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Boron accumulation in *Puccinellia frigida*, an extremely tolerant and promising species for boron phytoremediation

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ABSTRACT

Excessive concentrations of boron in soils and water are common problems in arid and semiarid regions, reducing both crop yields and the variety of species that can be cultivated. Current boron-removal technologies are either expensive or impractical for arid regions; hence, the use of boron-tolerant plants for phytoremediation of soils and water has gained much attention. Northern Chile is characterized by its arid climate and the Lluta River is an important water resource within the region, but it presents high boron concentrations that limit local agricultural activities. Hydrothermal springs located in the Colpitas River sub-basin are one of the Lluta River's main sources of boron. A native plant species—*Puccinellia frigida*—spontaneously colonizes the banks of these hydrothermal springs. To evaluate its phytoremediation potential, we analyzed several plant, soil, and water samples taken along a hydrothermal stream. This article presents and discusses the characteristics of the study site soil and water, molecular identification of *P. frigida*, and boron distribution within the plant's tissue. We found that the hydrothermal springs where this species grow are highly toxic to plants (As > 5 mg/L, B > 400 mg/L, EC > 20 mS/cm). We also found that *P. frigida* is tolerant to extremely high levels of available boron in soil (>4000 mg/kg) and shoots (>4900 mg/kg DW). In addition, the boron plant accumulation coefficient (PAC) and translocation factors (TF) were >1 in most samples (PAC: 0.9 - 2.8 and TF: 1.1 - 3.6); hence, *P. frigida* acts as a boron accumulator within the study site. Thus, it is established that *P. frigida* is one of the most boron-tolerant species known and that it has potential for boron phytoextraction purposes or pollution phytomanagement strategies.

1. Introduction

Even though boron is essential for plant growth in low concentrations (Dell and Huang, 1997), excessive concentrations in soils and water are very toxic to plants (Nable et al., 1997). Visual symptoms of boron toxicity include leaf burn, i.e., chlorotic and/or necrotic patches often at the margins and tips of older leaves (Nable et al., 1997), and reduced root and shoot elongation (Lovatt and Bates, 1984; Nable et al., 1990).

Toxic concentrations of boron occur naturally in many arid and semiarid regions throughout the world, e.g., South Australia (Brennan and Adcock, 2004), West Asia and North Africa (Yau et al., 1994), Turkey (Gemici et al., 2008; Türker et al., 2013), Italy (Tassi et al., 2011), the USA (Stiles et al., 2011), Argentina (Franco et al., 2008), and Chile (Torres and Acevedo, 2008). This seriously hinders agricultural activities within these areas by reducing both crop yields (Cartwright et al., 1984) and the variety of species that can be cultivated. In addition, during recent years, boron concentrations in surface waters have significantly increased (Wolska and Bryjak, 2013) due to natural factors (weathering of rocks and leaching of salt deposits) and anthropogenic activities (e.g., industrial processes such as the production of glass, ceramics, and detergents). Therefore, the removal of boron from water has become a critical problem for many highly developed countries (Wolska and Bryjak, 2013). Unfortunately, current technologies for the removal of boron from soils and water are not cost effective and therefore, new technologies are needed.

Boron toxicity in soils cannot be treated easily because of its sorption onto soil surfaces (Goldberg, 1997). The most common treatment practice is leaching (Leyshon and Jame, 1993), but this presents several limitations: (1) large volumes of water are required (Bingham et al., 1972), restricting its application in arid and semiarid regions; (2) removal is often incomplete, i.e., only the soluble fraction is removed, which leaves the sorbed fraction that can recharge the soil solution later (Peryea et al., 1985); and (3) soils must have good drainage capacity (Nable et al., 1997). In addition, the collection of the boron-enriched leachate is necessary to prevent contamination of adjacent sites (Bañuelos et al., 1993b). Another treatment method is to replace the surface soil with soil that has low boron levels; however, this treatment is expensive

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and the boron from subsurface layers can migrate and recharge the overlying non-contaminated soil (Nable et al., 1997).

Boron removal from water is also a complex procedure. This is because boric acid (H_3BO_3, the main species of boron in water) is extremely soluble, has relatively small size, and is found as uncharged molecules in neutral waters (pKa = 9.25 at 25 °C (Woods, 1994)). Conventional methods of water purification (sedimentation, precipitation, and coagulation or adsorption on clays) are ineffective for boron compounds (Wolska and Bryjak, 2013). Methods of reverse osmosis and ionic exchange are the simplest treatments for the removal of boron from water, but they require optimization because of the high costs involved (Dydo and Turek, 2013; Hilal et al., 2011). Even if these technologies were to become cheap alternatives for boron removal in the future, ion exchange resins and reverse osmosis membranes have limited lifespans and would become undesirable waste. Consequently, sustainable technologies must be developed for water treatment.

Phytoremediation is one such promising technology because it presents several advantages over physical and chemical remediation. Phytoremediation is clean, simple, cost effective, non-environmentally disruptive, and its by-products can be utilized in many ways (Doran, 2009; Sarma, 2011). As current technologies for boron removal are ineffective, considerable attention is being given to the use of boron-tolerant plants for boron phytoremediation. Different types of phytoremediation strategies for boron-polluted sites, soils, and water have been studied: phytoextraction, phytorestoration, evapotranspiration, and constructed wetlands.

Phytoextraction consists of planting tolerant plant species (sometimes with appropriate associated microorganisms) that can take up a contaminant and translocate it to their upper parts. The contaminant is then removed by harvesting the plant shoots. To phytoextract a metal or metalloid, plants must be able to develop in highly polluted soils and accumulate the element in their aerial tissue (McGrath and Zhao, 2003). For phytoextraction purposes, hyperaccumulator plants are preferred. Two ratios are used to define a plant as a hyperaccumulator: the bioconcentration factor (BF) and the translocation factor (TF). BF is the ratio of element concentration in the plant shoots to its total concentration in the soil. TF is the ratio of the element concentration in the plant shoots to its concentration in the plant roots. If BF > 1 and TF > 1, the species may be classified as a hyperaccumulator (McGrath and Zhao, 2003).

