Impacts of soil-soluble anions on wild and cultivated herbaceous species: implications for soil phytoremediation

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Abstract

This study addresses the growing problem of soil salinity. In Mediterranean regions, disused solid waste landfills have given rise to disturbed ecosystems. At such sites, both mobile soil anions and cations have toxic effects on plants. Thus, detailed knowledge of these effects is needed for the phytoremediation of soils facing the problem of salinization. In this study, high chloride levels were detected in soil samples from several sealed waste landfill sites showing metal pollution. We also examined the nutrition behaviour of six native and five cultivated herbaceous species (two forage species and three for human consumption) grown in soils containing zinc chloride. Based on the tolerance shown by *Lolium rigidum* to this salt in a first bioassay, we assessed its behaviour in the presence of other chlorides and other Zn salts (sulphates and nitrites). Besides providing plant anion concentrations (which are particularly novel results), our study identified *Lolium rigidum* as a species able to well-tolerate soil salinity and accumulate high levels of zinc chloride indicating its possible use for the phytostabilization of soils polluted with this salt.

Keywords: Soil and plant anions, mineral nutrition, phytotoxicity, phytoremediation

1. Introduction

Many ecosystems are in weak equilibrium and highly vulnerable to human interference. Human actions give rise to problems such as reduced nutrient availability with the consequence of diminished soil productivity and soil pollution. In many disturbed ecosystems, the balance of mobile anions controls the entire ion equilibrium. Anion cycles are

fragile and constantly perturbed by abiotic, biotic and, above all, human factors. Hence, any study of mineral cycles should commence with exploring the proportions of soluble anions transported by water in disturbed and natural ecosystems.

Salinity has been one of the main impacts of leachates arising from uncontrolled waste landfills,

affecting soils and adjacent water systems in both the central region of the Iberian Peninsula (Hernández *et al.*, 1998; 1999; Pastor and Hernández, 2012) and other regions (Fatta *et al.*, 1999; Tatsi and Zouboulis, 2002; Cheng and Chu, 2007; Kalčíková *et al.*, 2012). Surface or subsurface leachates can affect both neighbouring soils and wet zones and water courses where such waste is usually deposited, making this the main route of pollution spread. The bioaccumulation of pollutants in the surrounding biota is another way that landfill leachates can affect the environment and its human or animal inhabitants.

Despite evidence of high salinity in this type of setting, most studies to date have focused on the impacts of heavy metals and organic compounds (Adarve *et al.*, 1998; Gutiérrez-Ginés *et al.*, 2014) or the effects of leachates on the growth of various plant species (Hernández *et al.*, 1999; Cheng and Chu, 2007; Kalčíková *et al.*, 2012) or animals (Pastor *et al.*, 1993). Further, while numerous studies have analysed the effects of soil salinity on cultivated plants, most have centred on the effects of NaCl, and results are presented from a physiological perspective of mineral nutrition or the biomolecular framework of the stress suffered by the plants (Cornillon and Palloix, 1997; Hall and Williams, 2003; Sharma and Dietz, 2009). Far less common have been studies centring on other chlorides or anions frequently found in soils affected by waste landfills, and anion concentrations in plant tissues have been rarely determined (Hussain and Alquwaizany, 2014; Kováčik *et al.*, 2014).

Despite such gaps in the available literature, the topic of phytostabilization of soil covers at landfill sites will require the study of species that will adapt well to the climate and eco-chemical conditions of each environment. This prompted our interest in investigating the polluting actions of salts often found in landfill soils. In effect, these soils are adequate substrates for the transfer of oligonutrients to plants with possible toxic

effects. As a consequence of crop irrigation mainly, soil salinization is a growing problem worldwide. There is therefore a need to improve current understanding of the impacts of salts other than sodium chloride on the plants these soils sustain.

This study was designed to identify the ions produced by surface leachates in the discharge zones of solid waste landfills that cause soil salinization and to determine the effects of different Zn and Cl salts on both grassland and cultivated plant species.

2. Materials y Methods

2.1. Landfill sites and sampling

The sites sampled for this study were 15 sealed solid waste landfills, which have been the target of on going work by our research group (Pastor and Hernández, 2010). The landfills overlie substrates representative of the central zone of mainland Spain, and their soils show an acidity-alkalinity gradient. These substrates are granite in 3 of the sites, arkoses in a further 3, limestone in 8 and gypsum in the remaining site. Despite their different locations, the landfills have in common that they were exploited during the second half of the 20th century in an uncontrolled manner. Waste materials (mostly mixed industrial/urban) were dumped without any form of pretreatment and the land receiving this waste had in place no impermeabilization or leachate collection system. In the 1980's and 1990's these landfills were gradually sealed. However, sealing consisted solely of applying a layer no more than 50 cm-thick of debris and soil from the surrounding area. No further restoration measures were adopted. Most of the landfills have steep slopes (usually more than 50%), flat areas at the top and leachate discharge areas which usually coincide with rivers, streams or even wetlands. After their capping, the landfill sites have been used for cereal cultivation, as grazing land,

as hunting grounds or even reused for further uncontrolled waste disposal.

For the present study, sampling areas were randomly selected from the landfills' lower slopes. These zones receive surface runoff and are those mostly grazed by sheep and rabbits. The number of soil samples obtained from each landfill was proportional to its size. Factors affecting vegetation such as orientation and slope gradient were also taken into account.

