Use of municipal solid wastes for chemical and microbiological recovery of soils contaminated with metal(loid)s

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Abstract

Iron-rich water treatment residues (Fe-WTRs) and municipal solid waste compost (MSWC) were added together at two different total rates (i.e. 0.5% Fe-WTRs+0.5% MSWC and 1% Fe-WTRs+1% MSWC) to a degraded sub-alkaline soil (pH 8.0) contaminated with Sb (~110 mg·kg-1 soil), Pb (~1,200 mg·kg-1), Cd (~23 mg·kg-1), and Zn (~5,400 mg·kg-1). A large number of chemical and biological endpoints were evaluated to assess the efficacy of the treatments after five months of incubation. Both treatments significantly reduced the labile fractions of the metal(loid)s in soil, especially Sb, while increasing the abundance of culturable heterotrophic bacteria, actinomycetes and fungi (i.e. up to 6.3-, 1.6- and 4.1-fold higher than control respectively). Soil enzyme activities, i.e. dehydrogenase, β-glucosidase and urease, were also significantly enhanced in the treated soils (i.e. up to ~12-, 3- and 2-fold higher than control respectively). The amendment addition affected the structure of the soil microbial community as highlighted by the higher metabolic potential and catabolic versatility of treated soils (Biolog CLPP) and by the significantly higher α-diversity values based on high throughput partial 16S rRNA gene sequencing. Moreover, analysis of the dominant operational taxonomic units (OTUs) showed differences in the microbial communities of untreated and treated soils. Plant growth (Helichrysum italicum) in the treated soils was greatly stimulated while metal(loid)s uptake was significantly reduced. Overall, the results indicated that the applied treatment could be ideal for the chemical and (micro)biological recovery of sub-alkaline soils contaminated with Sb and co-occurring metals, and H. italicum appears to be a promising plant species for aided phytostabilisation of such soils.

Keywords	municipal solid waste compost; water treatment residues; antimony; soil microbial community; enzyme activity; community level physiological profile.
Taxonomy	Soil Microbial Community, Soil Rehabilitation, Soil Enzyme Activity, Contaminated Soils, Soil Microbial Populations, Soil Inorganic Pollutant
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Dear Professor Kate Scow
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attached to this mail you will find our revised version (R2) of the manuscript titled:

"Use of municipal solid wastes for chemical and microbiological recovery of soils contaminated with metal(loid)s"

authored by

Giovanni Garau[1*], Margherita Silvetti[1], Sotirios Vasileiadis[2], Erica Donner[2], Stefania Diquattro[1], Salvatore Deiana[1], Enzo Lombi[2], Paola Castaldi[1*]

Please, note that in this revised version (R2), the original title was modified (as above) according to the reviewers suggestion. This was the only revision requested by the reviewers, and no other changes have been applied to the revised manuscript.

Best regards
Giovanni Garau

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Authors reply to comments from the editors and reviewers.
We wish to thank the anonymous reviewers and the journal editor for the time devoted to improve our manuscript.

The specific point raised by the editor and reviewers was addressed by the authors and the manuscript revised accordingly.

Editor and Reviewers suggestions.
The only comment is to shorten your title to one which is shorter and more general. The current title makes the work seem very specific and provincial, not of interest to a broader audience. I suggest "Use of municipal solid wastes for chemical and microbiological recovery of soils contaminated with metals".

Authors
We agree with the suggestion. The title in this revised version was changed to “Use of municipal solid wastes for chemical and microbiological recovery of soils contaminated with metal(loid)s”.
Given the co-occurrence in soil of both metalloids and metals, we used the word “metal(loid)s” instead of “metals”.

Best regards
Giovanni Garau

Use of municipal solid wastes for chemical and microbiological recovery of soils contaminated with metal(loid)s

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Abstract

Iron-rich water treatment residues (Fe-WTRs) and municipal solid waste compost (MSWC) were added together at two different total rates (i.e. 0.5% Fe-WTRs+0.5% MSWC and 1% Fe-WTRs+1% MSWC) to a degraded sub-alkaline soil (pH 8.0) contaminated with Sb (~110 mg·kg^{-1} soil), Pb (~1,200 mg·kg^{-1}), Cd (~23 mg·kg^{-1}), and Zn (~5,400 mg·kg^{-1}). A large number of chemical and biological endpoints were evaluated to assess the efficacy of the treatments after five months of incubation. Both treatments significantly reduced the labile fractions of the metal(loid)s in soil, especially Sb, while increasing the abundance of culturable heterotrophic bacteria, actinomycetes and fungi (i.e. up to 6.3-, 1.6- and 4.1-fold higher than control respectively). Soil enzyme activities, i.e. dehydrogenase, β-glucosidase and urease, were also significantly enhanced in the treated soils (i.e. up to ~12-, 3- and 2-fold higher than control respectively). The amendment addition affected the structure of the soil microbial community as highlighted by the higher metabolic potential and catabolic versatility of treated soils (Biolog CLPP) and by the significantly higher α-diversity values based on high throughput partial 16S rRNA gene sequencing. Moreover, analysis of the dominant operational taxonomic units (OTUs) showed differences in the microbial communities of untreated and treated soils. Plant growth (*Helichrysum italicum*) in the treated soils was greatly stimulated while metal(loid)s uptake was significantly reduced. Overall, the results indicated that the applied treatment could be ideal for the chemical and (micro)biological recovery of sub-alkaline soils contaminated with Sb and co-occurring metals, and *H. italicum* appears to be a promising plant species for aided phytostabilisation of such soils.