Phytoextraction of boron from water and soils has been studied by different researchers (e.g. Aydın and Çakır, 2009; Bañuelos et al., 1993a, 1993b; Del-Campo Marín and Oron, 2007; Santos et al., 2010; Tassi et al., 2011). Bañuelos et al. (1993a) found that Indian mustard (*Brassica juncea*), tall fescue (*Festuca arundinacea*), bird's-foot trefoil (*Lotus corniculatus*), and kenaf (*Hibiscus cannibinus*) reduced extractable soil boron by 52% in field experiments. In another study, *B. juncea* was capable of removing 45% of the initially available boron concentration from a contaminated soil, which was 40 mg/kg (Tassi et al., 2011). In Brazil, *Ricinus communis L.* was tested for its ability to remove boron (and other heavy metals) from an agricultural soil contaminated with automobile scrap shredder residues (de Abreu et al., 2012). In pot experiments, this species was able to accumulate 626 mg/kg in its shoots. The time required to phytoextract 50% of the initial boron concentration was calculated to be 12–16 years. Boron hypertolerant and hyperaccumulator native plant species have been investigated in various countries and analyzed for their possible use as phytoextractors (Babaoglu et al., 2004; Franco and de Viana, 2009; Franco et al., 2008; Marquis et al., 1984; Stiles et al., 2010; Tassi et al., 2011). Because experimental conditions differ widely, it is difficult to make valid comparisons of boron tolerance between the species studied (Stiles et al., 2010); however, the most boron-tolerant species found to date appears to be *Puccinellia distans* Turkish ecotype (Stiles et al., 2010). This species tolerated more than 1250 mg B/L in hydroponic culture and 6000 mg B/kg DW in its living shoots.

Phytorestoration of boron-polluted areas and the evapotranspiration of high levels of boron waste have rarely been studied. *P. distans* US ecotype was studied for its possible use in the phytorestoration of a boron-contaminated mine (Stiles et al., 2011). In pot experiments using the mine soil (with some amendments), the species was able to germinate and survive, and it was proposed as a possible initial vegetation cover for the mine in the study. However, the capability of the species to grow successfully under the environmental conditions of the actual mining site was not investigated. The ability of poplars to evapotranspirate high levels of boron waste was studied by Robinson et al. (2007). They found that the poplars accumulated boron and increased evapotranspiration, which reduced the levels of boron leached from the contaminated site into receiving waters.

The potential of constructed wetlands to remove boron from water has also been studied (Gross et al., 2007; Lizama Allende et al., 2012; Lizama Allende et al., 2014; Türker et al., 2013; Türker et al., 2014a; Ye et al., 2003). Constructed wetlands can remove boron with rates that range from 0–65%. The main processes responsible in the boron removal are sorption and plant uptake; the latter is very relevant under favorable environmental conditions (Türker et al., 2014b). One way to enhance plant uptake (and thus, the performance of constructed wetlands) is the use of boron-hyperaccumulator species. Currently, only a few such species are known and therefore, the phytoremediation potential of other wetland species must be investigated (Türker et al., 2014b).

For all of the phytomanagement strategies outlined above, new species of boron-tolerant plants need to be studied in order to establish suitable candidates for different applications and sites. Ideal places for finding such candidates are sites highly contaminated with boron.

1.1. The Lluta Valley

Northern Chile is characterized by scarcity of water. The Lluta River, located in the XV Region of Arica and Parinacota, is one of the main rivers in Northern Chile. This river presents high levels of salinity (electrical conductivity (EC) > 2 mS/cm), arsenic (0.1–0.6 mg/L), and boron concentrations (16–25 mg/L) (DGA, 2004). High EC and arsenic (As) values are due, in part, to high levels of dissolved heavy metals, metalloids (As: 3.6 ± 0.46 mg/L; Fe: 81.6 ± 13.5 mg/L) and sulfate (4678 ± 16.9 mg/L) discharged into the surface waters in the upper sections of the valley (Leiva et al., 2014).

The elevated levels of boron in the Lluta River reduce the agricultural productivity and the variety of species that can be cultivated within the valley (Albornoz et al., 2007; Torres and Acevedo, 2008). In addition, the agricultural soils also present high concentrations of boron because of the deposition of soluble boron from irrigation water (which comes from the river) and groundwater (Bañuelos et al., 1999), further restricting agricultural activities.

The Colpitas River is the largest source of boron and an important source of arsenic in the Lluta River (Fig. 1) (DICTUC, 2008; JICA, 1995). Hydrothermal springs, located in the Colpitas River sub-basin, contribute the majority of boron, arsenic, and chloride (Cl^-) to the river, reaching concentrations in excess of 400, 10, and 5000 mg/L, respectively. The removal of the hydrothermal discharges to the river could improve the water quality of both the Colpitas and Lluta rivers significantly (JICA, 1995). We found that a native plant species, *Puccinellia frigida*, spontaneously colonizes the margins of some of these hydrothermal springs, tolerating the extremely high boron concentrations. This makes *P. frigida* a possible candidate for the phytomanagement of severe boron toxicity in this valley (e.g., phytoextraction in agricultural soils or evapotranspiration of hydrothermal waters to reduce boron leaching to the Colpitas River) or at other boron-contaminated locations.

The aim of this work was to determine the potential of *P. frigida* for boron phytoremediation purposes. Specimens of *P. frigida* growing along a hydrothermal stream, together with samples both of their associated soil and of the hydrothermal water were collected. The soil and water samples were analyzed and the plant species identification confirmed by molecular analyses. Boron accumulation in the shoots

