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Phytoremediation Efficacy of *Salix discolor* and *S. eriocephela* on Adjacent Acidic Clay and Shale Overburden on a Former Mine Site: Growth, Soil, and Foliage Traits

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Abstract: Plants regularly experience suboptimal environments, but this can be particularly acute on highly-disturbed mine sites. Two North American willows—*Salix discolor* Muhl. (DIS) and *S. eriocephala* Michx. (ERI)—were established in common-garden field tests on two adjacent coal mine spoil sites: one with high clay content, the other with shale overburden. The high clay content site had 44% less productivity, a pH of 3.6, 42% clay content, high water holding capacity at saturation (64%), and high soil electrical conductivity (EC) of 3.9 mS cm^{-1}. The adjacent shale overburden site had a pH of 6.8, and after removing 56.5% stone content, a high sand content (67.2%), low water holding capacity at saturation (23%), and an EC of 0.9 mS cm^{-1}. The acidic clay soil had significantly greater Na (20×), Ca (2×), Mg (4.4×), S (10×), C (12×) and N (2×) than the shale overburden. Foliar concentrations from the acidic clay site had significantly greater Mg (1.5×), Mn (3.3×), Fe (5.6×), Al (4.6×), and S (2×) than the shale overburden, indicating that these elements are more soluble under acidic conditions. There was no overall species difference in growth; however, survival was greater for ERI than DIS on both sites, thus overall biomass yield was greater for ERI than DIS. Foliar concentrations of ERI were significantly greater than those of DIS for N (1.3×), Ca (1.5×), Mg (1.2×), Fe (2×), Al (1.5×), and S (1.5×). There were no significant negative relationships between metal concentrations and growth or biomass yield. Both willows showed large variation among genotypes within each species in foliar concentrations, and some clones of DIS and ERI had up to 16× the Fe and Al uptake on the acidic site versus the adjacent overburden. Genetic selection among species and genotypes may be useful for reclamation activities aimed at reducing specific metal concentrations on abandoned mine sites. Results show that, despite having a greater water holding capacity, the greater acidity of the clay site resulted in greater metal mobility—in particular Na—and thus a greater EC. It appears that the decline in productivity was not due to toxicity effects from the increased mobility of metals, but rather to low pH and moisture stress from very high soil Na/EC.

Keywords: acidic soil; foliar nutrient and metal concentration; *Salix*; site reclamation; species variation

1. Introduction

There has been longstanding interest in Europe, and more recently in North America, in using willows (*Salix* spp.) as a source of biomass for energy purposes [1–5]. More recently, there has also been a growing interest in using willows for various environmental applications and land reclamation, including phytoremediation and phytoextraction of contaminated soils [6–17]. Highly disturbed mine sites with very low pH and high levels of metals and electrical conductivity (EC) can be challenging for reclamation and revegetation [18]. Although reclamation and phytoremediation of heavy-metal-contaminated sites using willows have been investigated in Europe, these studies have been restricted to a limited number of species and a limited number of genotypes (or clones) within species [11,12,19–32]. Of special concern for bioenergy production in the context of land reclamation is the identification of well-adapted, native plant species that can tolerate highly acidic soil conditions that may contain potentially toxic levels of metals and high EC [8,13,14,21,33–36]. Soil pH changes the solubility and mobility of metals, thereby increasing potential toxicity as soil acidity increases [37–41]. For instance, soil acidification can induce zinc (Zn) phytotoxicity [37] and aluminum (Al) toxicity in plants [42,43], and although trees are generally tolerant of high Al concentrations, irreversible damage to tree roots can occur at pH < 4.2, resulting in decreased growth and biomass yields [30,41,44,45].

With more than 350 species worldwide, willows are widespread across the northern hemisphere, and Canada has 76 native willows [46]. Willows are among the first woody plants to colonize former mine sites [9,46–48]. Although they are most often associated with wetlands and riparian zones, some willows are also adapted to drier upland sites. *Salix discolor* Muhl. (DIS) and *S. eriocephala* Michx. (ERI) are widespread across eastern and central Canada and are being field tested in common-garden studies because both species show promise as fast-growing sources of biomass for bioenergy production [1,49]. Both DIS and ERI are shrub willows that occur in natural populations on disturbed wetland sites across eastern and central North America, but DIS can also be found colonizing drier upland sites throughout its botanical range and is a common, naturally occurring colonizer of the Salmon Harbour (SH) coal mine site near Minto, New Brunswick (NB), Canada (Lat. 46.07° N; Long. 66.05° W). Several other willows, including *S. bebbiana* Sarg. and *S. lucida* Muhl., as well as aspen (*Populus tremuloides* Michx.), balsam poplar (*P. balsamifera* L.), birches (*Betula papyrifera* Marsh. and *B. populifolia* Marsh.), pin cherry (*Prunus pensylvanica* L.), and black locust (*Robinia pseudoacacia* L.), the latter introduced from eastern USA seed sources, can also be found colonizing recently disturbed shale rock overburden at the former SH coal mine. *Salix eriocephala*, however, is more commonly associated with deeper, more fertile soils along stream banks [49] and is only rarely found as a natural colonizer of the SH coal mine spoils.

In a previous paper, we detailed the coppice biomass yield components, which ultimately showed 44% less productivity on acidic clay deposits versus adjacent shale rock overburden at the SH mine site [45]. In this paper, our objective was to assess soil properties in relation to foliar concentration of nutrients and metals in 1-year-old coppice regrowth from DIS and ERI clones and assess these traits in light of productivity differences between sites. In addition, we wanted to quantify these growth differences for selection of superior clones for phytoremediation, biomass production, and land reclamation purposes. In order to assess adaptation and utility of willows for growth on reclaimed mine sites and biomass production for bioenergy purposes, we hypothesized that there would be both species and clonal differences in response to the different soil conditions and that foliar concentrations of some of the absorbed metals would negatively affect aboveground growth response.

2. Materials and Methods

Periodic flash flooding and ponding of mine drainage water on the former SH coal mine has formed a patchwork of small clay deposits of varying depths scattered over the exposed shale rock overburden that dominates these coal mine spoils. A common-garden field test and a clonal gene bank of selected genotypes of DIS and ERI containing 15 genotypes in common (Table 1) were established in 2008 and 2009, respectively, on two adjacent sites separated by several hundred meters at the SH coal mine spoils near Minto, NB, on a property operated by NB Power, the local electrical power utility. These adjacent mine spoil sites consisted of recently landscaped shale rock overburden resulting from coal strip-mining operations. However, prior to our study or any reclamation activities, one of these sites had existed for approximately 3 years as a settling pond that was part of a watercourse draining both surface runoff and water pumped from flooded coal seams. This pond deposited a thick layer of clay, covering a uniformly flat rectangular area measuring approximately 70 m × 40 m over the shale rock overburden that dominates the mine site (see contour map in Figure 1). The depth of the clay deposit was determined by digging 44 holes with a soil auger in a grid pattern at 6 m × 6 m intervals across the area of the clone bank and measuring depth to the rock overburden. The clay layer ranged in depth from 11 cm to 48 cm, with an average depth of 35 cm. Six soil samples were taken by shovel to a depth of 15 cm from each of these two sites in August 2013. Soil analyses were conducted according to McKeague [50] as follows: available P-#TP-CSS-MSSA 4.41 (sodium bicarbonate extraction), exchangeable cations-#TP-CSS-MSSA 4.5, FCMM 15 (ammonium acetate extraction), pH-#TP-CSS-MSSA3.13 (pH in 1:1 water), texture-#TP-CSSMSSA 2.12 (Hydrometer method), organic matter, N, C and S-#TP-LFIM (total C by LECO induction furnace) by the Laboratory for Forest Soils and Environmental Quality at the University of New Brunswick (UNB) in Fredericton, NB. The water content at saturation parameter was measured using the disturbed soil sample, which provides an estimate for this quantitative value.