Key words: Municipal solid waste compost; water treatment residues; antimony; soil microbial community; enzyme activity; community level physiological profile.

1. Introduction

Antimony (Sb) is a non-essential plant element which occurs naturally in soil, with its main source being the weathering of soil parent materials containing minerals such as stibnite (Sb_2S_3) and valentinite (Sb_2O_3) (Kabata-Pendias, 2011). While natural background concentrations of Sb in soils range from 0.25 to 1.4 mg·kg^{-1} (Kabata-Pendias, 2011), much higher concentrations can be found in the proximity of mining sites, mineral processing facilities and/or shooting ranges (due to human activities). In recent years, heavily Sb-polluted sites have been identified all around Europe, e.g. in Italy (~15,000 mg·kg^{-1} soil), Germany (~500 mg·kg^{-1} soil), Switzerland (~17,000 mg·kg^{-1} soil), France (~5,700 mg·kg^{-1} soil) (Tschan et al., 2009) as well as worldwide (Wang et al., 2010 and Sanderson et al., 2014). These sites, often characterised by critical concentrations of co-occurring metal(loid)s (Wang et al., 2010, Wang et al., 2011, Okkenhaug et al., 2013 and Sanderson et al., 2014), can be of particular environmental concern since they represent hazardous multi-element contamination sources for neighbouring soils and water bodies and constitute a threat for soil functionality and fertility.

Bioavailable Sb and co-occurring metal(loid)s can severely compromise soil functionality by affecting the size, composition and activity of the resident microbial communities (Garau et al., 2011, Wang et al., 2011, Garau et al., 2014 and Wei et al., 2015) as well as plant growth (Castaldi et al., 2009, Kabata-Pendias, 2011 and Pan et al., 2011). For instance, previous studies have reported reduced growth and biomass of both grasses and legumes under Sb and/or heavy metal(loid)s stress (Castaldi et al., 2009, Pan et al., 2011 and Garau et al., 2014) as well as decreased abundance of culturable soil microbial populations and reduced soil enzyme activities (Garau et al., 2007, Wang et al., 2011 and Garau et al., 2014). Moreover, it was recently shown that elevated Sb reduced the diversity of arbuscular mycorrhizal fungi (Wei et al., 2015) and

increased the distribution, diversity and abundance in soil bacterial populations of selected genes involved in Sb detoxification (Luo et al., 2014).

Although the co-occurrence of critical concentrations of Sb and additional metal(loid)s represents a substantial threat for soil and ecosystem functioning, effective and reliable approaches for the remediation of these polluted sites are currently lacking. In particular, the different speciation, mobility and bioavailability of Sb with respect to selected co-occurring metal(loid)s (e.g. trace metal cations) makes the identification of suitable amendments a very challenging task.

Antimony is commonly present in the environment in the trivalent (III) and pentavalent (V) states with the inorganic antimonite [$Sb(OH)_3$] and antimonate [$Sb(OH)_6^-$] being the dominant species in aqueous systems across a wide pH range (i.e. pH 4-10) (Okkenhaug et al., 2013). In aerated soils, the anionic species $Sb(OH)_6^-$ is prevalent (if not exclusive) (Johnson et al., 2005 and Filella et al., 2009) and displays a high affinity to amorphous and crystalline Fe-(hydr)oxides with which it can form stable bidentate inner-sphere complexes (Guo et al., 2014). In the pH range of the majority of soils (i.e. pH 5-9), such interactions are particularly favoured by the high point of zero charge of amorphous and crystalline Fe-(hydr)oxides (e.g. pH_{PZC} 7.5-9.0 for goethite, 8.5 for hematite, 7.0-9.0 for ferrihydrite, 9.5-10 for akaganeite (Strawn et al., 2015)). However, in the same pH range (i.e. pH 5-9), heavy metals such as Pb, Cd and Zn behave quite differently, being commonly present in the soil solution as divalent and/or monovalent (hydroxylated) cations at acidic and circumneutral pH or as soluble SOM-metal(II) complexes at higher pH values (Kabata-Pendias, 2011). Moreover, at neutral and/or alkaline pH substantial amounts of heavy metals are immobilised as Me-hydroxides, -carbonates and/or -hydroxycarbonates. Due to their cationic nature, and in contrast to Sb(V), soluble heavy metals show a limited affinity for positively charged soil surfaces but interact more strongly with negatively charged components (e.g. soil organic matter and clay minerals).

