁻¹ dry soil on average over all levels; $F_{1,12} = 0.24$, $p =$
17 0.635). This was found in both climates (no significant zinc \times climate interaction, $F_{1,12} = 3.49$, p
18 $= 0.086$ and $F_{1,11} = 0.21$, $p = 0.657$ for *L. perenne* and *M. lupulina*, respectively). The Zn/Mg
19 concentration ratio did not differ between the climates (no zinc \times climate interaction, $F_{1,25} = 0.33$,
20 $p = 0.568$), for both species.

21

22 *Community and species biomass*

1 Above-ground biomass of the grassland communities decreased by 13% upon soil Zn addition
2 (from 1.50 ± 0.27 to 1.24 ± 0.13 (SE) kg m^{-2}). Root biomass, on the other hand, did not change,
3 despite the increasing Zn concentration in the roots (0.74 ± 0.23 (SE) kg m^{-2}) (Table 3a). To
4 determine whether the decrease in above-ground biomass was general, it was analysed by
5 species. A shift between the two most productive species was detected: *M. lupulina* declined,
6 while *L. perenne* increased upon Zn addition (significant zinc \times species interaction (Table 3b,
7 Fig. 3); a posteriori analyses, $t_{227} = -5.22$, $p < 0.001$ and $t_{227} = 1.99$, $p = 0.048$, respectively).
8 Apparently the increase of *L. perenne* did not completely compensate for the decrease of *M.*
9 *lupulina*. Similar to the plant and soil Zn concentrations, the future climate neither affected
10 above-ground or root biomass, nor their responses to Zn addition (climate and zinc \times climate
11 never significant (Table 3)).

12

13 *Gas exchange*

14 The shift between the two species upon Zn addition, as described above, strongly suggests that
15 *M. lupulina* is more sensitive to Zn addition than *L. perenne*. This was confirmed by a decrease
16 of its net CO₂ assimilation rate (A_{sat}) upon Zn addition, whereas the other species did not respond
17 (significant zinc \times species interaction (Table 4, Figs. 4 and S1); a posteriori analyses, $t_{343} = -2.89$,
18 $p = 0.004$ for *M. lupulina*, $p > 0.05$ for the other species). The future climate did change the
19 response of A_{sat} to Zn addition (significant zinc \times climate interaction (Table 4)). This is visible in
20 Figs. 4 and S1 where the advantage of a future climate for A_{sat} at low Zn levels disappears at
21 higher Zn levels.

22

23

1 **Discussion**

2 The tolerance of grassland communities to Zn addition could change in a future climate if future
3 climate conditions either change the influx of Zn to the communities or their sensitivity to Zn. If
4 the influx of Zn was altered by climate, it would be reflected in changed Zn concentrations,
5 provided that biomass did not alter comparably and in this manner diluted the enhanced Zn
6 uptake. In our experiment, Zn concentrations in the roots and shoots did not differ between the
7 climates (Fig. 1, Table 1, 2). In addition, the biomass response to Zn addition, i.e. no response of
8 root biomass and a 13% decrease of above-ground community biomass, did not differ between
9 the climates either (Table 3). Influx of Zn into plants could increase in a future climate if
10 availability or uptake of Zn is altered. Although pH has been found a good predictor for metal
11 bioavailability (Rieuwerts et al., 2006), other soil properties, such as organic C content and CEC,
12 contribute substantially in determining availability (Degryse et al., 2003). The low CEC in our
13 soil probably made Zn highly available in both climates (see further). Moreover, the fact that Zn
14 was given dissolved in water and the low organic C content of our soil most likely excluded
15 increased Zn release due to any enhanced mineralization in a future climate. To study effects of
16 climate change on Zn bioavailability, experiments that include biogeochemical analyses should
17 be conducted on soils where Zn is associated in more complex, organical forms. However, the
18 fact that bioavailability was not altered by climate change in our experiment, does not exclude an
19 altered uptake or sensitivity of the plant communities. If root growth is stimulated in a future
20 climate the increased root-soil interface could result in a higher Zn uptake. Except, the absolute
21 amount of Zn taken up would increase and not the Zn concentration, because of a dilution by the
22 increasing biomass. Then again, root biomass did not differ between the two climates (Table 3).
23 Besides, Zn uptake per unit root, which is characterized by Michaelis-Menten kinetics (Broadley

1 et al., 2007), could be stimulated by higher soil temperatures in a future climate, which would
2 result in higher root Zn concentrations. Nevertheless, no differences in root Zn concentrations
3 between the climates were found.