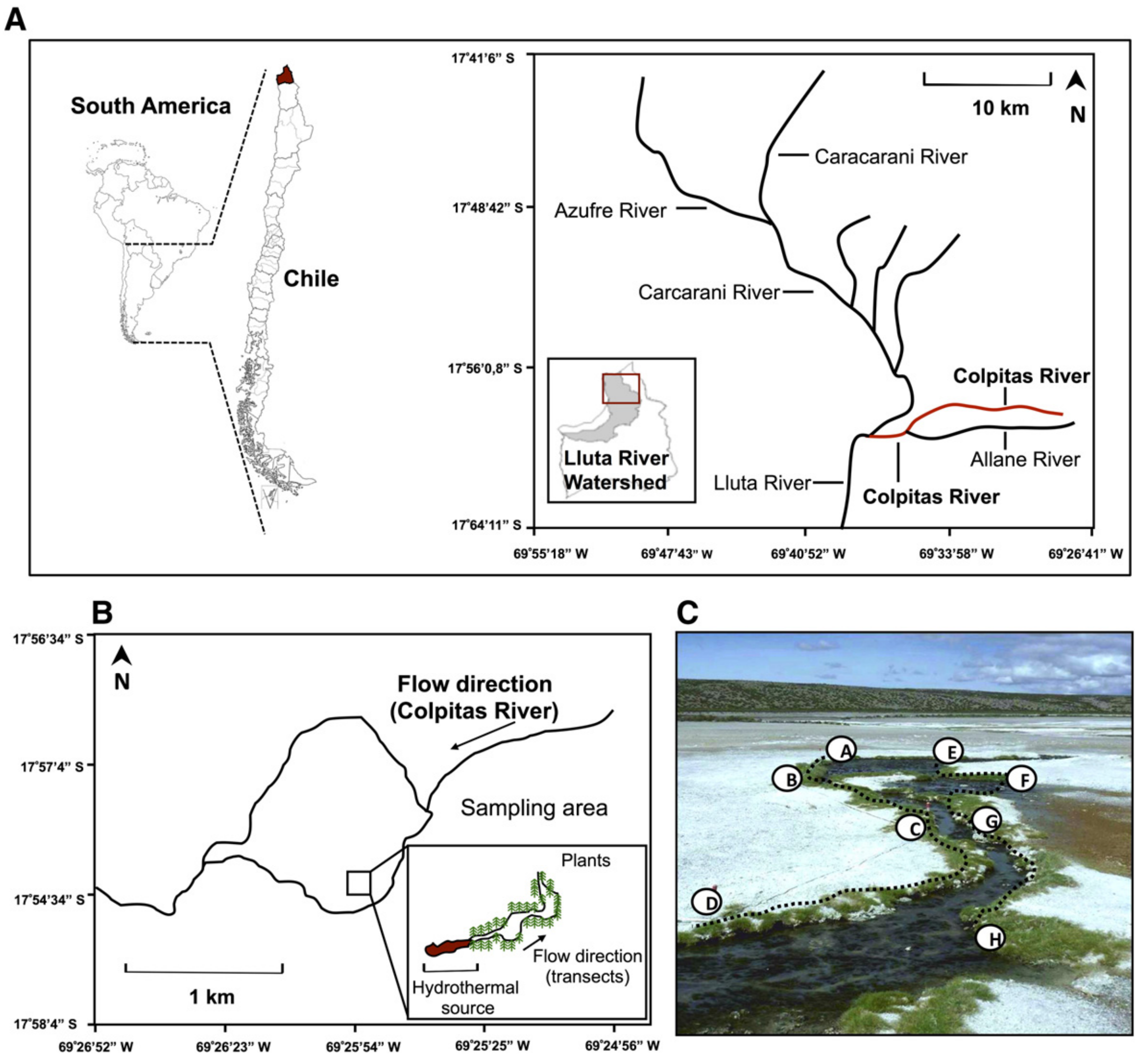

Fig. 1. Location of the study site. (A) Map of Chile highlighting the location of the Colpitas River sub-basin in the Lluta River watershed. (B) Study site location in the hydrothermal area of the Colpitas River sub-basin. (C) Sampling points along the hydrothermal spring; transect 1 (sampling points A–D) and transect 2 (sampling points E–H).

and roots of the plants and boron concentrations in the soil were determined. Finally, to establish whether the species acted as an accumulator in the study site, the TF, BF, and a modified BF (plant accumulator coefficient: PAC) were calculated.

2. Materials and method

2.1. Site description

The Colpitas River sub-basin is located in the XV Region of Arica and Parinacota, Northern Chile, forming part of the Lluta River watershed (Fig. 1). The 3378-km^2 Lluta River watershed extends between 17°39'–18°30'S and 70°20'–69°22'W. It presents an arid climate with average annual rainfall of 0.4 mm in the low areas of the valley and 237.7 mm in the high mountain areas. Rainfall occurs mainly as intense thunderstorms from late December to late February. The average annual temperature in the low areas of the valley is 19.1 °C and 8.4 °C in the upper basin (DGA, 2004). The city of Arica, the capital of the region, and many small villages depend on the water resources of the Lluta River.

The 219-km^2 Colpitas River sub-basin extends between 17°59'44"–17°54'16"S, 69°37'46"–69°23'25"W. The elevation ranges from 3600 to 4560 masl. The Colpitas River originates from the mountains in the east from two main tributaries: the Colpitas River and the Allane River. At the beginning of the Colpitas River, there is an area of 0.85 km^2 where hydrothermal springs dominate (Fig. 1). This is an important area because the hydrothermal waters flow into the Colpitas River increasing its boron, chloride and arsenic concentrations. At its end, the Colpitas River is joined by the Caracarani River, forming the Lluta River.

2.2. Water sample collection and on-site water quality measurements

Water samples from the hydrothermal spring area were collected between December 2007 and December 2011. Sixteen different sampling points were chosen to screen the area. To evaluate seasonal variations, some points were sampled in more than one campaign (supplementary data). One of these hydrothermal springs was selected for further analysis (Fig. 1). Four water samples were collected in February 2011 along the selected hydrothermal stream (17°57'30"S, 69°25'53"W; 4150 masl). Samples were collected from the midpoints between the plant and soil sampling locations A–E, B–F, C–G, and D–H (Fig. 1). Measurements obtained at each point are presented in Table 1. These values show no significant changes when analyzing water samples collected in different seasons (supplementary data).

Prior to the collection of the water samples, several on-site water quality parameters were measured: temperature (PHC301, HACH), pH (PHC301, HACH), dissolved oxygen (DO) (IntelliCAL LDO101 Standard

Table 1
Water geochemical parameters at sampling points along the selected hydrothermal stream. Water presented high boron, arsenic, chloride, and electrical conductivity levels, which might be considered toxic for most plant species (T: temperature, DO: dissolved oxygen, EC: electrical conductivity).