Given the large presence at the sites of Gramineae (Pastor and Hernández, 2008), specimens of 3 species of this family were taken from the same collection sites as the soils to examine their element composition. These data were compared with prior data obtained in the same species growing in grasslands of the same region that lacked landfills (Pastor *et al.*, 2012).

2.2. Greenhouse bioassays

As described in prior work (Pastor *et al.*, 2014), chloride is the anion that has been mostly related to soil salinity. This anion is not a plant's basic nutrient as are nitrates, nitrites and phosphates. For this reason we chose chloride to study its possible effects on plants. Zinc was selected as the main metal detected in many landfill soil samples (Pastor *et al.*, 2014). Zinc chloride is a soluble salt that may be easily incorporated into soil to provide different concentrations of the metal.

The species selected for the bioassays were wild species present in abundance at the sealed landfills grazed by sheep in late winter/early spring: the grasses *Lolium rigidum*, *Bromus rubens* and *Hordeum murinum*, which grow in both acidic and basic soil covers, the leguminous species *Trifolium subterraneum* and *Lupinus angustifolium*, which grow on lower slopes with acidic soils; and *Juncus buffonius*, which is often abundant in early spring, especially following copious autumn rains. The early leaf stages of this species are appetizing

for itinerant sheep that graze the lower landfill slopes. We also selected species that are usually sown on high flat areas and discharge areas of landfills such as wheat (*Triticum aestivum*, variety Anza) and barley (*Hordeum vulgare*, variety Volley), along with sunflower (*Helianthus annuus*), which is often sown following cereal harvesting in this Mediterranean setting. Also included in our study were the forage leguminous species white lupin (*Lupinus albus*) and vetch (*Vicia sativa*), also often grown in this region. The seeds of wild species were collected from plants spontaneously growing at the experimental station "La Higuera" (Toledo, Spain), which belongs to the Spanish Research Council, Consejo Superior de Investigaciones Científicas (CSIC). The seeds of cultivated species were also obtained from this station, such that they were all well-adapted to the soil and climate types.

Since acidic soils determine a greater availability of Zn for plants, the soil selected was an acidic soil of similar grain size and properties to the landfill soils (Pastor and Hernández, 2010) also obtained from "La Higuera" station. Soil properties are provided in Table 1.

The soil collected was dried at room temperature and sieved through a 2 mm sieve. To this soil, ZnCl_2 was added as one application of a solution prepared in deionized water to give final concentrations of the salt of 0, 100, 300, 500 or 700 mg/kg. The microcosms were set up in pots containing 1 kg of soil as three replicates per treatment. Seeds were germinated in Petri dishes in a growth chamber and 3 seeds introduced into the pots once germinated. The bioassay was run in a greenhouse (temperature 17.4-24.5 °C and humidity 60-70%) for 3 months in the case of the cultivated species and for 5 months for the wild species. The pots were watered daily with 100 ml of deionized water.

At the end of the bioassay, the plants were cut at ground level, washed in deionized water, dried in an oven at 70 °C for 48 h and their dry weights determined.

Table 1. Chemical properties of the soil used in the bioassays

Variable	Value	Nutrient	Value
pH	5.3	Ca (T)	743
OM	1.5	Ca (B)	725
EC	86	K (T)	2282
NO ₂ ⁻ (a)	1.1	K (B)	194
NO ₃ ⁻	7.8	Mg (T)	1273
Cl ⁻	11	Mg (B)	109
F ⁻	1.3	Fe (T)	5665
PO ₄ ³⁻	14	Fe (B)	40
SO ₄ ²⁻	39	Zn (T)	11
Na (T)	94	Zn (B)	2.0
Na (B)	8.6		

(a) anion concentration in mg/kg

OM = organic matter (%); EC = electrical conductivity ($\mu\text{S}/\text{cm}$); T = pseudototal concentration (mg/kg); B = exchangeable/bioavailable concentration (mg/kg).

According to the results of this bioassay in which *Lolium rigidum* grew well in all the treatments, in a second bioassay we subjected this species to higher ZnCl₂ concentrations (900, 1100, 1300 and 1500 mg/kg). The species was also grown in the control soil containing 700 mg/kg of other Zn salts (Zn (NO₃)₂ and ZnSO₄) and other chlorides (CaCl₂, FeCl₃, KCl, MgCl₂ and NaCl) to determine whether effects on growth and Zn and Cl accumulation depended on the salt. This second bioassay was run for 3 months in the same manner and under the same greenhouse conditions as for Bioassay 1.

