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Phytostabilisation with Mediterranean shrubs and liming improved soil quality in a pot experiment with a pyrite mine soil

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ABSTRACT

Phytoremediation can be a suitable option to manage derelict mine soils. A pot experiment was carried out under semi-controlled conditions with a mine-impacted soil. A further contamination event was mimicked by applying 5% of pyritic sludge. Four species were planted in pots (*Myrtus communis*, *Retama sphaerocarpa*, *Rosmarinus officinalis* and *Tamarix gallica*), and some pots remained unplanted as a control. The substrates were moderately to highly contaminated, mainly with arsenic and zinc. The strong acidification induced by the pyritic sludge was buffered with lime and plants survived in all the pots. Liming provoked an effective immobilisation of metals and arsenic. Plant establishment decreased labile As in the substrate by 50%, mainly *M. communis*, although the levels of extractable metals were not affected by the plants. *R. sphaerocarpa* and *M. communis* increased the levels of C and N in the soil by 23% and 34% respectively, and also enhanced enzymatic activities and microbial respiration to the double in some cases. The low transfer of trace elements to shoots limited the phytoextraction rate. Our results support the use of phytostabilisation in Mediterranean mine soils and show how plants of *R. sphaerocarpa* and *M. communis* may increase soil health and quality during revegetation.

1. Introduction

Several soils all over the world have been contaminated by trace elements since early stages of the Industrial Revolution, mainly due to mining activities [1]. In some cases, the contamination has been provoked by sudden events in which a large amount of metallic residues (i.e. sludge) covered land surface and the soil matrix became clogged with contaminant particles [2,3]. As a result, many of these soils should be remediated to decrease the environmental risk associated with the contamination [4]. For their reclamation there are different chemical, physical, and biological processes available. Amongst them, phytoremediation includes a wide range of phytotechnologies which aim to improve the environmental status of a polluted soil [5]. Phytoextraction is a technique where exceptional amounts of contaminants are accumulated in the harvestable tissues that are removed from the site, while phytostabilisation aims to improve soil quality and to confine the contaminant in a safer form in the soil. However, prior to implementing a phytotechnology the results of the interaction between the contaminated soil and the selected plant(s) species should be evaluated [6]. Here a pot experiment has been used because it allows obtaining valid results with a certain control of randomized factors.

There are many metal(loid) mine sites that have been closed down, but the surrounding soils remain contaminated [7–9]. These areas are commonly derelict and, in many of them, the main attributes of interest to be saved are environmental, where the aim is to recover the capacity of the ecosystem to function and provide services [10]. For this purpose, phytotechnologies, with moderate impact on the soil and the environment, are particularly welcome as the other alternatives are too expensive and are more invasive [11]. In these mine soils, contaminant and nutrient availability is usually (very) limited, and plants establishment is difficult [7], mainly in Mediterranean environments [8]. Therefore, the use of native plants is desirable not only to increase the chances for survival, but also to fulfil environmental guidelines [4].

To evaluate the efficiency of phytoremediation, many authors have studied the effects of plant growth and management in total/available content of contaminants in the soils and plants [12], but other aspect has been traditionally missed in phytoremediation prospects: the improvement in soil quality [13,14]. In those sites where the environmental functions and services must be preserved or improved, obtaining healthy soils is a main target and our studies

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should include the effects of plants on traditional indicators of soil quality (such as organic matter, nitrogen or biological activity). In a parallel experiment under field conditions, phytostabilisation of the studied soil was reported as the most realistic phytotechnology [15], although soil quality was not addressed.

The aim of the present work is to study the suitability of four Mediterranean shrubs for phytoremediation, with moderately contaminated soil which has been artificially polluted by a pyritic sludge to simulate a toxic spill.

2. Materials and methods

2.1. Experimental set up

The upper layer of soil (0–20 cm depth) was collected (~400 kg) in the experimental plot "El Vicario" (Aznalcóllar, Sevilla), in a zone affected by a pyritic sludge flooding 8 years before the soil collection. This soil is a Typic Xerofluvent with sandy-loam texture, low percentage of carbonates, and low organic matter and nitrogen. The soil was sieved to <4 mm and homogenized with a cement mixer. For treating the pots, toxic pyritic sludge from the Aznalcóllar spill was used (<2 mm), after being stored for 8 years at −20 °C. A fine mesh and a layer of quartz gravel (5 cm) was placed in the bottom of plastic pots (5 L), which were afterwards filled with 6 kg of soil:sand (quartz 4–6 mm, pH 6) 1:1 (m:m) mix. Toxic sludge (5% on a weight basis, equivalent to 6000 t km^{-2}) was added to half of the pots. The toxic sludge percentage is similar to the remaining fraction in some of the soils of Aznalcóllar after the ecological disaster [16]. The soil had the following initial characteristics: pH 6, 2% of organic matter, 0.07% of N, <0.1% carbonate, 2% Al, 80 mg As kg^{-1}, 2 mg Cd kg^{-1}, 105 mg Cu kg^{-1}, 3% Fe, 577 mg Mn kg^{-1}, 301 mg Zn kg^{-1}; for the pyrite sludge: pH 2.4, 0.6% Al, 3800 mg As kg^{-1}, 17 mg Cd kg^{-1}, 767 mg Cu kg^{-1}, 32% Fe, 970 mg Mn kg^{-1}, 3370 mg Zn kg^{-1}. Beneath the pots, a plastic plate was placed to collect the drainage. A layer of cotton cloth allows the flow up of this drainage by capillarity, thereby preventing losses from leaching during the experiment. Four shrub species were selected between those chosen to revegetate The Guadiamar Green Corridor (Anzalcóllar): *Myrtus communis* L., *Retama sphaerocarpa* L., *Rosmarinus officinalis* L., and *Tamarix gallica* L.; these were previously studied under hydroponics to understand their interaction with trace elements [17,18]. One year old plants were obtained from the nursery of the Andalusian Government (San Jerónimo Greenhouse, Sevilla, Spain), with a similar size to plants routinely used in the revegetation of degraded lands (20–50 cm of shoot length, 15–40 g $plant^{-1}$). One plant was inserted in each pot and the pots were placed under a cover inside the UAM campus (Madrid, Spain). The experiment was running for 2 years, with temperatures ranging −1 to 40 °C and plants were watered at a rate of 700–800 mm $year^{-1}$ with tap water, without any fertilisation. Lime (pure $CaCO_3$ reagent at a rate of 6% m:m) was added to the spiked pots to increase the pH after 3 months of interaction between the soil and the pyritic residue. Carbonate was added to the surface and the top 10 cm was carefully tilled to mix the substrate and the carbonate but avoiding damages in the root. Afterwards, pots were top-watered once a day for 5 days in order for the lime to move down the soil profile.