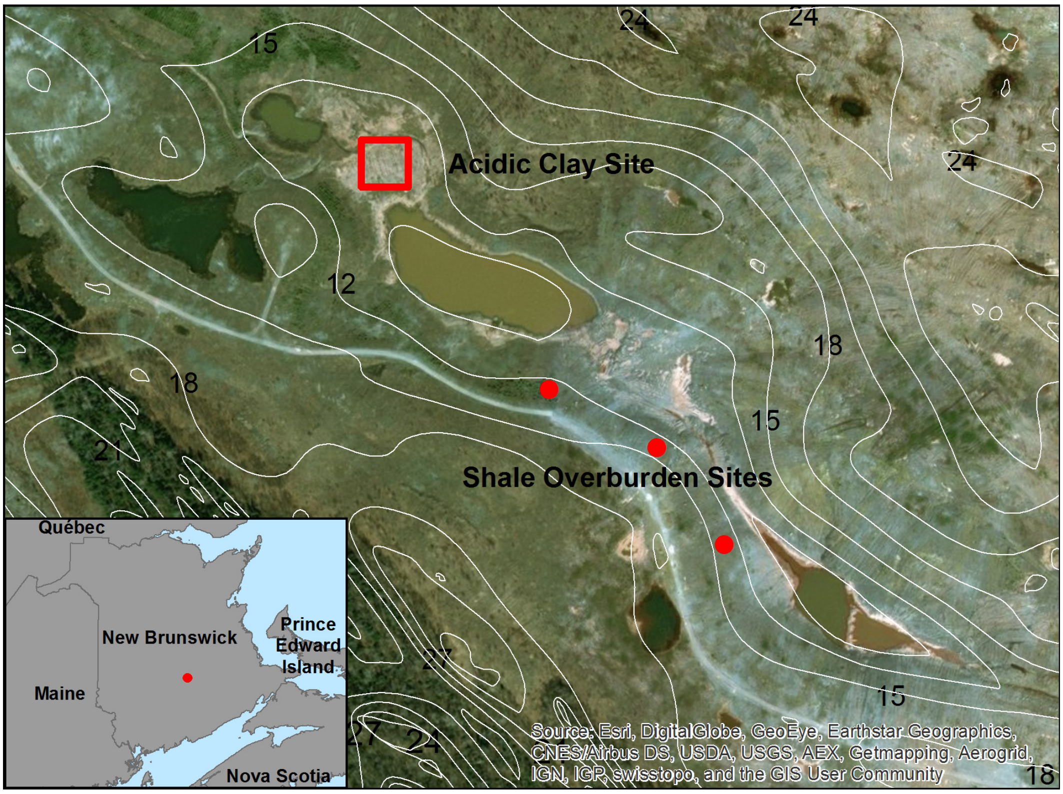

Figure 1. A map of the former Salmon Harbour coal mine site showing the locations of the acidic clay site (red square) and the three sampled sites (red dots) on the adjacent shale overburden. The insert shows the location of the coal mine site in New Brunswick, Canada.

The common garden experiment on the shale overburden site was established in May 2008 using unrooted stem cuttings of 15 DIS and ERI clones and was located approximately 300 m from the clone bank (acidic clay deposit), extending for a distance of approximately 180 m along the watercourse that drained the mine site (see Figure 1, Table 1)). This experiment was established along a gentle slope that had been landscaped to minimize soil erosion and surface runoff into the adjacent watercourse. Each genotype in the common-garden test was established as a linear, five-ramet (clonal) row plot running perpendicular to the watercourse, with the five ramets spaced at 0.5 m within the plot and 2 m between plots. Each clonal row plot was replicated three times in a randomized complete block test design.

The clay deposit site was established in May 2009 with unrooted stem cuttings to maintain 31 of the best-performing genotypes of DIS and ERI that had been selected from a common-garden field test established at the Montreal Botanical Gardens (MBG) in Montreal, PQ, Canada, in 2005. Also included on both the acidic clay deposit and the shale overburden sites was a check clone of *S. viminalis* clone #5025 (VIM), introduced from the Swedish willow breeding program [51], and which has been widely used in short-rotation willow biomass trials across Canada [3,52]. On the acidic clay site, each genotype was randomly established as a single linear row plot consisting of 21 plants (ramets) per genotype (clone) spaced at 1 m between ramets within the row plot and 2 m between adjacent clonal row plots, giving each plant approximately 2 m^2 of growing space.

Table 1. Origins of 15 *Salix discolor* and *S. eriocephala* genotypes used for measurements of foliar uptake of nutrients and metals from two common-garden studies at the Salmon Harbour mine site near Minto, New Brunswick (NB).

Species	Population Location *	Latitude North	Longitude West	Selected Clones
S. discolor	Levis, QC	46°78′	71°18′	LEV-D3, LEV-D6
	Lower Anfield, NB	46°92′	67°49′	ANF-D1
	Hawkesbury, ON	45°39′	74°75′	HAW-D5
	Montmagny, QC	46°94′	70°60′	MON-D1
	Mud Lake, ON	45°88′	76°78′	MUD-D4
	Richmond Fen, ON	45°13′	75°82′	RIC-D2
S. eriocephala	Ste. Anne de la Perade, QC	46°56′	72°20′	ANN-E6
	Bristol, NB	46°47′	67°58′	BRI-E2
	Fosterville, NB	45°78′	67°76′	FOS-E1
	Fredericton, NB	45°94′	66°62′	FRE-E1
	Green River, NB	47°34′	68°19′	GRE-E1
	Norton, NB	45°67′	65°81′	NOR-E10
	Shepody Creek, NB	45°71′	64°77′	SHE-E3
	Rivière au Saumon, QC	47°21′	70°35′	SAU-E3

* province of QC (Quebec), ON (Ontario), NB (New Brunswick).

The linear clonal plots of each species were randomly assigned on each site. However, on the acidic clay site, each of the 15 clonal plots of interest was established as a clone bank consisting of a single linear row plot containing 21 ramets per clone. Therefore, the clones on the acidic clay did not represent a true block effect, but the site was flat, uniform, and confined to a small area. The 21-ramet linear row plot on the clay deposit was divided into three *post hoc* blocks, with each block assigned a single, seven-ramet sample plot per clone per block. Thus, clonal variation could not be precisely determined due to this constraint on randomization, and estimates of within-clone variation should be interpreted with caution. Nevertheless, the clay site consisted of a 62 m by 22 m area of clay deposit, which differed greatly from the adjacent shale overburden (Table 2).

On both study sites, willow clones were established using 20 cm long, rootless stem cuttings collected during the dormant season from vigorous 1- and 2-year-old stem sections (as per [53]) from coppiced plants in a common garden established at the MBG. Each plant on the shale overburden had 1 m^2 of growing space versus 2 m^2 of growing space on the acidic clay deposit. However, the 1-year-old coppice growth of the willow clones on both sites was quite poor relative to more fertile sites such as the MBG from where these clones had been collected [53], and none of the plants on the mine site fully occupied the growing space available to them nor were they in competition with each other for growing space. The aboveground biomass was harvested from both sites in the fall of 2011 as 2-year-old coppice growth [45]. The green mass of the biomass of each harvested plant was measured to the nearest 10 g using an electronic weigh scale (Electronic Infant Scale, model ACS-20A-YE, Peoples' Republic of China). Green mass in t ha^{-1} was calculated by converting the harvested biomass per plant to biomass production per hectare to a standard 1 m^2 by multiplying by 10 (e.g., multiplying by 10,000 plants per ha divided by 1000 kg per ton). Overall yield was the product of green mass and survival.

Table 2. (**a**,**b**) Soil properties (Mean ± SE) from two sites, an acidic clay deposit and a shale rock overburden, at the Salmon Harbour mine site. Sites with different letters are significantly different at p = 0.05.

(**a**)

Site	Carbon (g kg^{-1})	Nitrogen (g kg^{-1})	Potassium (ppm)	Calcium (ppm)	Magnesium (ppm)	Phosphorus (ppm)	Sodium (%)
Acidic clay	53.1 ± 2.3a	2.07 ± 0.17a	116.1 ± 14.5a	2968 ± 116a	352.4 ± 14.6a	10.75 ± 0.6a	0.443 ± 0.031a
Shale overburden	4.6 ± 2.3b	1.02 ± 0.11b	91.1 ± 14.5a	1466 ± 116b	80.2 ± 14.6b	3.98 ± 0.65b	0.022 ± 0.031b

Exchangeable cations extracted using ammonium acetate, phosphorus was extracted by sodium bicarbonate.