2.3. Sample preparation and chemical analyses

Dried and sieved soil samples both from the land-fill sites and those used in the bioassays (control

soil) were analysed to determine their chemical properties. For the control soil, we determined pH in slurry and organic matter by potassium dichromate reduction (Hernández and Pastor, 1989). Soil anion concentrations (NO₂⁻, NO₃⁻, Cl⁻, F⁻, PO₄³⁻ and SO₄²⁻) were measured by ion chromatography. The remaining elements were quantified by ICP-OES. Soil samples were subjected to extraction with a 4:1 mixture of HNO₃ and HClO₄ (Walsh and SSSA, 1971) to determine pseudototal metal concentrations; to extraction with 1 M ammonium acetate at pH 7 for exchangeable Ca, Mg, K and Na; and to extraction with EDTA and ammonium acetate at pH 4.5 for bioavailable concentrations of the remaining metals (Lakanen and Ervio, 1971). Dry biomass samples were ground in an IKA-WERKE Yellow Line A10 grinder. Concentrations of Ca, K, Mg, Na, Fe and Zn were determined by ICP-OES following their prior extraction with a mixture of HNO₃ and HClO₄ (Walsh and SSSA, 1971). To measure plant anion concentrations, the method of Ghosh and Drew (1991) was modified as: 100 mg plant material were mixed with 10 ml of deionized water and 0.1 ml of a 1% Tween-20 solution and left to rest for 30 minutes. The suspension was then mixed for 3 minutes in an ultrasound bath, placed on a hot plate and brought to the boil for 2 minutes. Once cool, the suspension was filtered through a nylon 0.45 μm sieve and the extract made up to 25 ml and subjected to ion chromatography on a Dionex 4500i.

Plant specimens were also subjected to scanning electron microscopy (SEM) to visualize possible effects on leaf tissue morphology and estimate cell element contents using the method described in Gutiérrez-Ginés *et al.* (2012).

2.4. Data analysis

Plant element data were compared between the landfill and control sites using the Student's *t*-test. The results of the bioassays were compared between treatments by ANOVA. Data showing a non-normal distribution or not fulfilling the requirement of homoscedasticity were log transformed. Ad hoc comparisons were performed by the Bonferroni method. All statistical tests were conducted using the software package SPSS 22. In addition, nutrient ratios (N/P, Ca/P, K/Na, Mn/Fe and Fe/Zn) were calculated for the above-ground plant samples.

3. Results

3.1. Landfill soil salinity and plant nutrients

Table 2 shows the soluble anion levels detected in the capping soils of the fifteen landfill sites. Although only landfills 14 and 15 overlaid high salinity substrates, most landfills showed large differences between minimum and maximum EC values and anion concentrations. Of note, maximum EC values for landfills 3, 7 and 14 were more than 50 times higher than the minima, and Cl^- , NO_3^- and SO_4^{2-} were the anions whose minimum and maximum concentrations varied most (300 to 7000 times higher). At some sites F^- , NO_2^- and PO_4^{3-} levels were also elevated. However, at all landfill sites, Cl^- , NO_3^- and SO_4^{2-} were the anions of most concern in terms of causing high salinity.

Element composition differences between specimens growing in landfills and those inhabiting grasslands were in many cases significant (Table 3). Despite the important problem of salinity in most of the landfills (Table 2), plants did not reflect higher concentrations of Na or Ca in their

leaves. Most notable, were the greater Zn contents of landfill plant samples, followed by Mn and Fe concentrations. In addition, the N and Ca concentrations detected in above-ground masses of these species were higher in the grasslands.

3.2. Effects of Zn chloride on wild and cultivated species

Only two of the 11 plant species tested (*B. rubens* and *L. rigidum*) were unaffected by increasing ZnCl_2 in the soil (Table 4). The plants *H. murinum*, *V. sativa*, *T. subterraneum*, *J. buffonius*, *H. annuus* and *L. angustifolius* were unable to grow in the soils showing the highest ZnCl_2 concentrations. Although *T. aestivum*, *H. vulgare* and *L. albus* did manage to grow at this high concentration of the salt, their growth was fairly compromised as indicated by the dry weights recorded at the end of the experiment.

Zinc and chloride levels in the above-ground biomass of the species examined were considerably higher when these were exposed to higher concentrations of these elements (Tables 5 and 6), indicating their bioavailability and easy translocation to stems and leaves. *T. aestivum* and *H. vulgare* showed the greater accumulation of Zn and Cl^- , though also showed signs of toxicity. This was also observed in *L. albus* and even more evidently in *H. annuus*, which showed above-ground levels of 3700 mg/kg of Zn and 41 g/kg of Cl^- when exposed to 300 mg/kg of the salt, and in *L. angustifolius*, showing close to 2500 mg/kg of Zn and 41 mg/kg of Cl^- when exposed to 500 mg/kg of ZnCl_2 .

It should be highlighted that the species *L. rigidum* showed no signs of toxicity despite accumulating a mean of 3600 mg/kg of Zn and 37 g/kg of Cl^- when exposed to the maximum concentration of the salt. In contrast, *B. rubens* was the species that was least able to accumulate Zn and Cl^- .

Table 2. Electrical conductivity (EC, $\mu\text{S}/\text{cm}$) and anion concentrations (mg/kg) of soil samples from 15 landfills