2.2. Sample processing and analysis

Soils and plants were monitored throughout the experiment. Small soil samples were regularly collected, always maintaining plant integrity, and processed as described for the final sampling (see below). To analyse the influence of the liming procedure, an aliquot was taken before (14 weeks from the beginning of the experiment) and after the calcium carbonate addition (18 weeks). Weakly-retained fraction was monitored by extracting metals and As with 0.1 M $(NH_4)_2SO_4$ [19]. Arsenic fractionation was analysed as [20] described: (1) $(NH_4)_2SO_4$-extractable fraction, (2) $NH_4H_2PO_4$-extractable fraction, (3) $(NH_4)_2C_2O_4$-extractable, (4) $(NH_4)_2C_2O_4$-extractable at 96 °C, and (5) residual phase. Percentage of recovery of As after sequential extractions ranged 73–113%, with 5 replicates from each treatment. Plant length was measured after the summer growth period in 2006 and 2007, and a small amount of young shoots was sampled from each plant to analyse element concentration and plant biomarkers: lipid peroxidation, thiols and chlorophylls [17,18]. Lipid peroxidation in plant tissues was based on malon dialdehyde (MDA) concentration. For MDA determination, a 0.1 g FW aliquot of tissue was extracted with 1 mL of colorimetric reactive trichloroacetic acid (15%)–thiobarbituric acid (0.37%)–HCl (0.25 M), heated at 90 °C for 30 min and then cooled. After centrifugation at 11,000 × *g* for 10 min, the absorbance of supernatant was measured at 532 nm and 600 nm, using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. Acid soluble thiols were extracted and determined: 0.1 g fresh weight (FW) was extracted with 0.4 mL of NaOH (0.1 M) + $NaBH_4$ (25 mg mL^{-1}) and 0.2 mL of distilled water, then centrifuged at 11,000 × *g* for 5 min. The resulting supernatant (0.5 mL) was diluted with 0.2 mL HCl (35%) and centrifuged at 11,000 × *g* for 5 min. Then 0.5 mL of 300 μM 5,5′-dithiobis(2-nitrobenzoic acid) in phosphate buffer 0.5 M (pH 7.5) was added to 0.5 mL of supernatant and heated at 30 °C for 2 min. Absorbance was determined at 412 nm, and quantified using a calibration curve of glutathione. Chlorophylls were extracted by homogenizing 0.5 g FW leaf tissue in 80% acetone. After filtering and diluting to 50 mL with 80% acetone, absorbance was determined at 645 and 663 nm. Chlorophyll concentrations were estimated according to Wellburn's method [18].

For the final sampling, the soil from pots was sampled by vertically inserting a cylinder of 30 cm. Plants were uprooted, and the soil particles were manually removed. Afterwards, roots and shoots were divided, and plant material rinsed under tap water for 5 min. Then, plant material was submerged in distilled water for 2 min, dried at 60 °C for 3 days, and milled to a fine powder in a grinder. Soils were dried in a glasshouse (~30 °C) for 7 days, and sieved to <2 mm.

Plant material (0.5 g) was digested in an autoclave (Autoester-G, Selecta) with 3 mL HNO_3 (65%), 2 mL H_2O_2 (33%), and 10 mL mili-Q water at 125 °C under 1.25 kPa for 30 min, afterwards the samples were filtered and diluted to 25 mL with mili-Q water [9,10].

Soil pH was measured in a 1:2.5 (m:v) suspension, both in water and in 0.1 M KCl [24]. Total N and organic-C were determined in an automatic microanalyser (EuroVector).Total concentration of elements in soil samples were processed by autoclave digestion of soil (0.5 g) with 6 mL HNO_3, 4 mL H_2O_2, and 6 mL of mili-Q water; the extracts were filtered and diluted to 50 mL with mili-Q water [9,10]. For weakly retained As in soil samples, 2 g of soil was mixed with 20 mL of 0.1 M $(NH_4)_2SO_4$, shaken for 4 h, and the resultant suspension filtered [20]. Metals were not detectable in the previous extract, so they were extracted by a low molecular weight organic acid (LMWOAs) solution: 2 g of soil was mixed with 20 mL of LMWOAs solution (total concentration of acetic, lactic, citric, malic and formic acids was 0.01 M), shaken for 16 h, and the resultant suspension filtered [19].