(**b**)

Site	Sand (%)	Silt (%)	Clay (%)	pH	C:N ratio	Sulfur (%)	EC [1] (mS cm^{-1})	WC at Sat. [2] (%)
Acidic clay	12.9 ± 2.4b	44.9 ± 2.5a	42.3 ± 1.9a	3.6 ± 0.2b	25.9 ± 1.4b	0.079 ± 0.012a	3.89 ± 0.12a	64.0 ± 1.4a
Shale overburden	67.2 ± 2.4a	23.4 ± 2.5b	9.4 ± 1.9b	6.8 ± 0.2a	4.6 ± 1.4a	0.008 ± 0.012b	0.88 ± 0.12b	22.9 ± 1.4b

EC [1] = electrical conductivity; WC at Sat. [2] = water content at saturation.

Healthy foliage samples were harvested on 8 September 2014 from 1-year-old coppice stems of up to five ramets within each clonal row plot from three blocks (replications) on each of the two sites. Foliage from the five plants (ramets) per plot was bulked for analysis, resulting in a total of 90 foliage samples as experimental units. The foliage was stored in paper sampling bags, and placed in drying ovens at 65 °C for 48 h. Foliage was ground to a fine powder, and the grinder was washed with ethanol between samples. The Laboratory for Forest Soils and Environmental Quality at the University of New Brunswick used standard protocols (e.g., Method numbers TP-SSMA 15.3.1, 15.3.3, and 15.4 from [50]), for foliage analysis of C, N, P, K, Ca, Mg, Mn, Fe, Na, Zn, Al , and S. Foliage dry ashing is done by muffle furnace 500 °C for 4 h (slow ramp up), and extracted with 8N HCl at 90 °C for 30 min. and filtered. K, Ca, Mg, Mn, Al, Fe and Zn (cations) were run on the Varian SpectrAA 400 (Varian Techtron Pty. Limited, Mulgrave, Victoria, Australia) P is run on the Technicon Traaccs Autoanalyser (Technicon Instruments Corp., Tarrytown, New York, USA). Method #TP-LFIM (Total carbon by LECO induction furnace (LECO Corp. St. Joseph, Michigan, USA)) was used for N, C, and S [46].

Statistical Analysis

Foliar analysis data from both study sites were subjected to analyses of variance (ANOVA) in which site, species, and clones were considered fixed effects. The ANOVA model used was as follows:

$$Y_{ijklm} = \mu + B_{i(j)} + T_j + S_k + \mathrm{C}_{l(k)} + S_kT_j + \mathrm{C}_{l(k)}T_j + e_{ijklm},$$

where Y_{ijklm} is the dependent ramet trait of the i^{th} block, of the j^{th} site, of the k^{th} species, of the l^{th} clone of the m^{th} ramet and where μ is the overall mean. $B_{i(j)}$ is the effect of the i^{th} block (i = 1, ... 3) nested within the j^{th} site , T_{j} is the effect of the j^{th} site (j = 1, 2), S_k is the effect of the k^{th} species (k = 1, 2), $C_{l(k)}$ is the effect of the l^{th} clone (l = 1, ... 8) nested within the k^{th} species , and S_kT_j is the effect of the species by site interaction, $\mathrm{C}_{l(k)}T_j$ is the effect of the clone nested within species by site interaction, and $e_{ijkl\mathrm{m}}$ is the random error component.

The impact of nutrient and metal concentrations (mean values) on growth, using total aboveground green mass (mean values) from [45] were analyzed using analysis of covariance (ANCOVA). In these analyses, three sources of variation were studied: (1) covariate (i.e., nutrient or metal), (2) independent effect (species), and (3) independent effect × covariate. The analyses were based on the following model:

$$Y_{ij} = B_0 + B_{0i} + B_1X_{ij} + B_{1i}X_{ij} + e_{ij}$$

where Y_{ij} is the dependent trait of the j^{th} tree of the i^{th} site or species, B_0 and B_1 are average regression coefficients, B_{0i} and B_{1i} are the site or species-specific coefficients, X_{ij} is the independent variable, and e_{ij} is the error term. Results were considered statistically significant at p = 0.05. The data had satisfied normality and equality of variance assumptions. The general linear model from Systat (Version 12, Chicago, IL, USA) was used for analysis.

3. Results

3.1. Growth

The clay site had 44% less green mass productivity than the overburden site, with 1.27 and 1.83 t ha^{-1}, respectively (p = 0.012, Figure 2a), and there were no differences in productivity by species on either site. However, there was a significant species, site, and species × site interaction for survival. Overall, *ERI* and *DIS* had 93% and 65% survival, respectively (Figure 2b). The significant species × site interaction was a result of rank change with site. *Salix eriocephala* had greater survival on overburden vs. the clay site, at 99% and 86%, respectively; whereas, *DIS* had lower survival on the overburden vs. clay at 62% and 68%, respectively. Overall yield, which includes a survival factor, showed that the overburden had 51% greater overall yield than the clay site, with 1.54 and 1.01 t ha^{-1}, respectively

(Figure 2c). In contrast to plant green mass yields, overall yield (yield × survival) had a species effect with 48% greater yield for *ERI* compared with *DIS*, at 1.52 and 1.03 t ha^{-1}, respectively.

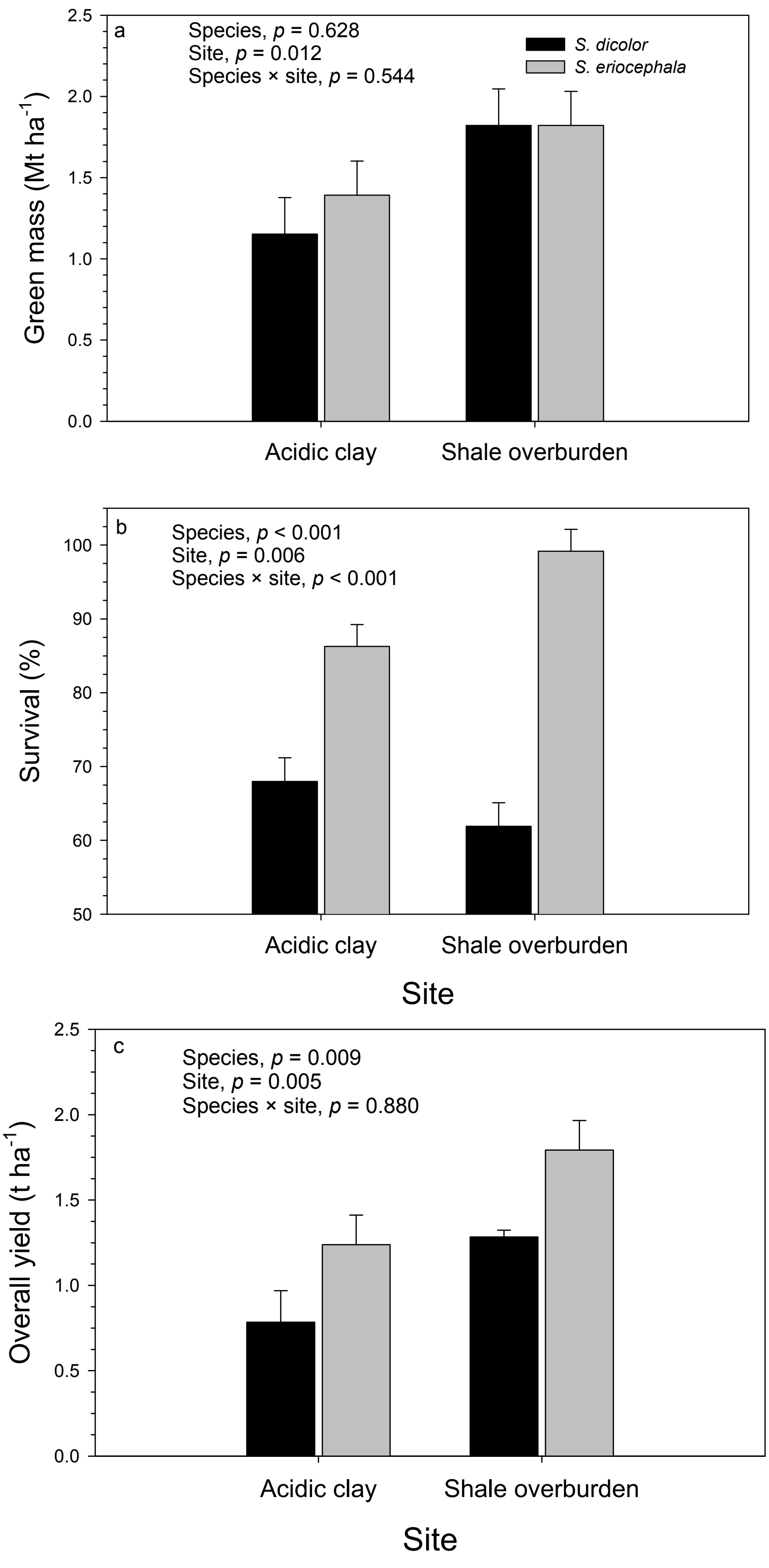

Figure 2. (**a**) Green mass, (**b**) survival, and (**c**) overall yield (mean ± SE) of *Salix discolor* and *S. eriocephala* on acidic clay and shale overburden sites at the Salmon Harbour coal mine near Minto, NB. Reproduced from Mosseler and Major [45].