Landfill		EC	F ⁻	Cl ⁻	NO ₂ ⁻	NO ₃ ⁻	PO ₄ ³⁻	SO ₄ ²⁻
Granite substrate								
#1	M $\pm$ sd (a)	510 $\pm$ 73	0.3 $\pm$ 0.0	12 $\pm$ 3.3	2.1 $\pm$ 1.2	491 $\pm$ 199	9.2 $\pm$ 4.1	62 $\pm$ 8.6
N = 4	Range	431 - 606	0.3 - 0.4	7.9 - 15	0.8 - 3.7	363 - 783	5.1 - 13	53 - 73
# 2	M $\pm$ sd	525 $\pm$ 126	0.3 $\pm$ 0.0	9.1 $\pm$ 0.5	1.6 $\pm$ 0.7	365 $\pm$ 144	10 $\pm$ 5.0	33 $\pm$ 9.7
N = 4	Range	401 - 669	0.3 - 0.4	8.5 - 9.7	1.1 - 2.6	202 - 544	6.2 - 17	25 - 46
# 3	M $\pm$ sd	1158 $\pm$ 1909	1.3 $\pm$ 2.2	777 $\pm$ 1866	0.6 $\pm$ 0.5	381 $\pm$ 556	4.9 $\pm$ 7.3	219 $\pm$ 354
N = 12	Range	129 - 6690	0.0 - 8.1	19 - 6310	0.0 - 1.4	4.1 - 1848	0.0 - 22	22 - 1157
Arkosic substrate								
# 4	M $\pm$ sd	434 $\pm$ 522	0.8 $\pm$ 1.0	26 $\pm$ 22	2.0 $\pm$ 2.4	336 $\pm$ 702	16 $\pm$ 16	49 $\pm$ 72
N = 5	Range	96 - 1346	0.0 - 2.5	5.0 - 59	0.0 - 6.1	0.8 - 1591	7.3 - 46	14 - 178
# 5	M $\pm$ sd	486 $\pm$ 422	1.0 $\pm$ 1.7	59 $\pm$ 47	1.4 $\pm$ 3.9	14 $\pm$ 30	4.4 $\pm$ 6.9	592 $\pm$ 932
N = 53	Range	63 - 2480	0.0 - 12	6.1 - 182	0.0 - 28	0.0 - 162	0.0 - 31	10 - 6060
# 6	M $\pm$ sd	173 $\pm$ 89	1.3 $\pm$ 1.1	36 $\pm$ 36	1.9 $\pm$ 0.9	37 $\pm$ 73	2.0 $\pm$ 4.3	67 $\pm$ 46
N = 5	Range	99 - 273	0.4 - 3.1	4.8 - 87	1.3 - 3.0	1.1 - 168	0.0 - 9.8	18 - 133
Limestone substrate								
# 7	M $\pm$ sd	1061 $\pm$ 1580	1.5 $\pm$ 1.9	63 $\pm$ 106	8.9 $\pm$ 26	193 $\pm$ 474	5.1 $\pm$ 7.4	1239 $\pm$ 1750
N = 50	Range	145 - 10230	0.3 - 13	6.1 - 422	0.0 - 126	0.5 - 2674	0.0 - 31	10 - 7133
# 8	M $\pm$ sd	556 $\pm$ 711	7.2 $\pm$ 13	23 $\pm$ 28	1.2 $\pm$ 0.9	23 $\pm$ 40	0.6 $\pm$ 1.3	710 $\pm$ 1234
N = 16	Range	88 - 2610	0.4 - 55	5.5 - 94	0.0 - 3.4	0.0 - 158	0.0 - 4.6	1.4 - 4549
# 9	M $\pm$ sd	577 $\pm$ 348	0.7 $\pm$ 0.2	74 $\pm$ 113	20 $\pm$ 44	209 $\pm$ 217	14 $\pm$ 11	229 $\pm$ 341
N = 7	Range	212 - 1210	0.4 - 0.9	6.5 - 319	0.0 - 120	4.9 - 458	0.0 - 28	14 - 973
# 10	M $\pm$ sd	534 $\pm$ 247	3.8 $\pm$ 1.4	26 $\pm$ 25	1.3 $\pm$ 0.8	48 $\pm$ 75	0.5 $\pm$ 1.4	413 $\pm$ 283
N = 36	Range	237 - 1248	0.0 - 6.3	7.7 - 107	0.0 - 4.1	0.9 - 299	0.0 - 6.3	91 - 1194
# 11	M $\pm$ sd	664 $\pm$ 514	0.8 $\pm$ 0.3	24 $\pm$ 21	2.6 $\pm$ 1.5	2313 $\pm$ 6556	1 $\pm$ 1	480 $\pm$ 1015
N = 9	Range	292 - 1947	0.5 - 1.4	7.6 - 64	0.0 - 5.6	64 - 19795	0.0 - 3.2	23 - 3164
# 12	M $\pm$ sd	283 $\pm$ 208	2.8 $\pm$ 1.2	12 $\pm$ 7.4	2.7 $\pm$ 1.9	73 $\pm$ 106	2.9 $\pm$ 2.8	93 $\pm$ 169
N = 16	Range	75 - 759	0.9 - 4.6	4.7 - 31	0.6 - 8.2	0.8 - 409	0.0 - 8.3	1.2 - 677
# 13	M $\pm$ sd	188 $\pm$ 47	3.1 $\pm$ 0.3	17 $\pm$ 11	1.4 $\pm$ 0.7	52 $\pm$ 50	1.1 $\pm$ 1.1	14 $\pm$ 3.1
N = 9	Range	137 - 289	2.5 - 3.5	7.6 - 46	0.7 - 2.3	7.0 - 158	0.0 - 3.1	10 - 18
# 14	M $\pm$ sd	1253 $\pm$ 2508	6.2 $\pm$ 6.4	309 $\pm$ 1281	1.5 $\pm$ 1.1	74 $\pm$ 167	0.9 $\pm$ 1.7	2096 $\pm$ 4956
N = 63	Range	60 - 17290	0.0 - 31	1.1 - 7570	0.0 - 7.2	0.0 - 1152	0.0 - 8.0	8.0 - 27816
Gypsum substrate								
# 15	M $\pm$ sd	2315 $\pm$ 90	0.8 $\pm$ 0.3	12 $\pm$ 5.5	1.3 $\pm$ 0.8	139 $\pm$ 57	1 $\pm$ 1.4	3958 $\pm$ 91
N = 6	Range	2170 - 2440	0.5 - 1.3	6.5 - 20	0.6 - 2.7	40 - 189	0.0 - 3.8	3791 - 4065

(a) M = mean; sd = standard deviation.