Metal concentration was determined by AAS in soil and plant extracts, and arsenic by AFS. Certified reference materials (CTA-VL2 for plant and CMR048-050 for soil) were also digested and analysed, with recoveries >83% for metals and As. Detection limits for each elements were: 0.1 μg As L^{-1}, 20 μg Cd, Cu, Mn and Zn L^{-1}, 100 μg Fe and Al L^{-1}.

The urease activity in the soil was analysed using the method proposed by Sastre-Conde and Lobo [21], using urea as substrate

and further determination by a selective ammonium electrode. The phosphatase activity was determined according to Trasar et al. [22], using *p*-nitrophenol phosphate as a substrate. The *p*-nitrophenol values were measured in a UV–vis spectrophotometry at 400 nm. β-glucosidase activity was determined according to Jimenez de Ridder and Bonmatí [23], after soil incubation with β-D-glucopirasonide substrate at 37 °C and pH 6 during 1 h and further measurement of the *p*-nitrophenol concentration by UV–vis spectrophotometry at 400 nm.

Glucose-induced soil respiration was determined in triplicate samples of soil at 30 °C by monitoring the CO_2 production for 24 h using the μ-Trac 4200 system (SY-LAB, GmbH P.O. Box 47, A-3002 Pukersdorf, Austria), based on the variation in conductivity of an aqueous 0.2% KOH solution [24].

2.3. Data processing and statistical analysis

Data were processed with Excel and SPSS softwares. Two-way ANOVA was used for mean comparison, with pyrite treatment (PT), plant species (Sp) and their interaction (PT × Sp) as factors.

Effect of plants on soil respiration was calculated as the increase of released CO_2, in percent, with regard to each treatment without plant (control). The transfer factor (TF) was calculated as the ratio $[TE]_{shoot}:[TE]_{soil}$.

3. Results and discussion

3.1. Changes in soils and plants during the experiment

3.1.1. Soil

Chemical parameters in non-spiked soils remained stable over time, but the changes in those soils with pyritic sludge application was particularly interesting since many changes occurred in the first stages of the experiment. The pH in pyrite-spiked soils decreased from 5 to <3 in only 14 weeks, derived from the strongly acidic oxidation of pyrite [16]. Such pH is itself hazardous for plant survival, but pyrite oxidation additionally results in the mobilisation of associated metals and As [25] and the acid environment releases Al and Mn from the soil components [26]. Fig. 1 shows this remarkable increase in metals and As availability 14 weeks after the acidification. This release of trace elements occurred, according to Aguilar et al. [27] findings, with Zn and Cu being more rapidly mobilised than As during the pyrite oxidation. The combination of very low pH and high contaminant availability suggested that liming with $CaCO_3$ was the best alternative. The dose of calcite was similar to the one suggested by Aguilar et al. [28] for soil of Aznalcóllar after pyrite contamination. The liming provoked an increase in soil pH to 6–7 in 4 weeks (18 weeks after the start point). At the same time, the labile fraction of metals and As in soil decreased in pyrite-spiked pots, even below extractable levels in non-spiked pots (0.3 mg Al kg^{-1}; 0.03 mg As kg^{-1}; 0.02 mg Cd kg^{-1}; 0.35 mg Cu kg^{-1}; 20 mg Mn kg^{-1}; 3 mg Zn kg^{-1}). Lime application is a well-known, effective amendment to immobilise metals [1,29], but many authors have described As mobility increases following pH increments [30]. Contrarily to this, As was also effectively immobilised by $CaCO_3$. This fact has been also reported in pyrite-contaminated mine soils [28,31,32], and may be attributed to *in situ* formation of Fe-oxides, retention in jarosite, gypsum and carbonate, or precipitation of arsenate salts (e.g. $CaAsO_4$) [16,31,32]. Contrastingly, other authors have reported how liming may induce As solubilisation in mine tailings [33], and thus the effects of lime application on As in a singular soil should be investigated in the laboratory previous to field application. To assess the changes in As geochemistry, a specific sequential extraction was performed (Fig. 2 for limed pots). At 14 weeks, there was a strong increase of