Sampling point	pH	T (°C)	DO (mg/L)	EC (mS/cm)	Fraction	Cl^- (mg/L)	SO_4^{2-} (mg/L)	As (mg/L)	B (mg/L)
A–E	6.4	28	4.5	23.3	Dissolved	7390	225	7.71	428
					Total			9.19	444
B–F	6.5	25	8.8	23.2	Dissolved	7810	287	7.10	438
					Total			9.37	446
C–G	6.6	25	11.9	22.8	Dissolved	8070	314	7.28	440
					Total			13.42	440
D–H	6.6	24	12.8	22.7	Dissolved	7530	222	5.64	429
					Total			10.55	446

Luminescent Dissolved Oxygen LDO Probe, HACH), and EC (CDC40, HACH) (Hq40d Multi, HACH, Loveland, CO, USA).

Water samples were collected in 300-mL high-density polyethylene bottles. For the dissolved fraction samples, aliquots were filtered immediately (0.45-μm nylon filters), whereas for the total fraction samples, aliquots were not filtered. Aliquots of each fraction were acidified to pH 2 with HNO_3 and stored at room temperature (~20 °C) until later analysis of B and As. Filtered, non-acidified water samples were stored at 4 °C and analyzed later for Cl^- and sulfate (SO_4^{2-}).

2.3. Water analysis

Water Cl^- and SO_4^{2-} concentrations were determined by ion chromatography (IC) using an 882 compact IC plus ion chromatograph (Metrohm Inc., Herisau, Switzerland). Boron was determined using a high-resolution continuum source atomic absorption spectrometer (contrAA® 700 from Analytik Jena, Jena, Germany). Arsenic was determined by total X-ray reflection fluorescence (S2 Picofox, Total X-ray reflection fluorescence spectrometer, Bruker AXS, Billerica, MA, USA) (Borgese et al., 2009). Arsenic and B concentrations in the water samples collected in December 2007, April 2008, and October 2009 were determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, PerkinElmer Optima 7300 DV ICP-OES, Shelton, CT, USA).

2.4. Plant and soil sample collection

Samples of plants and soils were collected in February 2011 from the selected hydrothermal site. Two transects were conducted along both sides of the hydrothermal stream (Fig. 1). Each transect consisted of four sampling points (eight sampling points in total: A, B, C, D, E, F, G, and H) separated by distances of 3.5 m. Samples from one square of plants (225 cm^2) and from 0–20-cm soil depth were collected at each point. Plants were immediately separated into shoots and roots and stored at 4 °C in open plastic bags until their pre-treatment and analysis in the laboratory. Soil samples were immediately sliced into 0–5-cm and 5–20-cm strata and stored in plastic bags at 4 °C until their analysis in the laboratory.

2.5. Soil analysis

Soil samples were dried at 40 °C for three days. The dried soil samples were then ground and sieved (≤2 mm), and analyzed for available boron concentration, physical and chemical properties at an accredited laboratory (Laboratorio de Servicio Agronomía, Pontificia Universidad Católica de Chile, Santiago, Chile). The physical and chemical properties of the eight samples analyzed are shown in Table 2. EC and pH were determined in a 1:2.5 soil:water suspension (Van Reeuwijk, 2002). Organic matter was determined using the chromic acid oxidation colorimetric procedure involving heat of dilution (Schulte, 1995). P-Olsen was determined by extraction with sodium bicarbonate (Olsen et al., 1954). Available K and exchangeable Ca, Mg, Na, and K were determined using the ammonium acetate method (Brown and Warncke, 1988) and the extracts were analyzed by ICP-OES (Liberty RL, Varian Inc., California, USA). Available S was extracted using the calcium phosphate extraction method (Singh et al., 1995) and determined by turbidimetry. Available Cu, Fe, Mn, and Zn were extracted using the diethylenetriaminepentaacetic acid extraction method (Lindsay and Norvell, 1978) and the extracts were analyzed by ICP-OES (Liberty RL, Varian Inc., California, USA). Cation exchange capacity (CEC) was determined using the sodium acetate method (Rhoades, 1982), and soil texture was determined using the hydrometer method (Sheldrick and Wang, 1993). Finally, available boron ($B_{available}$) was extracted by the hot 0.01 M $CaCl_2$ method (Bingham, 1982) and analyzed by ICP-OES (Liberty RL, Varian Inc., California, USA).

Total boron (B_{total}) and total arsenic (As_{total}) analyses were performed after microwave digestion (Microwave Accelerated Reaction Systems, model MARS; CEM Corp., Mattheus, NC, USA) using the USEPA 3051A digestion method (USEPA., 1998). The boron content of the digests was determined using a high-resolution continuum source atomic absorption spectrometer (contrAA® 700, Analytik Jena, Jena, Germany) and arsenic was determined by ICP-OES (Perkin Elmer Optima 7300 DV ICP-OES, Shelton, CT, USA).

2.6. Tissue analysis

Shoot tissue was separated into green and yellow categories and only green tissue was analyzed. This was done to determine the amount of boron that could be tolerated by the species, i.e., the amount of boron that would not produce any visible symptoms of boron toxicity (e.g., chlorosis). In other species subjected to high concentrations of boron, chlorotic and necrotic tissues presented higher concentrations of boron than green tissues (Rees et al., 2011); therefore, measuring the boron content of yellow tissue only could overestimate the plant's boron tolerance. The plants in this study were growing naturally in the field and therefore, we were unable to distinguish between natural senescent tissue and chlorotic tissue produced by boron toxicity. Thus, we did not analyze any yellow tissue in order to avoid overestimating the boron tolerance of *P. frigida*.

Roots were washed with tap water at high pressure until all soil was removed. Shoots and pre-treated roots were rinsed once in HNO_3 (0.05 N) and three times in deionized water. The tissue was dried at 60 °C until constant weight, and then ground and sieved (≤0.5 mm). The sieved tissue was stored at 4 °C in darkness until the digestions were performed.

2.6.1. Boron analysis

Boron was extracted from the tissues using a modified version of the "high temperature oxidation: dry ashing" method (Kalra, 1998). First, 0.5-g samples of sieved material were burned in a furnace at 500 °C for 4 h and then allowed to cool in the furnace. Subsequently, 2 mL of deionized water were added to the crucibles and 5 mL of HCl (6 N) were added to the wet samples. The crucibles were heated gently to boiling point and left for five minutes. Finally, the digestions were

Table 2
Chemical and physical properties of soil samples. Soil samples were strongly alkaline, saline-sodic, and sandy loam. High boron and arsenic concentrations were found in every soil sample (N.D.: not determined, OM: organic matter, CEC: cation exchange capacity).