Table 3. Element composition (%) of grass species collected from landfill sites compared with values recorded in the same species collected from reference grasslands

Species	# samples		N	P	Ca	Mg	K	Na	Fe(a)	Mn(a)	Zn(a)	Cu(a)
<i>Bromusrubens</i>												
Grassland	4	M	0.99	0.065	0.32	0.125	0.45	0.022	1485	58	21	2.0
		sd	0.20	0.015	0.08	0.055	0.15	0.002	15.0	12	1.0	2.0
Landfill	15	M	1.39	0.11	0.56	0.24	0.88	0.02	865	79	90	1.2
		sd	0.71	0.05	0.34	0.12	0.59	0.01	448	30	49	3.1
F									*	***		
<i>Hordeum murinum</i>												
Grassland	8	M	0.25	2.09	0.05	0.27	0.39	0.13	45	28	0.0	0.4
		sd	0.18	0.82	0.04	0.22	0.14	0.04	21	12	0.0	0.5
Landfill	15	M	1.46	0.27	0.47	0.20	0.88	0.05	585	66	70	0.9
		sd	0.72	0.41	0.39	0.11	0.37	0.07	513	42	39	3.0
			***	***	**	***		**	**	F	***	
<i>Lolium rigidum</i>												
Grassland	8	M	1.90	0.22	1.22	0.18	1.19	0.12	673	106	73	5.5
		sd	0.48	0.04	0.36	0.06	0.35	0.11	688	72	39	2.2
Landfill	14	M	1.46	0.15	0.47	0.18	1.33	0.08	408	85	78	0.8
		sd	0.51	0.06	0.31	0.09	0.42	0.09	379	44	43	2.2
F					***		***					

(a)concentration in mg/kg.

* $p < 0.05$ ** $p < 0.001$ *** $p < 0.0001$

F = difference at the confidence level 90%; M = mean; sd = standard deviation

Table 4. Dry weights (g) per individual plant specimen (above-ground) collected at the end of the experiment

Treat.	Wild species						Others
	Gramineae			Fabaceae		<i>J. buffonius</i>	
	<i>B. rubens</i>	<i>H. murinum</i>	<i>L. rigidum</i>	<i>L. angustifolium</i> (b)	<i>T. subterraneum</i>		
Control	0.43 ± 0.35 (a)	2.90 ± 0.24	1.8 ± 1.4	1.61 ± 0.26	a	0.70 ± 0.05	0.22 ± 0.16
100	0.30 ± 0.06	2.98 ± 1.63	1.6 ± 0.8	1.16 ± 0.27	b	0.48 ± 0.08	0.35 ± 0.10
300	0.54 ± 0.31	-	2.0 ± 1.3	0.29 ± 0.17	c	0.53	0.28 ± 0.12
500	1.12 ± 0.68	-	2.1 ± 1.6	0.14	-	-	-
700	0.49 ± 0.54	-	1.7 ± 0.5	-	-	-	-
Treat.	Crop species			Fodder species			
	<i>T. aestivum</i>	<i>H. vulgare</i>	<i>H. annus</i>		<i>L. albus</i>		<i>V. sativa</i>
Control	0.54 ± 0.07	1.08 ± 1.07	3.13 ± 0.59	a	2.13 ± 0.46	a	0.38 ± 0.11
100	0.62 ± 0.15	1.15 ± 0.91	2.24 ± 0.77	a	2.02 ± 0.85	a	0.27 ± 0.07
300	0.55 ± 0.25	1.06 ± 0.39	0.23 ± 0.20	b	0.67 ± 0.23	b	-
500	0.33 ± 0.07	0.76 ± 0.53	-		0.50 ± 0.34	bc	-
700	0.06	0.19 ± 0.15	-		0.16 ± 0.08	c	-

- (a) Results expressed as means ± standard deviation.
(b) Different letters indicate significant differences between treatments (Bonferroni, 95%)

Table 5. Zinc concentrations (mg/kg) recorded in the above-ground biomass of the species tested