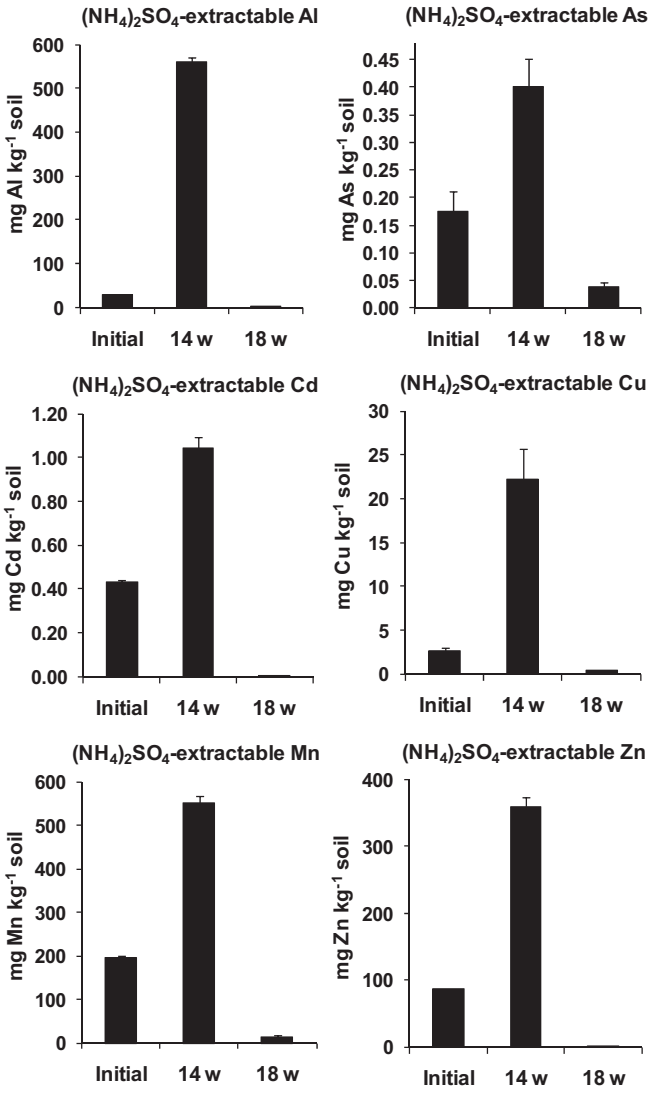

Fig. 1. Extractable Al, As, Cd, Cu, Mn and Zn concentrations in pyrite-spiked pots during the first 4.5 months of experiment. Mean ± SE (n = 5).

As in weakly retained fractions (F1 and 2), which is usually linked with the currently mobile/available element in soil [34]. However, after liming, As in these fractions decreased and was re-distributed to the other fractions. Arsenic in fraction 3 increased during the acidification and remained constant after the liming. Arsenic in fraction 4 decreased during the acidification but was doubled after the liming procedure. Fraction 4 is attributed by Wenzel et al. [20] to crystalline Fe-oxides, although this extraction may also be able to extract As retained in salts since, for instance, Kreidie et al. [32] demonstrated the dissolution of jarosite by this extraction step. Arsenic in fraction 5 decreased with time, indicating the progressive oxidation of pyrite.

After the liming, the pH and extractability of trace elements remained constant over time (from 18 weeks onwards).

Arsenic fractionation in non-spiked soils did not show noticeable changes during the experiment, accounting each fraction <0.1% in F1, 6% in F2, 19% in F3, 39% in F4 and 36% in F5 (data not shown).

3.1.2. Plant

Plants grown in soils spiked with pyrite reflected the changes in the pyrite-spiked soils. After 4 months, plants exposed to strong acidification, available metals and As due to the pyritic sludge showed higher concentration of As and Cu in young shoots

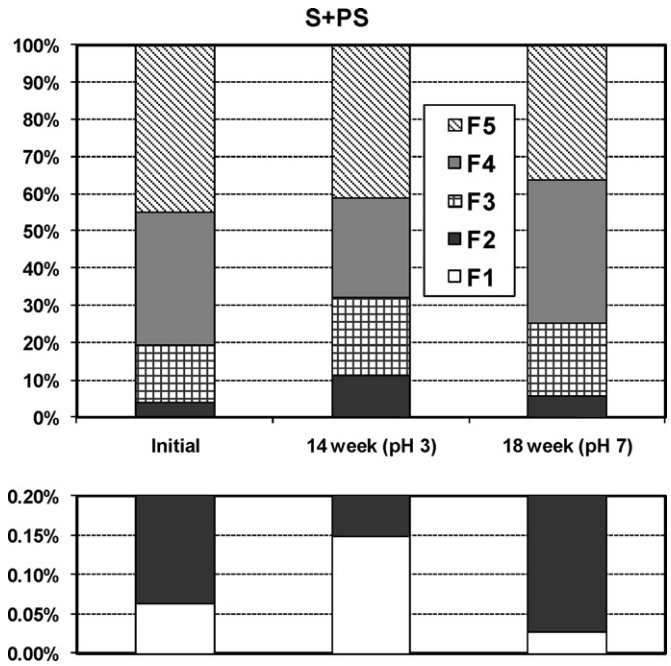

Fig. 2. Arsenic fractionation (percentage in each fraction, mean) after a stage of acidification (14 weeks from start) and a subsequent lime application (18 weeks). A detailed view of the lower part of the figure is presented below.

compared to non-spiked pots (Fig. 3, $p < 0.05$), similarly Zn, Al and Mn were also accumulated in plants at the same time (data not shown). Furthermore, significant increases of the stress bioindicators MDA (concentration), thiols (concentration) and chlorophyll *a* ($p < 0.05$) indicated plant stress. Pyrite-induced stress symptoms in *R. sphaerocarpa* were less evident than in the other plant species. The second sampling (after 16 months) showed how liming was able to revert the previous stress: concentrations of trace elements (except for Zn and Mn) were similar independently of the presence of pyrite sludge. No statistical differences on stress bioindicators were obtained. Generally, physiological parameters that respond as biomarkers of trace element contamination are useful in the employment of vascular plants for environmental monitoring and assessment [35]. Excessive concentrations of trace elements in plants have been reported to result in oxidative stress, and levels of MDA increased after exposures to As, Cd, Cu and Zn [36,37]. At the same time, metals and As interfere with chlorophyll synthesis and deplete the concentration of this biomolecule [38,39]. To detoxify these trace elements, plants generate compounds that are able to inactivate their toxic action, and –SH levels increased to alleviate toxicity in plants, e.g. with As, Cu or Cd [37,40,41]. In previous experiments we demonstrated the use of bioindicators in the diagnosis of As and Hg phytotoxicity in the studied plants [17,18], but they have not been tested in soil conditions yet. In fact, these bioindicators have been intensively studied in hydroponic studies, but soil studies are desirable to ensure their feasibility *in situ*. Our current results support both the applicability of such bioindicators and the sampling of young shoots as sensitive tissues for biomonitoring pyrite soil contamination.