Sample	0-5 cm				5-20 cm			
	A	D	E	H	A	D	E	H
Soil properties								
pH	8.72	8.90	8.77	8.54	8.41	8.54	8.61	8.56
EC (mS/cm)	6.65	4.94	6.87	8.83	7.27	4.99	7.06	5.67
OM (%)	7.47	6.51	11.8	7.36	7.05	4.49	6.97	4.55
B_{total} (mg/kg)	8710	9310	7240	4630	11 910	6880	3900	3700
As_{total} (mg/kg)	1960	7330	1570	20 830	1530	15 430	7460	19 480
Clay (%)	17	7	7	N.D	N.D.	19	15	11
Silt (%)	22	37	41	N.D.	N.D.	22	24	20
Sand (%)	61	55	51	N.D.	N.D.	59	61	69
Available nutrients								
P-Olsen (mg/kg)	46	149	67	286	123	144	80	255
K (mg/kg)	1240	1690	1510	2070	1990	1370	1150	1490
Cu (mg/kg)	0.70	1.17	0.79	0.80	0.85	0.11	2.63	0.41
Fe (mg/kg)	35.7	39.3	31.9	255	182	49.5	18.1	226
Mn (mg/kg)	32.6	13.0	28.5	109	37.6	38.7	15.1	51.9
Zn (mg/kg)	1.19	0.83	0.84	1.33	1.18	0.60	1.57	0.57
B (mg/kg)	5380	5740	4060	2480	2160	4390	1820	2010
S (mg/kg)	107	66	154	187	193	334	114	158
Exchangeable cations								
Ca (meq/100 g)	31.2	28.4	24.9	28.3	23.9	30.7	31.3	23.1
Mg (meq/100 g)	1.03	0.88	1.77	1.74	0.98	0.55	0.81	0.95
Na (meq/100 g)	44.4	39.3	43.9	60.3	49.5	37.4	51.4	39.0
K (meq/100 g)	3.18	4.33	3.86	5.29	5.09	3.49	2.94	3.81
CEC (meq/100 g)	79.7	72.9	74.4	95.6	79.4	72.2	86.5	66.9

allowed to cool at room temperature and filtered through a Whatman filter No. 42. The boron concentration was determined using the azomethine-H method (Gupta and Stewart, 1975).

2.6.2. Arsenic analysis

Tissue was digested (Microwave Accelerated Reaction Systems, model MARS; CEM Corp., Mattheus, NC, USA) using the USEPA 3052 method (USEPA, 1996). Arsenic in the digests was determined by ICP-OES (Perkin Elmer Optima 7300 DV ICP-OES, Shelton, CT, USA).

2.7. Plant accumulation coefficient, bioconcentration, and translocation factors

A modified version of the BF was calculated. We calculated the PAC, which considers the available boron fraction instead of the total boron content. Therefore, PAC $= B_{shoot}/B_{available}$, which provides values that are more realistic than BF.

This approach has been used by several authors to determine the ability of a plant species to accumulate a metal (or metalloid) (e.g. Branquinho et al., 2007; Komárek et al., 2007; Murciego et al., 2007; Poschenrieder et al., 2012). The BF of plants growing in different soils, but with equal total metal concentrations, cannot be compared directly. Metals (metalloids) might form a part of minerals that will not release the element during the plant's lifetime. For example, the same species growing under equal total metal concentrations were found to show different BFs that depended on soil characteristics (Si et al., 2006). Hence, the BF is not only a function of the ability of the plant species to accumulate a particular element, but also of the soil composition. Using the available fraction to calculate a modified BF makes the factors of different species more comparable, and provides better indication of a species' ability to accumulate a particular element.

The PAC was calculated as the ratio of boron concentration in plant shoots to the available boron concentration in soil (5–20-cm stratum). The 5–20-cm stratum was considered for the PAC (and BF) calculation because the 0–1-cm stratum was very rich in evaporite salts (Fig. 3), which can solubilize in $CaCl_2$ extractions, but are not readily available for the plants in the study site. We found that the first centimeter of the 0–5-cm stratum presented almost 10 times the boron concentration of the next four centimeters (data not shown). The BF was calculated as the shoot to total soil element concentration (5–20 cm); TF was calculated as the shoot to root ratio of element concentration.

2.8. DNA isolation, amplification, sequencing, and analysis

To confirm the visual identification of the plant species, the internal transcribed spacer (ITS) region was sequenced and analyzed. Total DNA isolation was performed from dried leaf tissue using the FavorPrep 96-Well Genomic DNA Extraction Kit (Favorgen Biotech Corp., Kaohsiung, Taiwan). Polymerase chain reaction ITS-region amplification was performed with the ITS4 and ITS5 primers (White, 1990). Reactions were prepared using 40 ng of DNA, 1 mM of each primer, 1.25 U Taq polymerase (Invitrogen, Carlsbad, CA, USA), 2.5 μl 10× PCR buffer, 0.2 mM of each deoxynucleotide triphosphate, 100 ng/uL of bovine seroalbumin, 1 μL of DMSO, 2 mM $MgCl_2$, and 15.5 μL of distilled water for a total volume of 25 μL. Amplifications were run in an Applied Biosystems 2720 thermal cycler (Invitrogen, Carlsbad, CA, USA) and reactions were performed using an initial denaturation step of 5 min at 94 °C, followed by 39 cycles of 30 s at 94 °C, 30 s of annealing at 55 °C, and a 1-min extension at 72 °C. A final extension step at 72 °C was undertaken for 7 min.

Amplified products (~700 bp) were visualized by agarose gel electrophoresis. The PCR products were sequenced by capillary electrophoresis with ABI3730XL (Macrogen, Seoul, Korea) and DNA sequences were read using Vector NTI software (Life technologies, InforMax, Inc., Maryland, USA). The ITS1- 5.8S rRNA- ITS2 sequence obtained (599 bp) was aligned against the National Center for Biotechnology Information database using the nucleotide–nucleotide BLAST sequence analysis tool (BLASTn: http://www.ncbi.nlm.nih.gov/BLAST/) with default settings. Species identification was determined from the maximum score value of the BLAST output. Finally, the ITS1-5.8S rRNA- ITS2 sequence obtained (599 bp) was submitted to Genbank. The assigned sequence accession number is KF020797.