3.2. Soil

The two sites showed significant differences in soil characteristics (Table 2). Although the soils of both study sites were composed from similar shale rock overburden, the clay deposit (42% clay content) was highly acidic (pH = 3.6), had high water holding capacity at saturation (64%) and a high EC of 3.9 mS cm^{-1}. The adjacent shale overburden site had a pH of 6.8, and after removing 56.5% stone content following sieving using a 2 mm sieve, the soil had a high sand content (67.2%), low water holding capacity at saturation (23%), and EC of 0.9 mS cm^{-1}, with comparatively little clay (9.4%) (Table 2). The acidic clay soil had significantly greater Na (20×), Ca (2×), Mg (4.4×), S (10×), C (12×) and N (2×) than the shale overburden site.

3.3. Foliage

The acidic clay site had significantly greater foliar concentrations of Mg (1.5×), Mn (3.3×), Fe (5.6×), Al (4.6×), and S (2×) than the shale overburden site (Table 3). Foliar concentrations of ERI were significantly greater than those of DIS for N (1.3×), Ca (1.5×), Mg (1.2×), Fe (2×), Al (1.5×), and S (1.5×). Differences in both foliar C and N concentrations were small but significantly lower on the acidic clay soil despite having significantly greater values in the soil samples (Table 2). Foliar N concentration was significantly higher in ERI clones on both site types, particularly on the shale rock overburden (Table 4, species × site $p < 0.001$; Figure 3a). Foliar P did not differ significantly by site or species, but there was a near significant species × site interaction ($p = 0.088$) showing that ERI had greater foliar P on the acidic clay, whereas DIS had greater foliar P on the shale rock overburden (Table 4; Figure 3b). Foliar K^+ had a significant species effect (DIS > ERI) but no site or species × site interaction (Figure 3c). Foliar Ca concentration had significant species (ERI > DIS) and site (overburden > acidic clay) effects (Figure 4a). Foliar Mg concentration was again significantly greater for ERI than DIS on both sites (Figure 4b). Foliar Mn concentration did not differ significantly between the two species, but site differences were highly significant (acidic clay > overburden, 3.3×; Figure 4c). Similarly, foliar Fe concentration was 5.6× greater on the acidic clay than on the shale overburden, and ERI had significantly greater foliar Fe than DIS, particularly on the acidic clay site (species × site $p = 0.020$; Figure 5a). Differences for foliar Zn concentrations were not significant for species or site effects (not shown, Table 4). Foliar Al concentration was significantly greater (4.6×) on the acidic clay site than on overburden, and there were also significant species differences, particularly on the acidic clay site where ERI foliage had twice the Al concentration than DIS (Figure 5b). The same basic pattern and significant differences were found for foliar S concentration (Figure 5c). The foliar C:N ratio did not differ significantly between site types but was significantly greater in DIS than ERI on both the acidic clay and the shale overburden (not shown). At the species level, patterns of foliar metal concentrations varied as Mn > Zn > Al > Fe for DIS, and for ERI, Mn > Fe > Al > Zn.

Table 3. (**a**,**b**) Foliage concentrations of nutrients and metals (Mean ± SE) from two sites, an acidic clay deposit and a shale rock overburden, at the Salmon Harbour mine site. Sites with different letters are significantly different at p = 0.05.

(**a**)

Site	Carbon (%)	Nitrogen (%)	Carbon: Nitrogen Ratio	Phosphorus (%)	Potassium (%)	Calcium (%)	Magnesium (%)
Acidic clay	49.95 ±0.06b	1.66 ±0.03b	30.8 ± 0.45a	0.210 ± 0.004a	1.01 ± 0.02a	1.73 ± 0.04b	0.292 ± 0.007a
Shale overburden	50.26 ±0.06a	1.75 ± 0.03a	29.9 ± 0.45a	0.208 ± 0.004a	0.99 ± 0.02a	1.89 ± 0.04a	0.195 ± 0.007b

(**b**)

Site	Manganese (ppm)	Iron (ppm)	Sodium (%)	Zinc (ppm)	Aluminum (ppm)	Sulfur (%)
Acidic clay	609.5 ± 30.3a	250.8 ± 26.4a	0.89 ± 0.04a	243.3 ± 8.9a	258.4 ± 19.8a	0.433 ± 0.014a
Shale overburden	185.4 ± 30.3b	44.5 ± 26.4b	0.90 ± 0.04a	257.3 ± 8.9a	56.1 ± 19.8b	0.243 ± 0.014b

Table 4. (**a**–**c**) Analyses of variance (ANOVA) for foliar concentrations of nutrients and metals, including source of variation, degrees of freedom (df), mean square values (MS), p values, and coefficient of determination (R^2). p values < 0.05 are in bold print.

(**a**)

Source of Variation	df	Carbon (%)		Nitrogen (%)		C:N ratio		Phosphorus (%)	
		MS	p Value	MS	p Value	MS	p Value	MS	p Value
Block (site)	4	0.98	**0.001**	0.18	**<0.001**	56.3	**<0.001**	1.8×10^{-3}	0.096
Species	1	2.20	**0.001**	2.87	**<0.001**	899.3	**<0.001**	0.4×10^{-3}	0.508
Site	1	2.20	**0.001**	0.18	**0.021**	19.5	0.149	0.1×10^{-3}	0.849
Species × site	1	1.16	**0.017**	0.54	**<0.001**	142.0	**<0.001**	2.6×10^{-3}	0.088
Genotype (species)	13	0.06	0.991	0.12	**<0.001**	39.5	**<0.001**	2.5×10^{-3}	**0.002**
Genotype (species) × site	13	0.17	0.567	0.02	0.816	7.5	0.636	1.3×10^{-3}	0.155
Error	56	0.19		0.03		9.1		0.8×10^{-3}	
R^2			0.541		0.773		0.789		0.554

Table 4. *Cont.*

(b)

Source of Variation	df	Iron (ppm)		Zinc (ppm)		Aluminum (ppm)		Sulfur (%)	
		MS	*p* Value	MS	*p* Value	MS	*p* Value	MS	*p* Value
Block (site)	4	10.3×10^4	**0.017**	17.0×10^3	**0.002**	10.3×10^4	**0.039**	0.014	0.164
Species	1	24.8×10^4	**0.007**	2.1×10^3	0.443	24.8×10^4	**0.005**	0.202	**<0.001**
Site	1	95.4×10^4	**<0.001**	4.4×10^3	0.270	95.4×10^4	**<0.001**	0.812	**<0.001**
Species × site	1	17.9×10^4	**0.020**	1.4×10^3	0.533	17.9×10^4	**0.006**	0.095	**0.001**
Genotype (species)	13	8.5×10^4	**0.005**	24.5×10^3	**<0.001**	8.5×10^4	**<0.001**	0.011	0.268
Genotype (species) × site	13	8.8×10^4	**0.004**	5.5×10^3	0.120	8.8×10^4	**<0.001**	0.015	0.079
Error	56	3.1×10^4		3.5×10^3		3.1×10^4		0.008	
R^2			0.700		0.703		0.752		0.764

(c)

Source of Variation	df	Potassium (%)		Sodium (%)		Calcium (%)		Magnesium (%)		Manganese (ppm)	
		MS	*p* Value	MS	*p* Value	MS	*p* Value	MS	*p* Value	MS	*p* Value
Block (site)	4	0.060	**0.010**	0.021	0.880	0.682	**<0.001**	0.014	**<0.001**	25.5×10^4	**<0.001**
Species	1	0.396	**<0.001**	0.082	0.288	3.679	**<0.001**	0.029	**0.001**	0.9×10^4	0.647
Site	1	0.015	0.341	0.002	0.878	0.575	**0.007**	0.210	**<0.001**	402.8×10^4	**<0.001**
Species × site	1	<0.001	0.955	0.134	0.176	0.004	0.822	0.002	0.358	0.4×10^4	0.757
Genotype (species)	13	0.109	**<0.001**	0.143	**0.038**	0.242	**0.001**	0.012	**<0.001**	7.2×10^4	0.074
Genotype (species) × site	13	0.029	0.067	0.131	0.061	0.109	0.159	0.002	0.463	1.9×10^4	0.938
Error	56	0.016		0.072		0.074		0.002		4.1×10^4	
R^2			0.728		0.491		0.767		0.783		0.731