Plant growth also showed a similar pattern, indicating pyrite-induced phytotoxicity in the first stage of the experiment, linked to the acidification. Shoot elongation was notably lower in pots with pyrite sludge than in non-spiked pots until the first sampling, but in the second one toxic elements were immobilised by liming and the growth rate was recovered to similar levels than in non-spiked plants (supplementary material Fig. SM1). Inhibition of plant growth is commonly disturbed by exposure to high concentrations of a single trace element or mixture of contaminants [38].

3.2. Final sampling

3.2.1. Chemical and biological parameters in soils

The pH in water (or in KCl, not shown) ranged 6–7 in all the pots at the end of the experiment (Table 1). C and N were classically analysed to evaluate soil quality, their levels were within those described in degraded Mediterranean lands [41], except in

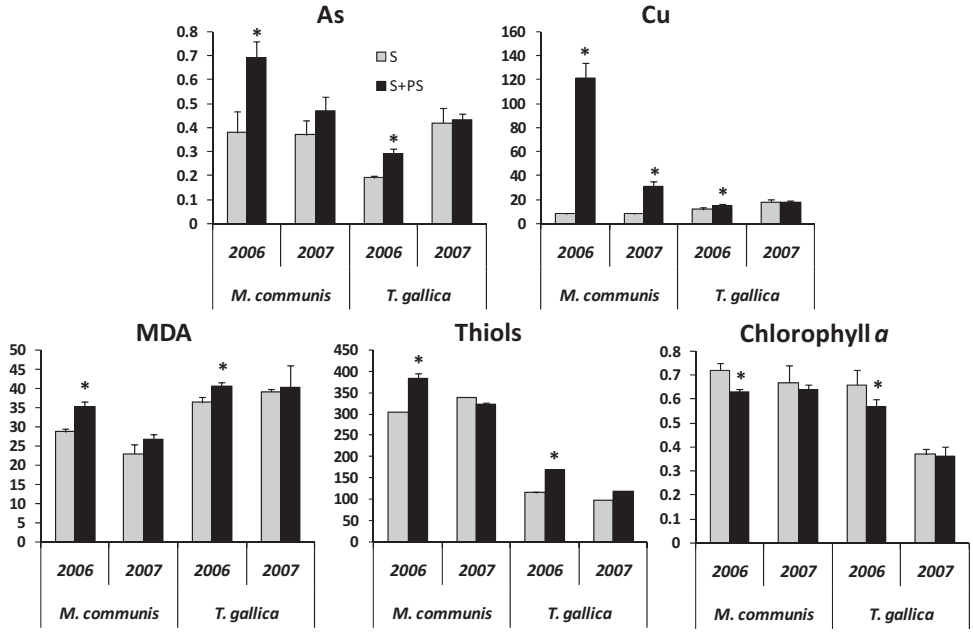

Fig. 3. Sampling of young shoots of *M. communis* and *T. gallica* during the experiment: As and Cu concentrations ($\mu g\,g^{-1}$ DW) and bioindicators: lipid peroxidation (nmol MDA g^{-1} FW), thiols (nmol –SH g^{-1} FW) and chlorophyll *a* (mg g^{-1} FW). Plants were growing in pots either with soil (S) or with soil spiked with 5% of pyrite sludge (S + PS), lime was applied slightly before the first sampling (2006). Mean ± SE. Asterisk indicates significant increase/decrease at $p < 0.05$ between soil and soil spiked with 5% of pyrite sludge.

Table 1
Chemical properties of the substrates: pH and levels of C and N. Mean ± SE ($n = 5$). Two-way ANOVA's result (below) indicates the statistical influence of the factors pot type (PT), species (Sp) and their interaction (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Pot type (PT)	Species (Sp)	pH	C (%)	N (g kg^{-1})
Soil				
	No plant	6.92 ± 0.05	0.98 ± 0.02	0.97 ± 0.12
	M. communis	6.88 ± 0.06	1.24 ± 0.08	1.13 ± 0.05
	R. sphaerocarpa	6.84 ± 0.07	1.13 ± 0.04	1.30 ± 0.15
	R. officinalis	7.26 ± 0.07	1.20 ± 0.03	0.92 ± 0.01
	T. gallica	7.45 ± 0.07	1.06 ± 0.01	1.02 ± 0.07
Pyrite-spiked (5%) soil				
	No plant	7.17 ± 0.08	0.88 ± 0.02	0.53 ± 0.03
	M. communis	6.34 ± 0.11	0.98 ± 0.02	0.67 ± 0.02
	R. sphaerocarpa	6.21 ± 0.25	0.95 ± 0.03	0.64 ± 0.01
	R. officinalis	6.91 ± 0.03	0.81 ± 0.04	0.50 ± 0.04
	T. gallica	6.77 ± 0.05	0.88 ± 0.04	0.47 ± 0.03
ANOVA	PT	<0.001***	<0.001***	<0.001***
	Sp	<0.001***	<0.001***	<0.001***
	P × Sp	0.001**	<0.001***	0.001**