	Wild species											
	Gramineae						Fabaceae				Others	
Treat.	<i>B. rubens</i> (a)		<i>H. murinum</i>		<i>L. rigidum</i>		<i>L. angustifolium</i>		<i>T. subterraneum</i>		<i>J. buffonius</i>	
Control	28 ± 3.1	a	33 ± 1.0	a	88 ± 36	a	29 ± 10	a	53 ± 21	a	80 ± 28	a
100	169 ± 21	b	313 ± 79	b	404 ± 136	b	321 ± 22	b	600 ± 100	b	160 ± 79	a
300	330 ± 55	c	-	-	1105 ± 749	c	2319 ± 1063	c	950	-	720 ± 85	b
500	507 ± 64	d	-	-	2090 ± 768	c	2475	-	-	-	-	-
700	482 ± 6.5	d	-	-	3634 ± 1594	d	-	-	-	-	-	-
	Crop species						Fodder species					
Treat.	<i>T. aestivum</i>		<i>H. vulgare</i>		<i>H. annus</i>		<i>L. albus</i>		<i>V. sativa</i>			
Control	77 ± 45	a	34 ± 11	a	43 ± 15	a	19 ± 5.6	a	80 ± 28			
100	80 ± 87	a	300 ± 76	b	453 ± 72	b	332 ± 175	b	407 ± 246			
300	1088 ± 1125	ab	1624 ± 666	c	3710	-	2504 ± 1329	c	-			
500	4207 ± 566	b	4990 ± 2062	d	-	-	3461 ± 1402	c	-			
700	8970	-	6141 ± 2150	d	-	-	3016 ± 1569	c	-			

- (a) Results expressed as means ± standard deviation. Different letters indicate significant differences between treatments (Bonferroni, 95%).

Table 6. Chloride concentrations (g/kg) recorded for the above-ground biomass of the species tested

Wild species										
Treat.	Gramineae						Fabaceae		Others	
	<i>B. Rubens</i>	<i>H. murinum</i>	<i>L. rigidum</i>				<i>L. angustifolium</i>	<i>T. subterraneum</i>	<i>J. buffonius</i>	
Control	4.5 ± 0.86	a	2.5 ± 0.6	a	2.3 ± 2.2	a	1.4 ± 0.24	a	0.56 ± 0.10	a
100	16.8 ± 1.2	b	13.4 ± 1.6	b	12.3 ± 3.5	b	20.9 ± 3.4	b	11.0 ± 1.8	b
300	20.4 ± 0.93	b	-		17.1 ± 6.6	b	42.1 ± 10.2	c	32.6	b
500	17.4 ± 5.4	b	-		34.0 ± 4.6	c	41.3	-	-	-
700	26.2 ± 3.1	b	-		37.3 ± 9.7	c	-	-	-	-
Treat.	Crop species						Fodder species			
	<i>T. aestivum</i>	<i>H. vulgare</i>	<i>H. annus</i>				<i>L. albus</i>	<i>V. sativa</i>		
Control	3.2 ± 1.0	a	3.5 ± 2.3	a	1.4 ± 0.15	a	2.5 ± 0.36	a	4.2 ± 0.40	
100	24.6 ± 3.6	b	12.9 ± 2.3	b	12.3 ± 1.4	b	15.9 ± 5.5	b	10.6 ± 10.3	
300	37.6 ± 11.2	b	45.6 ± 15.3	c	41.8		47.3 ± 2.3	c	-	
500	40.0 ± 10.4	b	70.1 ± 29.9	c	-		50.2 ± 12.9	c	-	
700	66.3		67.7 ± 23.1	c	-		35.7 ± 2.0	c	-	

(a) Results expressed as means ± standard deviation. Different letters indicate significant differences between treatments (Bonferroni, 95%).

Table 7. Dry weights (DW, g) per individual specimen of *L. rigidum* recorded at the end of the ZnCl₂ bioassay and above-ground plant anion and nutrient concentrations (g/kg)

Treat.		DW	Cl ⁻ (a)	NO ₃ ⁻	SO ₄ ²⁻	Ca	K	Mg	Na (b)	Fe	Zn
Control	M	1.7	1.9 a	0.28	4.4	5.8 a	16.0	2.3	346	385	53 a
	sd	0.5	0.65	0.16	2.8	1.7	5.9	0.82	127	393	17
700	M	2.3	24.2 b	0.30	1.6	12.1 ab	21.9	2.8	425	218	4987 ab
	sd	0.4	7.7	0.15	0.75	4.2	6.3	1.4	326	145	2014
900	M	3.5	33.8 b	1.0	1.8	17.5 b	15.9	3.8	651	236	12861 b
	sd	2.7	20.8	1.5	2.1	13.0	6.4	3.4	455	259	7503
1100	M	2.0	42.5 b	0.30	1.9	16.4 b	18.2	2.9	509	163	8616 ab
	sd	0.7	20.4	0.3	0.17	7.8	8.1	2.8	463	71	11498
1300	M	2.7	32.1 b	0.33	2.1	17.1 b	15.6	3.2	512	171	7216 ab
	sd	0.5	23.6	0.53	0.45	10.6	3.6	3.7	482	110	11530
1500		0.8	-	-	-	-	-	-	-	-	-

(a) Different letters in the same column indicate significant differences between treatments.

(b) Na, Fe and Zn concentration in mg/kg

M = mean; sd = standard deviation.