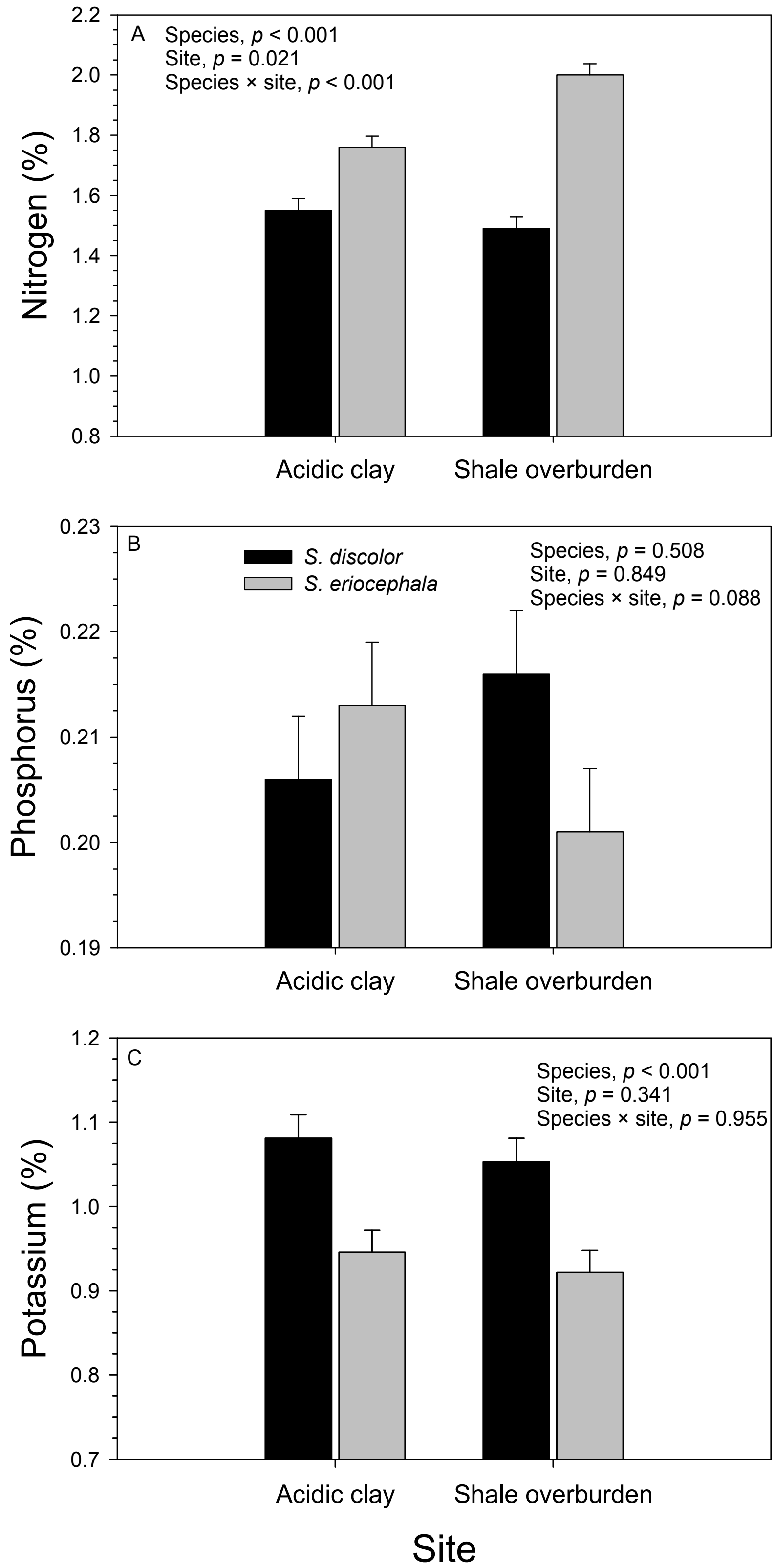

Figure 3. Foliar (**a**) nitrogen, (**b**) phosphorus, and (**c**) potassium concentrations (mean ± SE) of *Salix discolor* and *S. eriocephala* on acidic clay and shale overburden sites.

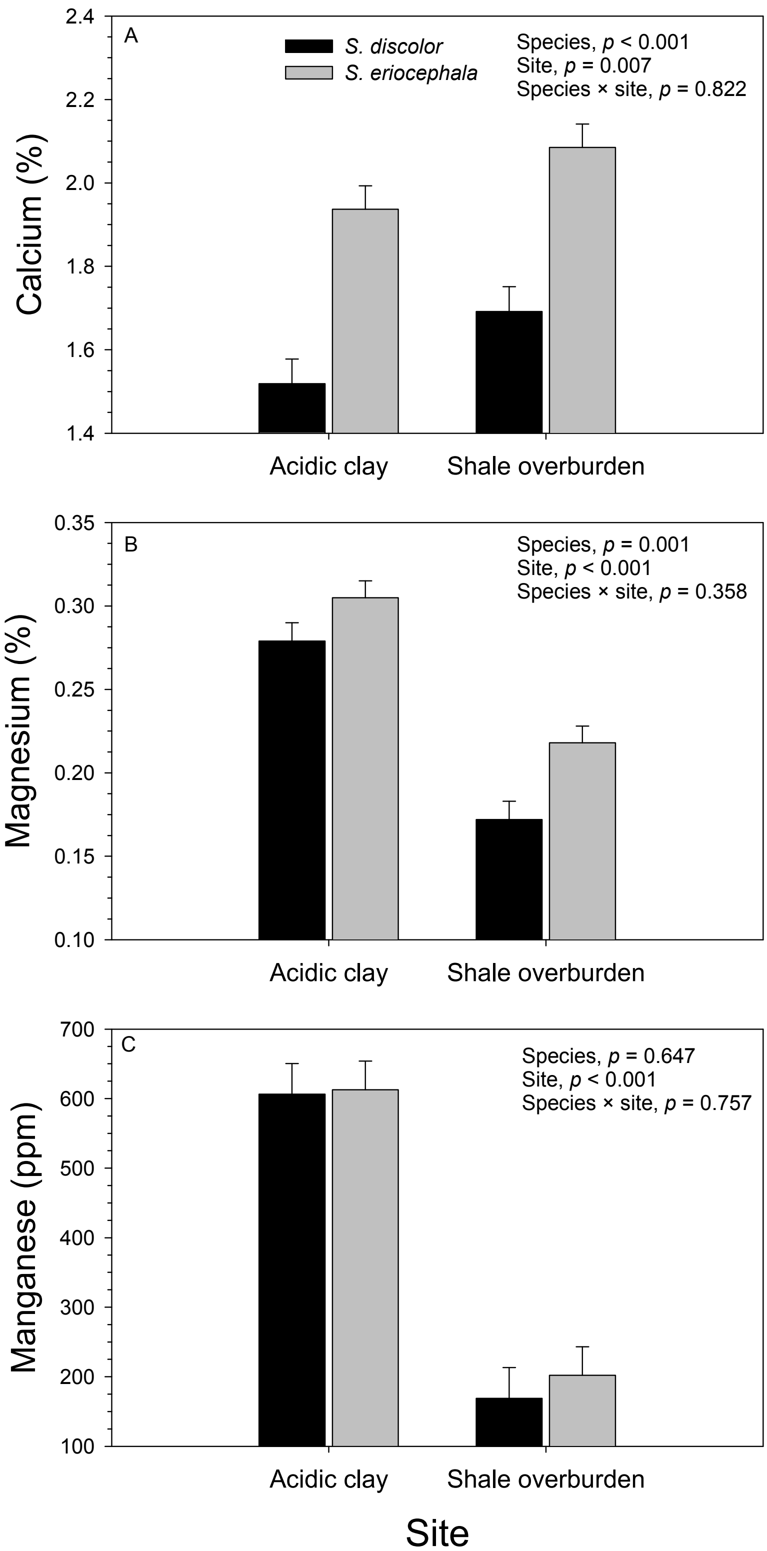

Figure 4. Foliar (**a**) calcium, (**b**) magnesium, and (**c**) manganese concentrations (mean ± SE) of *Salix discolor* and *S. eriocephala* on acidic clay and shale overburden sites.