some pots with plants, where the values were close to normal values in Mediterranean soils. Levels of C and N in soils were significantly influenced by pyrite spiking, by the plant species and by the interaction of both factors ($p < 0.001$ or $p < 0.01$). The presence of pyrite sludge reduced C and N in the soil in comparison with non-spiked pots, probably by slowing the incorporation rate in the presence of toxic elements and an inhibiting environment (very low pH) at the beginning of the experiment. Carbon concentrations in soil generally increased in those pots with plants, mainly when *R. sphaerocarpa* or *M. communis* were growing. This increase can be explained by the presence of root biomass in soil, root exudates, and the litter fall over the soil surface. Levels of organic matter in soil are directly correlated with soil fertility and sustainability, especially in Mediterranean ecosystems [42], and it is considered an efficient C reservoir [43]. N levels in soil were also enhanced by plant growth, again *R. sphaerocarpa* and *M. communis* being the plants where this effect was observed. The increase of N could be attributed to N-loaded material in soils and biological N_2 fixation. *R. sphaerocarpa* is a leguminous plant that can establish symbiotic relationships with *Rhizobium* ssp. to fix N_2, but *M. communis* cannot form nodules. In the latter case the plant might create favourable conditions to free-living N-fixing microorganisms [44].

Total As, Cd, Cu and Zn concentrations in soil were increased by the application of pyrite sludge ($p < 0.001$) (supplementary material, Table SM1). The substrates had levels around 80 and 300 mg As kg^{-1} and 300 and 500 mg Zn kg^{-1} without and with application of pyritic sludge respectively, these two elements being the most noticeable contaminants. Plant growth in the pot did not significantly influence total concentration of As, Cd or Cu, and only some slight decreases were observed for Zn and Mn (in a few cases); however we are not focussing on the effect on Mn and Zn since phytostabilisation does not aim to deplete total metal concentrations. Total trace element concentrations in all the pots, independently of the application of pyrite sludge, were in the interval reported by Cabrera et al. [45] in soils contaminated by a toxic spill, therefore our experiment mimicked a sudden pyrite contamination scenario. Although levels of metals are high in pyrite-spiked soils, only As in this treatment exceeded the intervention values for soils by the Andalusian environmental authority [46].

With respect to the labile fraction of trace elements in soil, there were differences between micronutrients and exclusively toxic elements. Ammonium sulphate-extractable As was higher in non-spiked pots, despite the higher total As concentration in spiked pots, and plant growth decreased this fraction in both substrates (Fig. 4, $p < 0.001$). *M. communis* was the most efficient plant in this immobilisation. LWOA-extractable Cd was depleted also by plant treatment ($p < 0.001$), with higher concentration in soil of spiked pots. Plants were not able to immobilise Zn (Fig. 4), showing a similar pattern to Cu or Mn (not shown), and in some cases plant induced an increase of LWOA-extractable concentration of these metals. The immobilising effect of liming was maintained to the end of the experiment. Although phytostabilisation aims to deplete available/mobile fraction of contaminants in soil [47], in our study this effect was only observed in some cases, e.g. As and Cd.

Enzymatic activity in soils has been a frequent indicator of biological soil quality to monitor the reclamation of degraded lands [48]. These biological indicators are proposed as tools to evaluate the efficiency of phytoremediation processes, because they are