4 Root to shoot translocation factors were smaller on Zn contaminated soils than on control soils
5 (Fig. 2). This reflects the existence of physiological mechanisms that regulate internal
6 translocation so that Zn is mainly accumulated in the roots (Santibanez et al., 2008). These
7 mechanisms worked equally well in both climates (Table 2a) and thus shoot Zn concentrations
8 did not differ between the climates (Table 2b).

9 Climate change clearly did not stimulate Zn uptake, but did it alter the plant or community
10 response to Zn tissue concentrations? In future climate, A_{sat} was more sensitive to Zn addition
11 than in current climate (Fig. 4, Table 4). An explanation for this increased sensitivity can be
12 found in the functioning of RubisCO. RubisCO is substrate-limited by the current atmospheric
13 CO_2 concentration; therefore, it has the potential to respond to increasing CO_2 and promote
14 photosynthesis (Long et al., 2004). However, under Zn pollution Zn replaces Mg at the RubisCO
15 carboxylation sites, which decreases the enzyme's affinity for CO_2 (Monnet et al., 2001;
16 Vanassche and Clijsters, 1986a). Increasing the Mg concentration has been found a protection
17 mechanism against excess of Zn (Monnet et al., 2001). Since the Zn/Mg concentration ratio did
18 not differ between the climates, relatively equal fractions of the active carboxylation sites were
19 inactivated by replacement of Mg in both climates. This means that if in future climate more
20 sites were activated by increased CO_2 (Long et al., 2004), the absolute amount of active sites
21 inactivated by Zn would be larger than in current climate, resulting in an increased sensitivity to
22 Zn addition in future climate. As mentioned above, the difference in response of A_{sat} to the
23 climates was not reflected in the species or community biomass response (Table 3, Fig. 3). This

1 can be because of different reasons, e.g. the response of A_{sat} is not necessarily translated in a
2 response of A at any moment and light intensity, assimilated carbon is not only invested in
3 growth but also in maintenance and protection, possible differences in community carbon losses
4 (e.g. respiration, exudates) are not taken into account.

5 The individual species of the grassland communities differed in their sensitivity to increasing soil
6 Zn concentrations. The above-ground biomass of *M. lupulina* declined upon Zn addition while
7 for four of the five other species it did not respond (Table 2b, Fig. 3). The positive response of *L.*
8 *perenne*, is most likely caused by the fact that the advantage of a larger resource availability, due
9 to the decline of *M. lupulina*, is stronger than any possible drawback by Zn addition.

10 Furthermore, A_{sat} decreased for *M. lupulina*, while it did not for *L. perenne* (Table 4, Fig. 4). The
11 increasing Mg concentration in *L. perenne* could have had a protective effect (Monnet et al.,
12 2001), while for *M. lupulina* the Mg concentration did not increase. The results of the individual
13 species indicate that *M. lupulina* is more sensitive to Zn addition than the other species. In other
14 words, *M. lupulina* will not thrive very well on Zn polluted soils, neither in current nor in future
15 climate, while the other species will be tolerant to Zn in both climates.

16 In this study, which is to our knowledge the only study that combines warming, elevated CO_2
17 and Zn addition, we found that, although the uptake of Zn did not change, the response of A_{sat} to
18 Zn addition did change in a future climate. However, this change in A_{sat} was not reflected in an
19 altered biomass response to Zn addition in a future climate.

20 Studies on Zn stress are mostly conducted in lab conditions with plants grown on hydroponic
21 solutions (e.g. Monnet et al., 2001), but Zn behaviour in soils is mainly determined by
22 environmental factors such as pH, soil texture, organic matter and CEC (Kabata-Pendias, 2004),
23 which determine the bioavailability for plants (Broadley et al., 2007). Therefore it is necessary to

1 study effects of Zn pollution under more natural conditions. For this study we used unpolluted
2 soils to which Zn was added, instead of polluted soils with different degrees of contamination.
3 This has the advantage that Zn can be added gradually and that Zn is the only variable which
4 determines the plant responses. Polluted soils, e.g. around metallurgical industrial activities or
5 with sewage sludge deposited grasslands, typically contain combinations of several heavy metals
6 (Bidar et al., 2009; Hillman et al., 2003; Santibanez et al., 2008).

7 The highest soil Zn concentrations achieved in this experiment were between 300 and 350 mg Zn
8 kg⁻¹ dry soil (Table 1). We realize that those concentrations are below the critical soil
9 concentration (above which toxicity is probable) of 400 mg Zn kg⁻¹ as considered by Kabata-
10 Pendias and Pendias (2001). In addition, they are not as high as most concentrations found in
11 polluted soils (Arienzo et al., 2004; Bidar et al., 2007; Maiz et al., 2000; Remon et al., 2005).