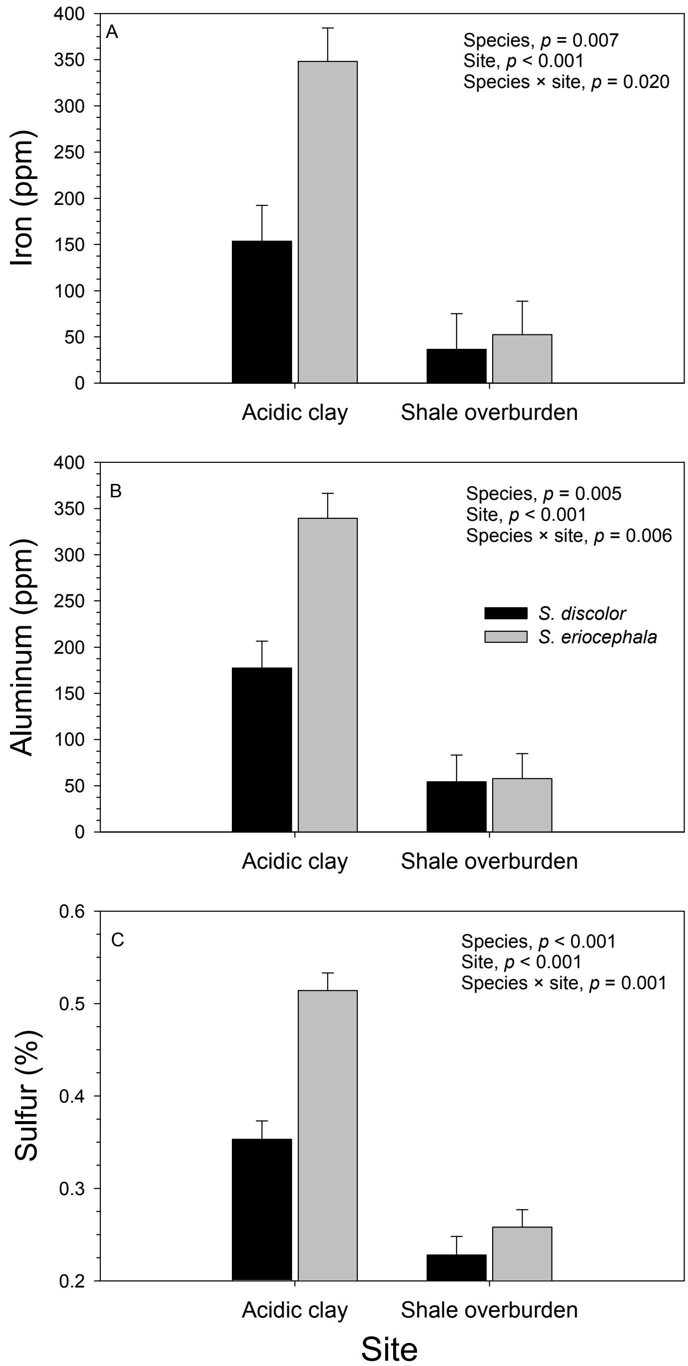

Figure 5. Foliar (**a**) iron, (**b**) aluminum and (**c**) sulfur concentrations (mean ± SE) of *Salix discolor* and *S. eriocephala* on acidic clay and shale overburden sites.

At the genotype level (clones within species), ANOVA showed significant differences among clones in foliar concentrations for most macronutrients and metals, including N, P, K, Ca, Mg, Na, Fe, Zn, and Al (Table 4), but only Fe and Al showed significant clone × site interactions. Foliar Fe and Al concentrations were consistently greater on the acidic clay site and showed some very strong genotypic variation up to eight times greater for Fe and Al in some DIS clones (e.g., clones ANF-D1, RIC-D2) (Figures 6a and 7a). For ERI, some clones also had up to eight times the Fe and Al concentrations than other ERI clones (e.g., clones FOS-E1, FRE-E1) (Figures 6b and 7b). Metal concentrations in certain ERI clones were up to 16× those of some DIS clones.

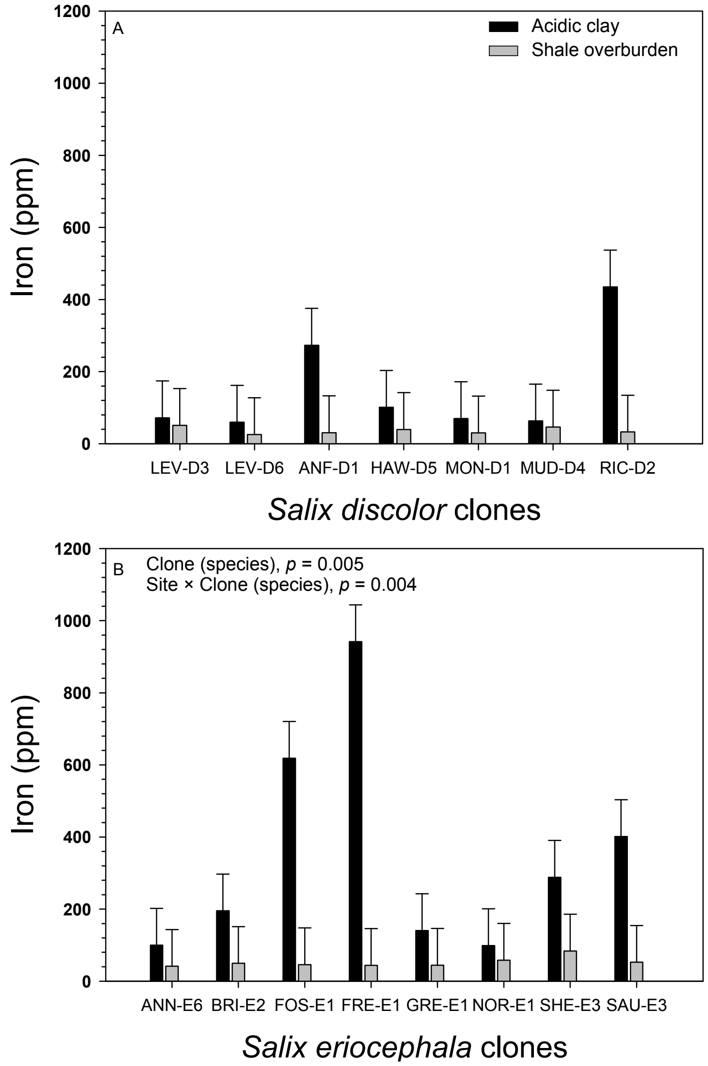

Figure 6. Foliar iron concentrations (mean ± SE) from 15 genotypes of (**a**) *Salix discolor* (seven) and (**b**) *S. eriocephala* (eight) on acidic clay and shale overburden sites.

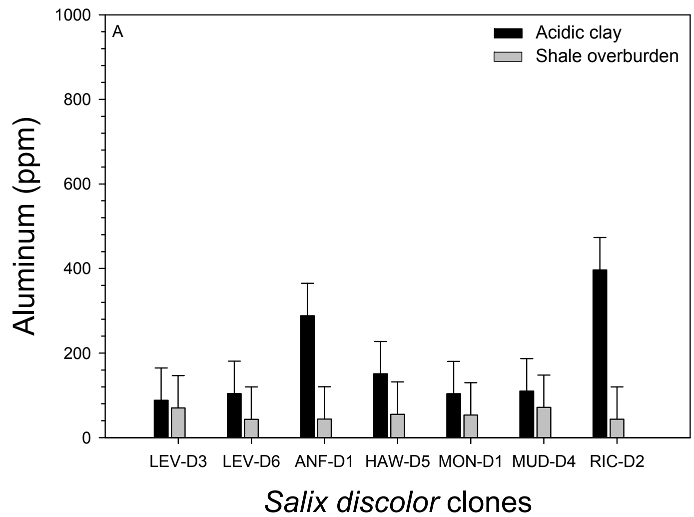

Figure 7. *Cont.*

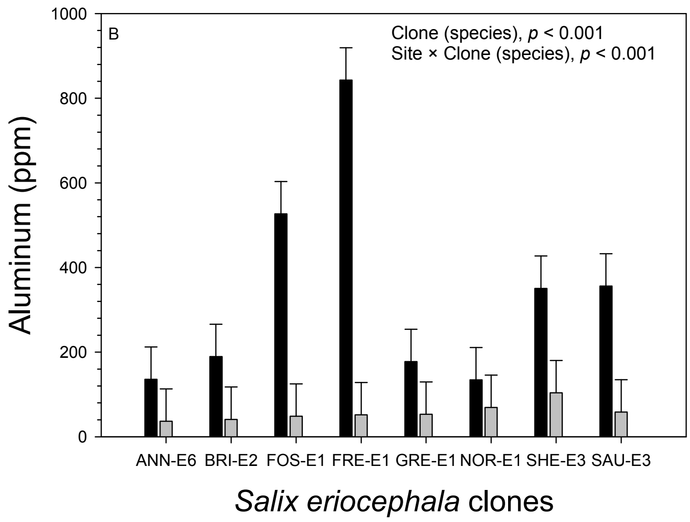

Figure 7. Foliar aluminum concentrations (mean ± SE) from 15 genotypes of (**a**) *Salix discolor* (seven) and (**b**) *S. eriocephala* (eight) on acidic clay and shale overburden sites.