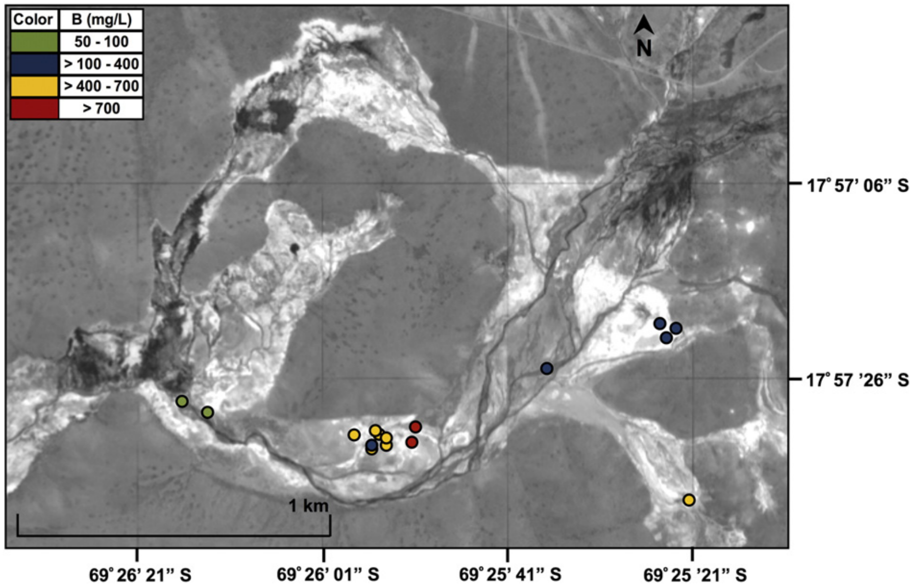

Fig. 2. Boron concentrations in hydrothermal springs located at the headwaters of the Colpitas River. High concentrations of boron are present throughout the area. Source: Google Earth.

3. Results and discussion

3.1. Hydrothermal springs have extremely high boron concentrations

The hydrothermal springs present extremely high concentrations of B (53–861 mg/L), As (1.1–17.8 mg/L) and Cl^- (820–18,000 mg/L), and elevated EC values (7.6–42.4 mS/cm) (supplementary data).

Although the hydrothermal springs exhibit spatial variability in boron concentration (Fig. 2), no significant seasonal variability is observed (supplementary data). All the area presented high concentrations of boron (>50 mg/L), reaching extremely high values in some of the sampling points (>700 mg/l). We found that only one plant species, *P. frigida*, was colonizing these hydrothermal springs, and it was present throughout the area. The results obtained through further study of one selected hydrothermal spring are presented in the following sections.

3.2. Water and soil of the selected hydrothermal spring present toxic conditions for plant establishment

Water from the selected hydrothermal stream presented EC, B, As, and Cl^- values in excess of 20 mS/cm and 400, 5, and 7000 mg/L, respectively (Table 1). The Food and Agriculture Organization (FAO) guidelines for irrigation water quality suggest severe restriction of use of water with EC > 3 mS/cm, B > 3 mg/L, and Cl^- > 350 mg/L, and recommend a maximum concentration of 0.1 mg/L for As (Ayers

Fig. 3. *Puccinellia frigida* growing on the banks of the hydrothermal stream (upper left) and in hydroponic culture (right). Evaporitic salts can be distinguished in the first centimeters of the study site soil (lower left).

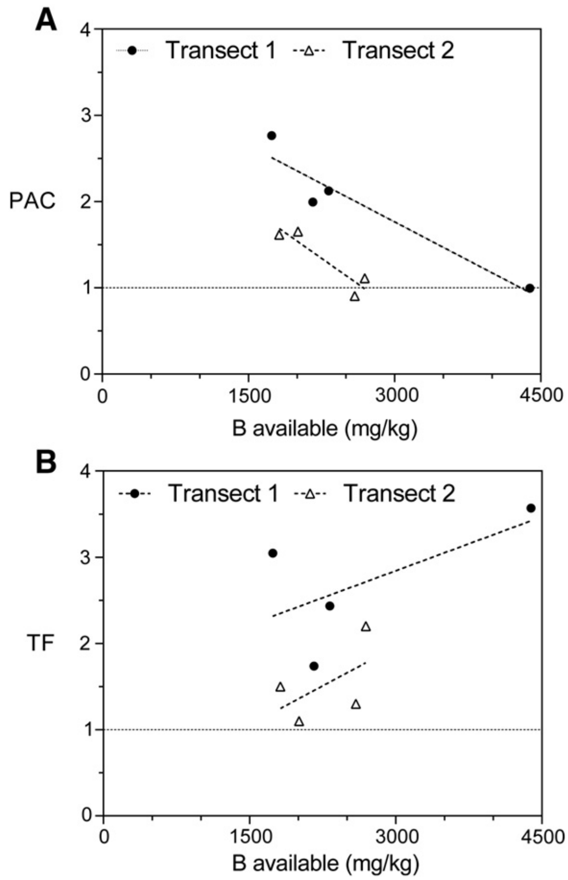

Fig. 4. Variations of plant accumulation coefficient (PAC) and translocation factor (TF) with soil available boron concentration within the study site. (A) PAC vs. B available for transects 1 and 2; PAC increases as B available decreases. (B) TF vs. B available for transects 1 and 2; TF increases as B available increases.

and Westcot, 1994). The concentrations found in the hydrothermal water of the study site largely exceed the FAO's water quality guidelines and therefore, they can be considered extremely toxic for plants.

Soil samples (Table 2) presented pH values of 8.4–8.9, EC within the range 4.9–8.8 mS/cm, and organic matter of 4.5–11.8%. The soil-particle sizes showed sand percentages of 51–69%, silt percentages of 20–41%, and clay percentages of 7–19%. Soil sodium levels were high with an exchangeable sodium percentage (ESP) that varied between 52–63%; therefore, this soil could be classified as a sodic soil (ESP > 15% (Bernstein, 1975)). The characteristics of the soils in both transects were strongly alkaline, saline–sodic, and sandy loam.