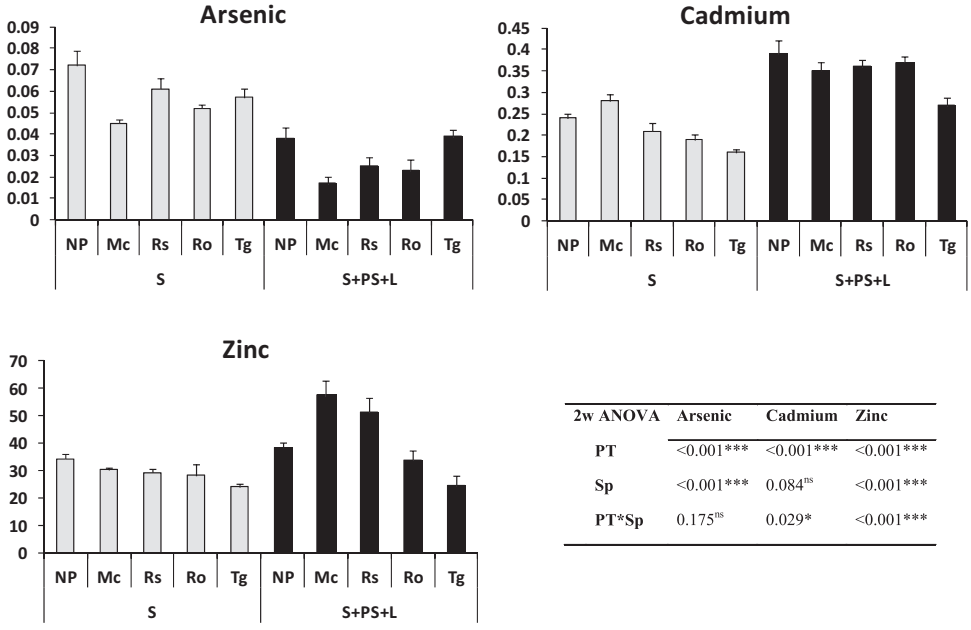

Fig. 4. Extractable trace element concentration in soils (mg kg^{-1}): arsenic extracted by $(NH_4)_2SO_4$ solution; cadmium and zinc were extracted by a mix of LMWOAs solution. Mean ± SE ($n = 5$). Two-way ANOVA's result (below) indicates the statistical influence of the factors pot type (PT), species (Sp) and their interaction (ns = non-significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

sensitive, integrative and their response is quick [49]. To obtain a complete overview in this study, we tested enzymes that are involved in the biogeochemical cycles of C (β-galactosidase), N (urease) and P (acid phosphatase), and the biological activity (respiration rate). In many cases, plant establishment, pyrite application and the interaction between both factors influenced both enzymatic activities and respiration in soils (Fig. 5; supplementary material Table SM2 for statistical differences). Pyrite application had no significant effect on either phosphatase or respiration rate, while urease and β-galactosidase were inhibited. Plant establishment induced a species-dependent effect. As a general rule, plant establishment enhanced enzymatic activities and the respiration rate independently of the application of pyrite. *R. sphaerocarpa* and *M. communis* seem to show the best results. *R. officinalis* only slightly activated some of them, while it did not provoke activation in urease. In a previous experiment, roots of *R. officinalis* were the more recalcitrant in soils [50], therefore its roots may not be a good substrate for microbial activity in soils.

The increase of microbial activities has been linked to improvements in soil quality, mainly in Mediterranean environments. The current work shows how plant establishment, mainly *R. sphaerocarpa* and *M. communis*, accelerate C, N and P cycles in soil, which may facilitate the soil to develop its functions (e.g. nutrient supply, recycle materials, establish a niche for microorganisms). The increase of enzymatic activity in the rhizosphere is a well-known phenomena: roots can exudate organic compounds and dead roots are a good substrate and supply available nutrients for microbiota. Therefore microbial populations are more active, but also rhizosphere populations are qualitatively and quantitatively different to those in the bulk soil [51]. Contaminant elements in soil inhibit enzymatic activities, but rhizosphere habitats protect microorganisms and partly alleviate the toxic effects [52]. The levels of enzymatic activity reported in our work are low [53], in agreement with the values reported by Hinojosa et al. [54] for degraded soils of Aznalcóllar. Also Sastre-Conde et al. [55] found similar urease and phosphatase activities in non-degraded Mediterranean soils, but these values are very low with respect to those in heavy metal-contaminated soils in North of Spain [56], where the climate, soil and vegetation differ from Mediterranean conditions. Epelde et al. [56] described that phytoextraction increased microbial activity in soil while amounts of trace elements decreased in soil, and roots explored more soil volume. In our study phytostabilisation caused similar benefits, improving soil quality.

The negative effects of pyrite waste on soil enzymes have been previously tested, reporting EC_{50} of 0.7–2.7% in a short experiment [57], with the higher sensitivity for β-galactosidase. EC_{50} in our conditions would be higher, since 5% of pyrite spiking depleted by 50% only urease in pots with *R. sphaerocarpa*. This may suggest certain adaptation of enzymes in long experiments. On the other hand, Hinojosa et al. [57] reported liming reverted pyrite toxicity in part, and in our case early $CaCO_3$ assisted either to maintain and/or to prime biological activity in the soils.

3.2.2. Plants

Plant biomass was affected by the pyrite spiking, the plant treatment, and the interaction between the factors (supplementary material Fig. SM2; $p < 0.001$). Plant growth inhibition during the acidification in pyrite-spiked soils was reflected in plant biomass at the end of the experiment, so that lower biomass was obtained in these pots. *T. gallica* showed the highest shoot biomass, while roots of *M. communis* had the highest belowground biomass. Levels of trace elements in shoots and roots at the end of the experiment showed also the strong acidification before the liming, showing higher concentrations in those plants growing in 5% of pyrite sludge. Concentration of As, Cd, Cu, Mn and Zn were generally higher or much higher in these plants, especially in roots (Table 2; $p < 0.001$). However, concentration of metals and As in this Mediterranean shrubs are lower than concentrations reported in previous similar experiments with pyritic waste application, using agricultural plants, e.g. lupin, soya, sorghum, maize and wheat [58,59]. The plantation of the studied Mediterranean shrubs in phytostabilisation of trace element-contaminated soils, therefore, posed a lower risk to be transferred through the food chain than cropping agricultural species due to both lower concentrations of contaminants and lower edibility for organisms. Transfer factors for all the metals and As were low, always <1, and for As <0.01. These TFs also support the use of these Mediterranean shrubs only for phytostabilisation strategies, in accordance to the results observed in a parallel field experiment [15].