Table 8. Dry weights (DW, g) per individual specimen of *L. rigidum* and above-ground anion and nutrient concentrations (mg/kg) recorded at the end of the bioassay in which 700 mg/kg of different salts were added to the soil

Treat.		DW	Cl ⁻ (a)	NO ₃ ⁻	SO ₄ ²⁻	Ca	K	Mg	Na	Fe	Zn
Control	M	1.7	1862 a	280 a	4418 ab	5837 a	16041	2278	346 a	385	53
	sd	0.5	654	162	2846	1664	5885	817	127	393	17
ZnCl ₂	M	2.3	24170 b	304 a	1629 a	12141 ab	21941	2838	425 a	218	4987
	sd	0.4	7705	149	746	4157	6257	1424	326	145	2014
CaCl ₂	M	1.9	23206 b	769 a	1645 a	17898 b	17493	3967	883 ab	190	270
	sd	0.2	1254	48	418	3681	1828	771	22	26	66
FeCl ₃	M	2.3	15401 b	685 a	1295 a	10553 ab	15727	2640	619 a	482	84
	sd	0.3	6063	10	336	2590	2926	831	69	526	34
KCl	M	2.1	24938 b	543 a	1430 a	8360 ab	23405	2161	592 a	103	67
	sd	0.3	1621	26	364	1412	1368	190	197	26	16
MgCl ₂	M	1.6	11797 b	689 a	3337 ab	11103 ab	14922	2998	1162 ab	404	83
	sd	0.1	8807	32	1213	2125	2812	1591	44	474	7
NaCl	M	1.3	13328 b	747 a	2229 a	10765 ab	12276	2201	5243 b	376	82
	sd	0.3	4895	21	995	1237	1352	440	1899	156	7
Zn(NO ₃) ₂	M	2.5	2356 a	4157 b	1236 a	12830 ab	10879	3040	961 ab	641	4060
	sd	0.3	77	2658	860	528	1430	173	57	376	490
ZnSO ₄	M	2.5	1185 a	692 a	8232 b	6506 ab	13096	1472	639 a	295	2286
	sd	2.2	439	29	3516	1712	1155	404	183	108	587

(a) Different letters in the same column indicate significant differences between treatments. M = mean: sd = standard deviation.

Table 9. Element percentages observed in the leaf tissue of *Lolium rigidum* growing in soil containing 700 or 1100 mg/kg of ZnCl₂

Element (%)	ZnCl ₂	
	700 mg/kg	1100mg/kg
Mg	4.38	-
Cl	0.91	15.37
K	22.99	30.94
Ca	0.73	-
Fe	68.61	-
Zn	2.37	-
Cu		44.77
Na		8.91

Ratios between nutrients provide information on the nutritional state of a plant species. Thus, deviations from values observed in controls could indicate stress induced by the high concentrations of ZnCl₂. Nutrient ratios (N/P, Ca/P, K/Na, Mn/Fe and Fe/Zn) were calculated but results are not shown since they were not conclusive. Only the Fe/Zn ratio showed a clear decreasing trend in plants exposed to the greater Zn concentrations. In all species, a reduction was observed in the K/Na ratio, especially in the higher ZnCl₂ treatments. This could be attributed to the notable increase produced in Na concentrations in the above-ground mass of all species, which was around double in plants exposed to higher concentrations of ZnCl₂ than control plants, and even 14 times higher in the case of *T. aestivum*. Variations in the remaining nutrient ratios differed according to species. The ratio N/P seemed higher for the greater

ZnCl₂ concentrations in the cases of *L. rigidum* and *H. vulgare*. The Ca/P ratio increased slightly for *B. rubens*, and this seemed more evident in *L. rigidum* and *T. aestivum*.

In Bioassay 2, *L. rigidum* only showed growth problems in response to the maximum soil ZnCl₂ concentration tested (1500 mg/kg). No signs of toxicity were observed for the remaining treatments (Table 7). Leaf Zn and Cl⁻ concentrations increased when the salt concentration was higher in the soil, but stabilized for the 900 mg/kg ZnCl₂ treatment (Table 7). Apart from Zn and Cl, Ca was the only element in leaves affected by the soil ZnCl₂ treatment. Thus, while the Ca/P ratio increased with increasing ZnCl₂ levels, as observed for several species in Bioassay 1, this ratio was reduced in plants grown in soils containing more than 1300 mg/kg of ZnCl₂. This also occurred for K/Na though less evidently than in the first bioassay.

3.3. Response shown by *Lolium rigidum* to non zinc chloride-induced soil salinity

The growth of *L. rigidum* was unaffected by any of the salts added to the soils at 700 mg/kg (Table 8). Despite supplementing *L. rigidum* with different salts, the concentrations of these salts recorded in the plant's above-ground mass did not always reflect this. Thus, while Zn, Na, and the anions SO₄²⁻, NO₃⁻ and Cl⁻ showed increasing concentrations in the above-ground parts of the plants exposed to these salts, Fe, Mg, K and Ca did not show this behaviour (Table 8). Nutrient ratios also varied with respect to controls according to the salt added. The SEM results obtained are provided in Table 9.

4. Discussion

The problem of soil salinity in capped landfills is an important problem that needs to be addressed if we

are to restore these sites. Soil is considered saline and to compromise plant growth when EC is higher than 4000 µS/cm (Marschner, 2012). This was confirmed in the case of 3 landfills examined here. To best of our knowledge, this is the first detailed study of the salinity and ion composition of capping soils used for landfills. Most similar studies on landfill salinity problems have focused mainly on leachates and drainage (Fatta *et al.*, 1999; Tatsi and Zouboulis, 2002; Kalčíková *et al.*, 2012; Hussain and Alquwaizany, 2014). As in leachates, the main anions found hereto cause soil salinity in landfills were Cl⁻, SO₄²⁻ and NO₃⁻.