12 However, the low CEC capacity of our soil allowed us to keep Zn (ZnCl₂) addition low, to avoid
13 concurrent salt stress. The low CEC ensured a high Zn bioavailability, which is more important
14 for plants than bulk [Zn_{soil}]. Moreover, according to Maiz *et al.* (2000), the concentration found
15 in plant material is a good indication for the metal bioavailability in the soil. In our experiment
16 the [Zn_{shoot}] values (299 ± 50 (SE), average over all species) were comparable with or even
17 higher than those found in similar species on soils with higher Zn pollution levels in the
18 previously mentioned studies (e.g. Arienzo et al., 2004; Bidar et al., 2007; [Zn_{shoot}] 93 ± 6 and
19 296 ± 15 (SE), respectively). This indicates the value of our experiment as a screening study of
20 future plant responses to Zn addition. However, to predict ecosystem responses on more complex
21 soil types, further validation with swards from polluted soils exposed to combined CO₂
22 enrichment and temperature increase, should be conducted. These studies should include
23 determination of soil processes, such as the microbial control of SOM, since these are likely to

1 alter under climate change (Zak et al., 2000). Such studies should be conducted on the long term
2 since soil microbes could adapt to future climate conditions (Bradford et al., 2010), which would
3 counteract any increased metal availability. Furthermore, arbuscular mycorrhizal fungi (AMF),
4 which have been found to provide protection against heavy metal stress (Leyval et al., 1997),
5 ought to be taken into account too. Warming and elevated CO₂ both stimulate AMF colonization
6 (Rillig et al., 1999; Rillig et al., 2002), which could increase stress tolerance. In addition, long
7 term studies should determine whether the altered response of A_{sat} in a future climate might be
8 reflected in an altered biomass response on the longer term. Besides, the response of plant
9 communities to Zn stress might change if the species composition alters over time, especially if
10 the abundance of N₂-fixers would decrease.

11

12

13

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1 Table 1: Zinc concentration in the soil ($[Zn_{soil}]$, $\mu\text{g Zn g}^{-1}$ dry matter) and the roots ($[Zn_{roots}]$,
 2 mg Zn kg^{-1} dry soil) for five levels (0 to 4) of Zn added (g Zn L^{-1}) as ZnCl_2 in two climates
 3 (current climate and future climate with elevated CO_2 and higher air temperatures). Means (1
 4 SE) ($n = 3$).

level	Zn added	$[Zn_{soil}]$		$[Zn_{roots}]$	
		current	future	current	future
0	0	25.7 (2.4)	22.4 (1.3)	33.1 (4.4)	31.1 (3.1)
1	12.5	94.2 (2.4)	101.7 (0.2)	253.1 (44.6)	272.6 (15.4)
2	20.8	136.2 (17.1)	206.2 (14.6)	469.7 (169.7)	325.8 (71.2)
3	29.2	189.7 (23.0)	180.3 (8.3)	554.1 (104.3)	568.2 (146.6)
4	41.7	354.4 (60.0)	309.7 (56.7)	967.3 (217.0)	899.7 (119.0)

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1 Table 2: Statistical analyses of a) Zn concentration in the soil ($[Zn_{soil}]$) and the roots ($[Zn_{roots}]$)
 2 and translocation factors (TF; the ratio of Zn concentration in the shoot ($[Zn_{shoot}]$) to $[Zn_{root}]$) and
 3 b) $[Zn_{shoot}]$, of grassland communities subjected to five levels of Zn addition (between 0 and 354
 4 mg Zn kg⁻¹ dry soil) and two climates (current climate and future climate with elevated CO₂ and
 5 higher air temperatures). Zn concentrations were analysed with ANCOVA, TF with ANOVA.
 6 $[Zn_{shoot}]$ and TF were determined for levels 0,2 and 4. $[Zn_{shoot}]$ was analysed by species. Degrees
 7 of freedom (DF), F values and significance levels (p); p-values are presented in bold when
 8 significant (< 0.05).