With respect to available nutrients, only the Zn content was deficient for some soils (<1.0 mg/kg), whereas K and P-Olsen concentrations were excessive for all of them, exceeding 800 and 40 mg/kg, respectively (Marx et al., 1996). The extremely high B_{total} concentrations found in every sample (3700–11 910 mg/kg) will be discussed in detail in Section 3.3.

Soil As_{total} concentration was between 1530 and 20 830 mg/kg (Table 2), largely exceeding the toxicity threshold concentration of 40 mg/kg for sandy and loamy soils (Sheppard, 1992). Thus, it is clear that *P. frigida* is also an arsenic-tolerant species. In fact, arsenic concentrations in shoots ranged from 16–170 mg/kg DW, and in roots from 500–4870 mg/kg DW. These concentrations are high considering that many species suffer from phytotoxicity when arsenic concentrations in shoots reach 5–20 mg/kg DW (Kabata-Pendias and Pendias, 1992).

Many places with boron toxicity problems share similar soil and water characteristics with the study site. Boron toxicity occurs mainly in arid and semiarid regions, associated with high salinity levels and alkaline soils (Yau and Ryan, 2008). For example, the boron-contaminated soils of the Rio Tinto borax mine (California, EEUU) have high levels of salinity and alkalinity (Stiles et al., 2011). Boron toxicity in Southern Australia is most commonly associated with alkaline sodic soils (Brennan and Adcock, 2004), and in the Kirka boron mine reserve area of Turkey, mine effluents with extremely high boron levels are also highly saline. In addition, some places with boron toxicity problems also suffer from arsenic contamination, as is the case for our study site. In Turkey, the springs in the mining area of the Bigadic district have high levels of boron, arsenic, and salinity (up to 391 mg/l, 0.9 mg/l, and 2.85 mS/cm, respectively) (Gemici et al., 2008). Therefore, finding a plant species tolerant of arsenic and salinity and able to grow in alkaline soils in an arid environment is important for successful implementation of phytoremediation strategies in boron-contaminated locations.

3.3. *Puccinellia frigida* spontaneously colonizes the hydrothermal area

We found only one plant species colonizing the banks of the hydrothermal springs. The species was visually identified as *P. frigida*, which was confirmed by ITS sequencing. When comparing the ITS1- 5.8S rRNA -ITS2 sequence obtained (Genbank accession number KF020797) using BLASTn, five species from the *Puccinellia* genus presented the same maximum score (1061), expect value (0.0), identity (99%), and gaps (0). Of these, only *P. frigida* would be expected to grow in the studied area (Nicora, 1999), which confirmed our visual identification.

P. frigida is a Chilean native perennial grass, 10–35-cm tall, which can also be found in Peru, Argentina, and Bolivia in meadows and salty soils at 3200–4500 masl (Nicora, 1999). To date, the tolerance of *P. frigida* to any metal or metalloid has not been reported and this is the first study in which its tolerance to boron and arsenic is evaluated.

Table 3
Boron concentrations in soils, shoots, and roots, bioconcentration and translocation factors (BF and TF), and plant accumulation coefficient (PAC) of *P. frigida* in the study site. Boron concentrations indicate a high tolerance to this element, and the species acts as a hyperaccumulator in the study site (PAC > 1 and TF > 1).

Sample ID	B_{shoot} (mg/kg DW)	B_{roots} (mg/kg DW)	B_{total} (mg/kg soil)	$B_{available}$ (mg/kg soil)	TF	PAC	BF
Transect 1							
A	4310	2480	11 910	2160	1.7	2	0.36
B	4810	1580	3300	1740	3.1	2.8	1.46
C	4930	2020	4690	2320	2.4	2.1	1.05
D	4370	1230	6890	4390	3.6	1	0.63
Transect 2							
E	2940	1990	3670	1820	1.5	1.6	0.80
F	2990	1390	4250	2690	2.2	1.1	0.70
G	2340	1760	4320	2590	1.3	0.9	0.54
H	3320	3100	3700	2010	1.1	1.7	0.90

3.4. Boron concentrations in the study site soil and tissues of P. frigida indicate high boron tolerance

3.4.1. Study site soil presents high boron levels

The available boron of the 5 - 20 cm stratum of the soil in the study site exceeded 1700 mg/kg at every sampling point, and reached values higher than 4000 mg/kg (Table 3). These concentrations are extremely high, considering that concentrations over 5 mg/kg are toxic for most plants (Ponnamperuma et al., 1979). Moreover, no other species able to tolerate the available boron concentrations found in the soil in this study have been reported. These results indicate that *P. frigida* is an extremely boron-tolerant species.

Boron tolerance in plant species has been studied in both controlled and field conditions. Based on species studied under controlled conditions, the most tolerant are two species studied in hydroponic culture: *Distichlis stricta*, able to withstand 600 mg/L with a 50% of reduction of its growth (Marquis et al., 1984) and *Puccinellia distans* Turkish ecotype, which tolerated 1250 mg/L with a ~50% reduction of its growth (Stiles et al., 2010). In the field, the highest soil boron concentrations where plants have been reported is 227 mg available boron per kg (Babaoglu et al., 2004). However, it is important to note that the boron tolerance of the different species cannot be compared directly because of the differences in experimental and site conditions. Some important soil and site characteristics known to affect boron tolerance are soil pH, soil salinity, and climatic conditions. In our study site, soil pH was around 8.6 and thus, approximately 82% of total available boron could be readily absorbed by roots, because boron is absorbed by plants as an uncharged species (Brown and Shelp, 1997). The soil in the study site presented high salinity levels, which can either increase or decrease boron uptake depending on the plant species, boron concentration, and salinity levels (El-Motaium et al., 1994; Ismail, 2003; Wimmer and Goldbach, 2012; Wimmer et al., 2003; Yermiyahu et al., 2008). Climatic conditions (temperature, wind velocity, illumination, and relative humidity) also affect transpiration rates and consequently, boron uptake (Hu and Brown, 1997). On the other hand, $CaCl_2$ extractions cannot be converted directly to boron concentrations in soil solution, to make a valid comparison with hydroponic cultures, because this depends on adsorption/desorption and precipitation/dissolution reactions within the soils (Nable et al., 1997).