Gramineae clearly showed a greater abundance in the landfill sites than in the reference grassland communities (Pastor and Hernández, 2008). This is not surprising since many are frequently found in disturbed environments forming subnitrophilic communities of the order *Brometalia-rubenti-ectori*. The nutrient status of the three grass species sampled in the landfills was similar to that of those collected in the grasslands, indicating a tolerance of these species to this type of disturbed environment. We have already reported that species of the Gramineae family are tolerant to disturbed polluted soils, i.e. abandoned mines with high concentrations of heavy metals in soils (Gutiérrez-Ginés *et al.*, 2015). We thus propose that these species should be considered for remediation purposes for a wide range of disturbances.

When soil ZnCl₂ concentrations exceeded 300 mg/kg, most species had difficulties in growing. Apart from the loss of dry weight, they showed signs of toxicity when exposed to increasing ZnCl₂ concentrations (chlorosis and reduced growth). These symptoms have been also described for plants cultivated in soils containing NaCl (Rogers and Nobles, 1991). Salinity can affect plant growth because of modified osmotic pressure, ion toxicity, and/or nutrient imbalances (Marschner, 2012). The Zn and Cl concentrations observed in leaves, as well as changes in nutrient

ratios indicate that any of these mechanisms could be responsible for plant growth problems at higher ZnCl_2 concentrations.

In response to the 300 mg/kg soil ZnCl_2 treatment, Zn and Cl^- concentrations in plants were within the toxic range, i.e. 300 mg/kg plant dry weight for Zn, and 3.5 – 30 g/kg for Cl depending on the tolerance of the species (Marschner, 2012). Other authors have reported higher Cl concentrations in plant leaves when exposed to increasing soil Cl^- concentrations (Shi *et al.*, 2015; Pushpavalli *et al.*, 2016), and higher cation concentrations when plants grow in high salinity soils (Bhuiyan *et al.*, 2015).

The reduced K/Na ratio observed in most species when ZnCl_2 was added to the soil was surprising, since Na was not added. This nutrient imbalance may also explain the reduced growth of many of the species and has often been associated with a lower tolerance to salinity (Marschner, 2012). In effect, the correction of this imbalance is one of the main strategies used to improve the quality of saline soils (Hussain *et al.*, 2013). *L. rigidum* did not show this K/Na ratio decrease in any of the treatments.

The reader is referred to Marschner (2012) for a comprehensive review of mechanisms of plant tolerance to salinity. The two species able to tolerate ZnCl_2 in our experiment (*B. rubens* and *L. rigidum*) seem to show contrasting adaptation mechanisms to salinity: an exclusion mechanism indicated by low Zn and Cl uptake by *B. rubens* (the lowest of all the species tested) and an uptake mechanism indicated by high Zn and Cl^- concentrations observed in the leaves of *L. rigidum*.

Lolium rigidum proved tolerant to high salinity and also to the trace element Zn, since no growth problems were noted for levels up to 1500 mg/kg of ZnCl_2 in soil. Up to this soil ZnCl_2 concentration, the highest leaf Cl concentration recorded was 42 g/kg, or slightly exceeding the highest concentration found not to

cause toxicity in tolerant plants like barley, spinach, lettuce or sugar beet (Marschner, 2012). Noteworthy, Zn concentrations in leaves of up to 8000 mg/kg were recorded, which means a bioaccumulation index from soil to above-ground mass much higher than 1. According to McGrath and Zhao (2003), this species could be considered a hyper accumulator of this metal, although further research is needed to confirm this issue.

Different salts cause different deleterious effects on plant development (Shi *et al.*, 2015). None of the salts tested on *L. rigidum* hindered the growth of this species. Accordingly, *L. rigidum* could be an excellent candidate for remediating soils affected by salinity and trace elements in capped landfills. In future studies, the effects on this species of other types of pollution found in landfills, such as high levels of trace elements and organic compounds (Gutiérrez-Ginés *et al.*, 2014) need to be assessed. Zn concentrations in plant dry matter higher than 300 - 500 mg/kg can be toxic for livestock (Chaney, 1989). When *L. rigidum* is exposed to this metal as ZnCl_2 , $\text{Zn}(\text{NO}_3)_2$ or ZnSO_4 , it can accumulate Zn in concentrations even 15 times higher than those limits. Although the outstanding Zn accumulation capacity of this species is a useful characteristic for remediation purposes, this feature is at the same time a problem for trophic networks, since many landfills are given uses such as cattle grazing or hunting (Pastor and Hernández, 2010).

5. Conclusions

This study examines the chloride, nitrite, nitrate, fluoride, phosphate, and sulphate concentrations of 207 samples of soils taken solid waste landfills after their sealing. Based on the high levels of chlorides observed and known high concentrations of zinc at the landfill sites, we assessed the responses of several grass species to ZnCl_2 in a greenhouse bioassay.

The three grass species tested proved more tolerant to this type of salinity than leguminous species. Among these, *Lolium rigidum* was able to grow in soils containing ZnCl_2 concentrations of up to 1300 mg/kg. The nutrition responses of the 11 species analysed, both at the physiological and cytological level in the case of *L. rigidum*, revealed possible mechanisms whereby the plant is able to respond to the stress provoked by soluble anions.

Our study provides quantitative data on the anion levels of metal-polluted waste landfill soils and Zn concentrations of plants of nutritional interest to both livestock and humans, along with plant anion levels. The latter data, besides being novel in mineral nutrition studies, could serve as indicators of salt levels in the edible portions of plants.

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