Covariate analysis of total aboveground green mass, using macronutrients or metals as the covariate and testing species effect, showed no significant nutrient/metal effects, species effects, or species × nutrient/metal interaction for any of the 12 foliar nutrients, metals or C:N ratio (Table 3), except for Ca. Green mass showed a significant Ca × species interaction ($p = 0.019$), species ($p = 0.027$), and Ca ($p = 0.008$) effects. Green mass had a different positive slope in relation to increasing Ca concentration, with a steeper slope for DIS than ERI (Figure 8).

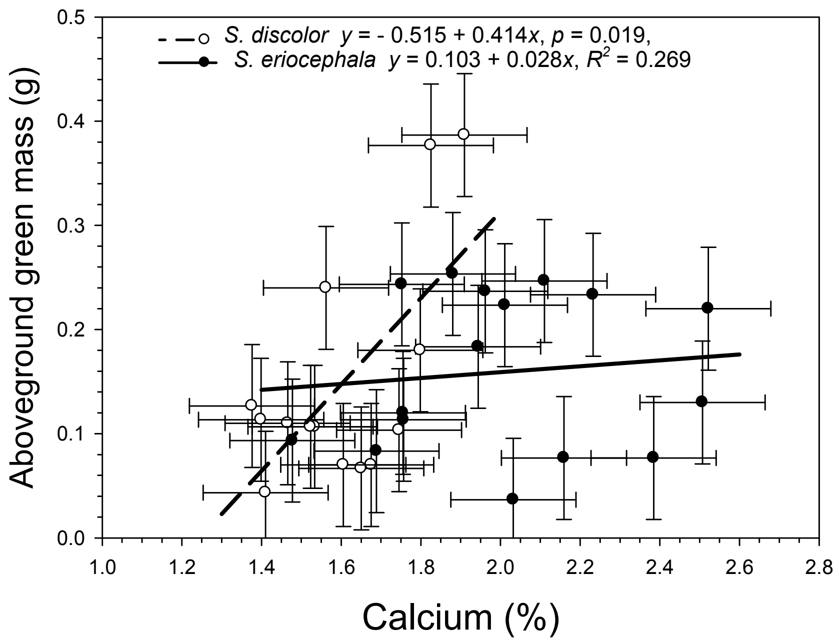

Figure 8. Covariate analysis of green mass vs. calcium concentration for *Salix discolor* and *S. eriocephala*.

4. Discussion

Willows are recognized for their phytoremediation potential and for phytoextraction of soils contaminated by heavy metals [11,22,23,25,26,31,32,54–56]. Both species and clonal differences have been observed for heavy metal concentrations, but these higher concentrations have sometimes had little apparent effect on growth and biomass accumulation in willows [30,54–57]. A limited number of European willows have been assessed for genetic variation both among and within species in their capacity for absorption of various soil contaminants under field conditions [19,21,23,25,27,58–60]. However, much less is known about such responses for North American willows [6,8,9,13,14], or their potential use in phytoremediation.

Both low and high pH soils can have high EC. Generally, acidic soils have lower EC compared with high pH soils. However, high soil EC under acidic conditions can be related to mobility of elements under acidic conditions, including some nutrients, Na and other metals [37,38,61]. In addition, clay itself has greater EC than sandy sites, generally due to greater cation exchange capacity [62]. The higher clay content of the acidic clay site could also result in increased binding of metals to clay mineral particles. Moreover, the acidic clay site had 20× the Na than the shale overburden, which presents another unfavorable condition, saline soils. The clay site that was formed as a settling pond and is often inundated after winter snow melt has a greater water content at saturation. Although high EC/saline soils may be physically wet, they can be a physiologically dry habitat for plants depending on the saline concentrations or EC. Increases in soil EC create lower soil water potential, which makes it more difficult for plants to extract soil water, creating an osmotic "drought" effect [63]. Salinity is customarily measured using EC, and the EC on the clay site was 3.9 mS cm^{-1}, which is considered high salinity for typical glycophytic plants. In a *Salix* species × salinity experiment examining response to control (0.45 mS cm^{-1}), "moderate" (1.5 mS cm^{-1}), and "high" salinity (3.0 mS cm^{-1}), DIS and ERI did not survive the high EC/saline conditions to the end of the experiment (day 160); however, *S. interior* Rowlee (INT) did show a 35% survival [64]. At moderate salinity, survival was 67%, 68% and 100%, for DIS, ERI and INT, respectively. Thus, despite the highly acidic, high EC/Na soil conditions, DIS and ERI managed to survive and even showed modest growth under these conditions. Note, the potential productivity of DIS and ERI on a fertile site such as the MBG was on average 24 and 45 Mt ha^{-1}, respectively. The only other species growing on this site naturally is coltsfoot (*Tussilago farfara* L.), despite a considerable diversity of invasive species surrounding the site and occupying the shale overburden site [53].

Despite the 20× higher Na concentration on the acidic clay compared with the shale overburden, there was no difference in foliar Na between sites. In almost all plant salinity studies, Na appears to reach toxic concentrations before Cl does, and so most studies have concentrated on Na exclusion and control of Na within plants [65]. In an analysis of foliar Na and other nutrients in a controlled salinity × willow species study, DIS and ERI had similar foliar Na; however, INT had a far greater capacity to incorporate the excess Na [66]. In a review of the physiological mechanisms of plant salinity/Na tolerance, there are generally three distinct adaptation mechanisms to salinity: cellular osmotic stress adjustment; Na or Cl exclusion; and tolerance of tissue to accumulated Na or Cl [65]. Salinity can cause the accumulation of high concentrations of ions such as Na, Cl, and SO_4 [67]. Some species do not show much increase in salt concentration over time and thus can avoid leaf mortality, whereas other species exclude or store salt in vacuoles, thereby controlling uptake and compartmentalization of ions such as Na, Cl, Fe, and S [68,69]. Among these ions, Na is considered the main ion negatively affecting plant growth. Thus, Na must be effectively partitioned and confined within cell vacuoles. Exactly how plants direct Na to the vacuole is unclear (69), but by doing so, plants osmotically adjust so they can extract water from a saline site.

Although we did not assess total metal concentrations in the soil, the soils on these sites were derived largely from the same shale rock parent material and total metal concentrations were assumed to be reasonably similar across our study sites. Foliar concentrations were greater on the acidic clay site for Mn (3.3×), Fe (5.6×), and Al (4.6×) compared with the adjacent shale overburden. The significantly greater foliar concentrations for metals such as Al, Fe, and Mn in plants on the acidic (pH = 3.6) clay site compared with an adjacent shale overburden (pH = 6.8), indicate that these metals were most likely mobilized by low pH [37,38,70] and taken up in higher concentrations by willow foliage. Whereas Zn concentrations did not vary significantly either by species or site (250 ppm), apparently unaffected by acidic conditions. Baseline concentrations of Zn in eight European willows assessed by Nissen and Lepp [21] (Table 3) ranged from 82 to 296 ppm. On shale soils, Alloway [70] suggested that Zn concentrations greater than 300 ppm approached phytotoxic levels. Rosselli et al. [36] showed that both *Salix* and *Betula* growing on contaminated sites had higher foliar Zn concentrations in their foliage than other tree species tested (*Alnus*, *Fraxinus*, and *Sorbus*).