3.4.2. P. frigida's tissue has high boron concentrations

Extremely high boron concentrations in the shoots of *P. frigida* were found; levels of boron exceeded 4000 mg/kg DW at every sampling point in transect 1 and exceeded 2000 mg/kg DW in transect 2 (Table 3). These shoot boron concentrations are the second highest reported values for shoots of any boron-tolerant plant species without visible damage, after *P. distans* (~6000 mg/kg DW) (Stiles et al., 2010). Generally, leaves with boron concentrations higher than 250 mg/kg exhibit symptoms of toxicity (Gupta, 1993). It should be noted that the shoot boron concentration in *P. distans* is the maximum tolerated by this species in hydroponic cultures, whereas *P. frigida* presented these levels under natural conditions. Furthermore, in the study of Stiles et al. (2010), the type of shoot analyzed is not specified (green or yellow); we found almost three times more boron in yellow tissue than green for some samples (data not shown).

The roots of *P. frigida* also presented high boron concentrations (>1000 mg/kg DW; Table 3), which are similar to those measured in *P. distans* in hydroponic culture (~2000 mg/kg DW at 1250 mg/L; Stiles et al. (2010)). Despite that boron could be overestimated in this case because soil particles could remain attached to the roots, results suggest that both shoots and roots possess an internal boron-tolerance mechanism.

3.4.3. Spatial and seasonal variability of boron concentration in P. frigida's tissue

To investigate the spatial and seasonal variability of boron absorption in *P. frigida's* tissue, we collected samples in December 2011. This date corresponds to the end of the spring season, which is also the end of the "dry season" in the Altiplano. The new samples were collected from an area of 130 m × 30 m. We collected and analyzed six samples of plants growing on the banks of other hydrothermal springs within the area, and six samples from the originally selected hydrothermal spring (supplementary data).

Boron concentrations in the samples of the study transects collected in spring (dry season) were similar to those found in summer (rainy season), being slightly higher for the case of shoots (3870–6080 mgB/kg DW) and lower for roots (760–1570 mgB/kg DW). Boron concentrations in the samples collected from other areas were also of the same order of magnitude as those found in the study transects in summer (3650–7030 mgB/kg DW in shoots and 850–2270 mgB/kg DW in roots), although different areas presented variations in the boron concentrations in the plant's tissues. For every sample collected in December 2011, the TF was > 1. In summary, no major spatial or seasonal variability was found in the analyzed hydrothermal springs.

3.5. Boron bioaccumulation and translocation in P. frigida indicate hyperaccumulation

At most sample points, PAC and TF were > 1 (Table 3) and therefore, *P. frigida* acts as a boron hyperaccumulator in the study site because it meets the outlined criteria: (a) it is able to develop in soils with high boron concentrations, (b) boron concentrations in shoots and roots largely exceed those considered toxic for plants, (c) PAC > 1 at many sampling points, and (d) TF > 1 at every sampling point.

If BF (instead of PAC) is used to classify this species, sampling points B and C presented BF > 1 (Table 3). Reeves and Baker (2000) proposed that if one hyperaccumulator plant is detected, this is sufficient to define the species as a hyperaccumulator. Therefore, based on this criterion, *P. frigida* can be classified as a hyperaccumulator.

To confirm this capacity and evaluate its possible uses in phytoextraction and other phytomanagement strategies, hydroponic and controlled environment studies must be conducted. Some reasons for this are: (1) although shoots and roots were washed thoroughly in the present study, some soil particles could remain attached, especially to the roots; (2) environmental conditions can influence boron tolerance within the study site (see Section 3.3); and (3) growth rate must be determined in order to investigate whether this plant could extract boron within a reasonable time, and whether boron tolerance and accumulation are accomplished by extreme growth reduction.

3.6. Plant accumulation coefficient depends on boron availability

Within the study site, PAC decreases as available boron concentration of soil increases (Fig. 4). This behavior is expected for hyperaccumulator plants, which are able to accumulate metals (metalloids) in their aerial parts, but lose this ability at very high soil metal (metalloid) concentrations (McGrath et al., 2000). On the other hand, this suggests that at different boron concentrations in the substratum, *P. frigida* might act as an accumulator or excluder. This provides the possibility of using *P. frigida* for phytoextraction or phytostabilization purposes depending on the available boron concentrations of the soil.

TF appears to increase with available boron concentrations (Fig. 4). However, no conclusions can be drawn because this correlation is not that strong, and these results might have great uncertainty due to the possible contamination of roots with soil particles. Unfortunately, we were unable to quantify root contamination. To examine it, we analyzed other metals in the soils and roots by ICP-OES (data not shown). Roots had high contents of iron and manganese, but these could be due either to soil contamination or to the formation of iron and manganese plaques, because different wetland plants form iron plaques (e.g. Blute et al., 2004; Otte et al., 1995). On the other hand, Al, Ba, and Cr contents in soil were too low to allow a valid analysis of root contamination.

4. Conclusions

P. frigida, a perennial grass, was found naturally colonizing a hydrothermal area in which soils and water present concentrations of As, B, and EC levels that are extremely toxic for plants. Soil and tissue analyses demonstrated that *P. frigida* is one of the most boron-tolerant species known. In the study site, it acts as a boron hyperaccumulator by accumulating boron in its aerial tissue. Therefore, *P. frigida* has the potential to be used for boron phytoextraction purposes, because it is a perennial species, hypertolerant to boron, and a hyperaccumulator able to grow in alkaline and extremely salty soils; conditions often present in boron-contaminated areas. Experiments to confirm and test this capacity need to be performed.

Finally, the extreme boron tolerance shown by *P. frigida* and its ability to colonize the hydrothermal springs makes this species a good candidate with which to reduce boron leaching from the hydrothermal area to the Colpitas River. However, this phytomanagement strategy should be investigated carefully because of the ability of *P. frigida* to accumulate boron in its shoots. Strategies of regular coppicing should be considered to prevent boron release from the decomposition of its leaves.

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Appendix A. Supplementary data

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