9 a)

	$[Zn_{soil}]$			$[Zn_{roots}]$			TF		
	DF	F	p	DF	F	p	DF	F	p
zinc	1,26	111.52	<0.001	1,5	70.96	<0.001	2,12	9.83	0.003
climate	1,26	0.05	0.826	1,4	0.00	0.995	1,12	0.79	0.391
zinc x climate	1,25	0.57	0.457	1,4	0.07	0.805	2,10	0.45	0.651

13 b)

	$[Zn_{shoot}]$		
	DF	F	p
zinc	1,5	171.18	<0.001
climate	1,4	0.11	0.759
species	5,73	0.53	0.753
zinc x species	5,73	13.26	<0.001
zinc x climate	1,4	0.20	0.679
climate x species	5,68	1.14	0.347
zinc x climate x species	5,63	0.67	0.645

1 Table 3: Statistical analysis (ANCOVA) of a) root and above-ground (shoot + stubble) biomass
 2 at community level and b) above-ground biomass by species, of grassland communities
 3 subjected to five levels of Zn addition (between 0 and 354 mg Zn kg⁻¹ dry soil) and two climates
 4 (current climate and future climate with elevated CO₂ and higher air temperatures). Degrees of
 5 freedom (DF), F values and significance levels (p); p-values are presented in bold when
 6 significant (< 0.05).

7 a)

	biomass					
	roots			above-ground		
	DF	F	p	DF	F	p
zinc	1,37	0.53	0.470	1,37	6.89	0.012
climate	1,37	0.00	0.962	1,37	0.52	0.477
zinc x climate	1,36	0.66	0.420	1,36	0.14	0.708

10 b)

	above-ground biomass		
	DF	F	p
zinc	1,227	4.11	0.044
climate	1,227	0.31	0.579
species	5,227	47.23	<0.001
zinc x species	5,227	5.77	<0.001
zinc x climate	1,221	0.03	0.862
climate x species	5,221	2.19	0.056
zinc x climate x species	5,216	0.45	0.814

14

1 Table 4: Statistical analysis (ANCOVA) of light-saturated net CO₂ assimilation rate (A_{sat}) of
 2 leaves of grassland species subjected to five levels of Zn addition (between 0 and 354 mg Zn kg⁻¹
 3 dry soil) and two climates (current climate and future climate with elevated CO₂ and higher air
 4 temperatures), measured at two moments in the growing season (20-24 August and 17-21
 5 September). Degrees of freedom (DF), F values and significance levels (p); p-values are
 6 presented in bold when significant (< 0.05). Interactions with measurement period were never
 7 significant and therefore not shown.

	A _{sat}		
	DF	F	p
zinc	1,343	4.56	0.033
climate	1,343	22.80	<0.001
species	5,343	16.90	<0.001
period	1,343	6.23	0.013
zinc × species	5,343	3.15	0.008
zinc × climate	1,343	4.48	0.035
climate × species	5,338	1.27	0.276
zinc × climate × species	5,326	0.81	0.540

9
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1 Figure 1. Zinc concentration in the aboveground biomass of the individual species of the
2 grassland communities for three of the five levels of Zn addition (between 0 and 354 mg Zn kg⁻¹
3 dry soil; level 0, 2 and 4) and in current (□, ---) or future (■, —) climate. Means ± 1 SE (n = 3
4 (chambers)). Individual values were used for linear regressions (n = 9).

5
6 Figure 2. Translocation factors (TF) of the grassland communities, calculated as the ratio of the
7 community shoot to root zinc concentration, for three of the five levels of Zn addition (between 0
8 and 354 mg Zn kg⁻¹ dry soil; level 0, 2 and 4 (shoot Zn concentrations were not determined in
9 levels 1 and 3)) and in current (□) or future (■) climate. Means ± 1 SE (n = 3 (chambers)).

10
11 Figure 3. Above-ground biomass of the individual species of the grassland communities
12 subjected to five levels of Zn addition (between 0 and 354 mg Zn kg⁻¹ dry soil) and to current
13 (□, ---) or future (■, —) climate. Means ± 1 SE (n = 3 (chambers)); at level 0, n = 9 (3
14 chambers, 3 replicates per chamber). Individual values were used for linear regressions (n = 21).

15
16 Figure 4. Light-saturated net CO₂ assimilation rate (A_{sat}) of leaves of grassland species subjected
17 to five levels of Zn addition (between 0 and 354 mg Zn kg⁻¹ dry soil) and to current (□, ---) or
18 future (■, —) climate. Only the results of the first measurement period (20 – 24 August, 2007)
19 are shown, for the second measurement period see Supplementary information (Fig. S1). Means
20 ± 1 SE (n=3). Individual values were used for linear regressions (n = 15).

21

1 **Supplementary information**

2 Figure S1. Light-saturated net CO₂ assimilation rate (A_{sat}) of leaves of grassland species
3 subjected to five levels of Zn addition (between 0 and 354 mg Zn kg⁻¹ dry soil) and to current
4 (□, ---) or future (■, —) climate. Results of the second measurement period (17 – 21
5 September, 2007) are shown. Means ± 1 SE (n=3). Individual values were used for linear
6 regressions (n = 15).