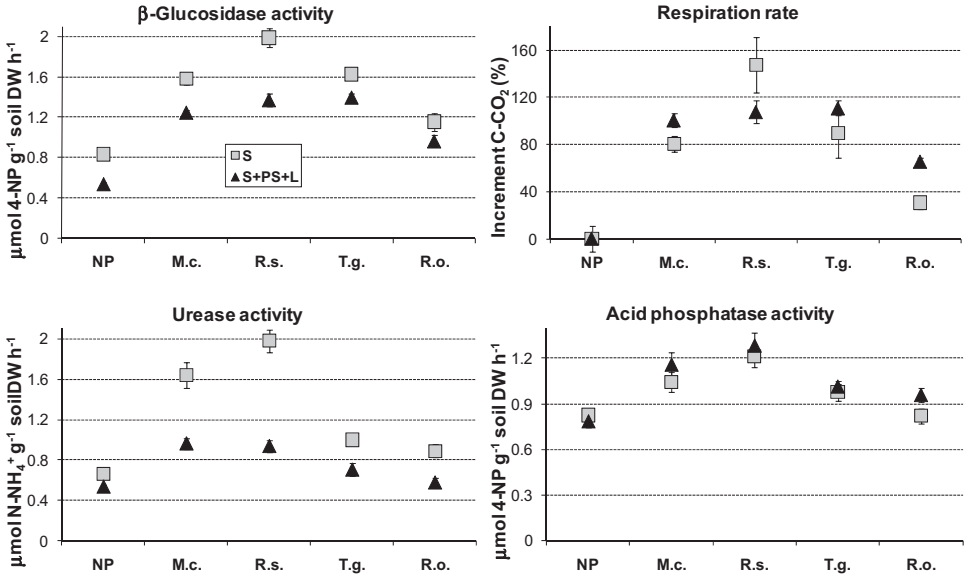

Fig. 5. Enzymatic activities: β-glucosidase, urease and acid phosphatase activities and respiration rate in soils (S, grey squares) or limed pyrite-spiked soils (S + PS + L, black triangles) with different plant species (NP = no plant; M.c. = *Myrtus communis*; R.s. = *Retama sphaerocarpa*; R.o. = *Rosmarinus officinalis*; T.g. = *Tamarix gallica*). Mean ± SE ($n = 5$ or 3 for respiration rate). Statistical information is shown as supplementary material (Table SM2).

Table 2
Trace element concentration in plant organs and transfer factor (TF), S = pots with soil; S + PS + L = pots with limed pyrite-spiked soils. Mean ($n = 5$). Two-way ANOVA's result (below) indicates the statistical influence of the factors pot type (PT), species (Sp) and their interaction (PT × Sp), ns = non-significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Pot type (PT)	Species (Sp)		Total concentration in plant ($\mu g\,g^{-1}$ DW)			
			As	Cd	Cu	Zn
S	*M. communis*	Shoot	0.65	0.04	5.98	26.7
		Root	2.53	1.27	41.0	55.9
		TF	0.008	0.03	0.06	0.09
	R. sphaerocarpa	Shoot	0.36	0.55	10.0	82.1
		Root	1.92	1.10	41.7	162.8
		TF	0.004	0.31	0.07	0.25
	R. officinalis	Shoot	0.34	0.14	12.5	46.5
		Root	4.42	1.30	72.9	123.9
		TF	0.004	0.08	0.12	0.15
	T. gallica	Shoot	0.36	0.44	12.1	29.6
		Root	2.24	1.17	32.5	51.6
		TF	0.005	0.27	0.13	0.10
S + PS + L	*M. communis*	Shoot	0.77	0.32	8.6	105.9
		Root	12.9	2.37	49.5	208.0
		TF	0.003	0.02	0.07	0.03
	R. sphaerocarpa	Shoot	0.37	1.19	14.9	214.9
		Root	7.23	2.63	54.3	316
		TF	0.002	0.60	0.12	0.60
	R. officinalis	Shoot	0.43	0.43	11.9	114.9
		Root	14.3	3.60	78.0	351
		TF	0.002	0.21	0.10	0.31
	T. gallica	Shoot	0.44	0.94	14.6	84.9
		Root	13.2	1.84	67.3	241
		TF	0.002	0.49	0.12	0.23
Two-way ANOVA	Shoot	PT	<0.001***	<0.001***	<0.001***	<0.001***
		Sp	<0.001***	0.023*	<0.001***	<0.001***
		PT × Sp	0.006**	0.184 ns	0.024*	0.008**
	Root	PT	<0.001***	<0.001***	<0.001***	<0.001***
		Sp	0.022*	<0.001***	<0.001***	<0.001***
		PT × Sp	0.638 ns	0.220 ns	0.003**	<0.001***
	TF	PT	<0.001***	<0.001***	0.082 ns	<0.001***
		Sp	<0.001***	<0.001***	<0.001***	<0.001***
		PT × Sp	0.038*	0.340ns	0.064ns	0.009**

4. Conclusions

The success of phytostabilisation may not only be restricted to immobilisation of contaminants, it also offers other environmental advantages such as [47]: increase of microbiota activity in soils, carbon sequestration, support to soil functioning and ecosystem services, etc. In our work, plant establishment had a positive effect on soil quality, improving C, N and biological activity in the soils, mainly for *R. sphaerocarpa* and *M. communis*. Therefore, revegetating contaminated lands with these species may improve soil health and aid in reinstating the ecosystem over time. In pyrite-contaminated Mediterranean sites, liming assisted effectively soil phytostabilisation. After experiments at different scales, *R. sphaerocarpa* has the best properties to achieve successful soil remediation

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.11.013.

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