Plants growing in highly acidic soil conditions can experience aluminum (Al) toxicity [43,44,71], of which the most easily recognized symptom is inhibition of root growth [72]. Foliage analysis indicated significantly greater Al (4.6×) levels on the acidic clay deposit than the shale overburden (Table 2), and green mass was significantly lower (44%) on the acidic clay than on the shale overburden, but increased levels of foliar Al did not influence growth on either site. From a land reclamation perspective, perhaps the more important finding was that willow species can grow roots from rootless stem cuttings on these sites and still maintain comparatively good growth under low pH and high EC and potentially phytotoxic soil conditions.

On the acidic clay site, Mn was 610 ppm; whereas, mean values of Mn at 850 ppm have been reported [68]. Bourret et al. [9] found foliar Mn concentrations ranging from approximately 350 ppm in *Salix geyeriana* Andersson, to 150 ppm in *S. monticola* Bebb growing in unsaturated mine tailings. At foliar Mn concentrations of 610 ppm on the acidic clay site, neither DIS nor ERI showed any foliar symptoms of phytotoxicity on the SH coal mine spoils. Manganese is an essential plant nutrient, but toxic levels of Mn have been reported in arid areas under irrigation and from metal-rich mine surface runoff and drainage waters [35]. Shanahan et al. [13] established toxicity thresholds of 2791 and 3117 mg L^{-1} for Mn, and 556 and 623 mg L^{-1} for Zn, in two willow species native to western North America. Bioavailability of both Mn and Zn in soils increases dramatically as soil pH declines, and this was also evident in foliar Mn concentrations in our study, which showed 3.3 times the concentration on the acidic clay site compared with the shale overburden. High levels of both Mn and Zn can result in nutrient deficiencies by interfering in the uptake of other essential elements, including C, Fe, and Mg [13], but no such effects were evident in the foliage of either DIS or ERI on the two sites assessed in our study. Other plants have shown a wide tolerance in foliar concentrations of Mn [73].

In Swedish field trials, VIM generally showed poorer growth on acidic clay soils [30], where low soil pH may have mobilized metals to an extent that led to increased foliar concentrations. Mine tailings from base metal mining operations can be highly acidic [33], resulting in potentially phytotoxic levels for metals such as Al, Mn, and Zn [9,14,37,38,70]. Although, the pH of the clay deposit at the SH coal mine was very low, none of the visible symptoms of foliar toxicity, such as leaf chlorosis or necrosis, bleaching, decreases in leaf sizes, leaf curling, etc. (see Marschner [74]), were evident in plants on either site. There was a small but significant site effect on survival, with 77.1% and 80.5% survival for the acidic clay and overburden sites, respectively, and biomass yield was 44% greater on the overburden compared with the acidic clay site [45]. On both sites, ERI had significantly greater survival than DIS but both species grew comparatively well, despite low pH on the clay site, demonstrating that both species have a reasonably high tolerance for such harsh site conditions. On the acidic clay site, mean biomass growth for the best DIS and ERI genotypes were comparable to the highly selected VIM check clone #5027 [45]. On the shale overburden site, the best DIS genotype exceeded the biomass of VIM by 12%, whereas the best ERI genotype underperformed VIM by 27%. As Pulford et al. [27] noted in their phytoremediation field trials, European willows have the ability to grow and produce high biomass yields capable of absorbing high metal concentrations, and this can be important where the aim of phytoextraction is the removal of metals from moderately contaminated sites.

In field trials of VIM in Switzerland that compared phytoextraction of Cd and Zn on an acidic site (pH = 5.2) versus a calcareous site (pH = 7.3), Hammer et al. [29] reported higher levels of Zn in the VIM foliage on the acidic site. In our study, significantly greater foliage concentrations of Al, Fe, Mn, S, and Mg were also found on the acidic clay site, but no differences between sites were found for foliage concentration of K, P, and Zn. Through application of elemental S to a calcareous site, Hammer et al. [29] were able to solubilize heavy metals, thereby increasing metals uptake by VIM. The highly acidic clay site in our study already had a very high S concentration, probably due to the presence of metal sulfides in floodwaters associated with the high S concentration of coal seams at the SH mine site. This high S concentration and related acidity may also have promoted high foliar concentrations of metals on the clay site. Both studies show that low pH and presence of high S concentration are associated with heavy metal uptake and lower biomass yields in willow [45].

Soil C was more than 10× greater on the acidic clay than shale overburden site and at first glance might indicate greater organic matter in the soil. However, there was only a scattering of coltsfoot on the site, and as this was a coal mine site, it would appear more likely that the greater C was from the settling of fine coal particles on the acidic clay site. Soil N was comparatively low (see Hansen et al. [75]) for the shale rock overburden and acidic clay sites (0.1% and 0.2%, respectively). However, foliar N concentrations were relatively high in both DIS and ERI considering the infertile site conditions and were greatest on the shale overburden despite low soil N. The relatively high foliar N concentration is puzzling in view of the low soil N concentration and supports recent research demonstrating the presence of endophytes in the stems of willows capable of fixing N on nutrient-poor sites [76,77].

There were some strong and significant differences in species responses with respect to uptake of both macronutrients (e.g., N, K, Ca, Mg, and S) and metals (e.g., Al, Fe, and Zn) (see also Pulford et al. [27]). The higher foliar concentrations of N, Ca, and Mg in ERI on both sites may reflect a higher nutrient demand in ERI. In natural habitats, ERI is generally associated with more fertile sites, whereas DIS is a natural colonizer of highly disturbed, drier, and rockier upland sites and is commonly found as a natural invader and colonizer of the shale overburden of the SH mine site [49]. ERI also had significantly greater foliar concentrations of metals such as Fe and Al, especially under the acidic conditions of the clay site, and the results suggest that selection of specific ERI genotypes may be useful in areas contaminated by these metals.

Landberg and Greger [19,59] described clonal variation in two European willows, VIM and *S. dasyclados*, for cadmium (Cd) uptake and noted variation in clonal tolerance for specific heavy metals, as well as a general tolerance in certain willow clones. Greger and Landberg [40] also showed that accumulation, transport, and tolerance for specific heavy metals can be highly clone specific and that this specificity can vary with site conditions. Our results showed strong clonal differences with respect to uptake of metals in both DIS and ERI, with a remarkable consistency in the capacity of certain willow clones to take up several different metals simultaneously. For instance, clones of either DIS or ERI able to take up the largest quantities of Al were the same clones that took up the largest quantities of Fe. These results suggest that certain genotypes may have a greater capacity for uptake or tolerance for metals and that clonal selection for increased metal uptake on contaminated sites looks promising.

Despite large variation in metal concentrations among species and genotypes, there were no significant negative (or positive) relationships with productivity, as might have been expected. In addition, there was no relationship between nutrient concentrations and productivity except for a positive relationship of increasing Ca concentration on productivity, particularly for DIS. Calcium may help neutralize very acidic soil conditions. The genotype with the greatest productivity and Ca concentration was the DIS clone MON-D1.

5. Conclusions

Phytoremediation efforts often require plant tolerance to a number of suboptimal conditions. On a former coal mine spoil, neither *Salix discolor* nor *S. eriocephala* showed any negative relationships between metal concentrations and plant growth, nor any visible effects on foliage health. Genetic differences among species and genotypes within these species in their biological capacity to take up and tolerate metals suggests that selection and breeding for willow clones with special capacities for uptake of heavy metals from moderately contaminated soils looks promising. *Salix eriocephala* showed particular promise for use in phytoextraction of heavy metals such as Fe and Al. Results showed that despite having a greater water holding capacity, the clay site was very acidic, resulting in greater metal mobility and EC, and it would appear that the decline in productivity on a highly acidic site was not due to toxicity of mobile metals but to low pH and moisture stress from very high soil Na/EC.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

DIS	*Salix discolor*
EC	electrical conductivity
ERI	*Salix eriocephala*
MBG	Montreal Botanical Garden
Al	Aluminum
C	Carbon
Ca	Calcium
Fe	Iron
INT	*Salix interior*
K	Potassium
Mg	Magnesium
Mn	Manganese
Na	Sodium
N	Nitrogen
NB	New Brunswick
ON	Ontario
P	Phosphorous
QC	Quebec
S	Sulfur
VIM	*Salix viminalis*
Zn	Zinc

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