In the last decades, a variety of potential sorbent materials for the in-situ remediation of metal(loid) polluted soils have been proposed and tested (Castaldi et al., 2009, Garau et al., 2011 and Garau et al., 2014), however Sb-contaminated (or co-contaminated) sites have been largely overlooked and neglected until recently. To date, only a few amendments, mostly based on Fe- and Al-containing materials/minerals, have been tested with variable success as Sb-immobilising agents. For instance, adding olivine and hematite to a contaminated soil was found to have no effect on porewater Sb concentration (Okkenhaug et al., 2012), while red mud (alumina industry residues high in Fe-oxides) addition reduced porewater Sb only in some cases (Sanderson et al., 2015). The addition of organic amendments (e.g. mussel shell, cow bone, chicken manure, sodium humate) have mostly been deemed ineffective at fixing Sb (Conesa et al., 2010, Ahmad et al., 2014 and Shtangeeva et al., 2014), although mussel shell and cow bone, and also red muds, reduced the labile co-occurring Pb from the soil solution (Ahmad et al., 2014 and Sanderson et al., 2015). Other research has shown that a 2% commercial Fe-oxyhydroxide, and very high rates of amorphous Fe- and Al-oxyhydroxides, could effectively stabilise the antimony in different polluted soils (Alvarez-Ayuso et al., 2013, Okkenhaug et al., 2013 and Okkenhaug et al., 2016). However, the mobilisation of co-occurring metals (e.g. Pb, Cu, and Zn) was detected after the addition of Fe-based materials to Sb-contaminated soils (Okkenhaug et al., 2013). These findings, together with the limited number of studies addressing the remediation of Sb-contaminated sites, highlight the need to deepen our knowledge on the physico-chemical factors governing the mobility of Sb (and co-occurring metal(loid)s) in soil and to further select for sorbents with ideal Sb-immobilizing capabilities. Such sorbents, other than reducing the mobility and phytoavailability of Sb and co-occurring metal(loid)s, should also ideally improve soil fertility as well as soil microbial abundance, diversity and functionality.

In this context, the aim of this work was to evaluate the suitability of the combined application of two low-cost and sustainable amendments for the chemical and biological recovery of a degraded alkaline soil contaminated with Sb, Pb, Cd and Zn. Iron-rich drinking-water treatment residuals (Fe-WTRs) and municipal solid waste compost (MSWC) were the selected amendments.

The presence in Fe-WTRs of an abundant mineral component rich in Fe-(hydr)oxides makes these residues potentially effective for the immobilisation of Sb in our alkaline soil (Guo et al., 2014), while earlier studies also suggest their suitability as metal-fixing agents (Castaldi et al., 2015). On the other hand, the application of MSWC together with Fe-WTRs was expected to contribute to soil recovery mainly by improving its nutritional and functional status.

The effectiveness of the combined application of Fe-WTRs and MSWC in a 1:1 ratio, at two different total rates (i.e. 1 and 2%), was assessed in this study using a comprehensive approach that spanned chemical investigation of metal(loid)-mobility, plant growth and pollutant phytoavailability, soil microbial abundance and bacterial diversity, community level physiological profiling, and soil enzyme activity.

2. Materials and Methods

2.1. Soil and amendment origins, characteristics and microcosm set up

Sixty topsoil samples (0-20 cm depth; ~ 2 kg each) were collected from an area of approximately 1 ha located in the abandoned mining site of Argentiera (N40°44’11’’, E8°8’54’’) in north-western Sardinia (Italy), where Pb, Ag and Zn were extracted for about one century (1867-1963) from silver-rich galena [(Pb, Ag)S] and sphalerite (ZnS). The site is characterized by the presence of Sb-

133 rich minerals such as boulangerite ($Pb_5Sb_4S_{11}$), tetrahedrite [$(Cu,Fe)_{12}Sb_4S_{13}$], freibergite
134 [$(Ag,Cu,Fe)_{12}(Sb,As)_4S_{13}$], bournonite ($PbCuSbS_3$) and pyrargyrite ($Ag_3SbS_3$) (Pirri, 1996). According
135 to particle-size analysis, carried out using the pipette method (Day, 1965), the soil in the sampled
136 area was a sandy-loam (USDA classification) with 68% coarse sand, 15% fine sand and 17% silt.
137 The collected soil samples were bulked together, air dried and sieved to <2 mm before being
138 employed for the set-up of different soil microcosms, each consisting of approx. 10 kg soil.
139 Triplicate microcosms were amended with 0.5% (w/w) Fe-WTRs + 0.5% (w/w) MSWC (1% total
140 amendment rate) or 1% (w/w) Fe-WTRs + 1% (w/w) MSWC (2% total amendment rate) or left
141 untreated as controls. These rates were selected based on the specific metal(loid)s immobilising
142 capabilities of Fe-WTRs and MSWC highlighted in previous studies (Manzano et al., 2016 and
143 Garau et al., 2014). Before addition to the microcosms, Fe-WTRs (provided by the Bidighinzu plant
144 of the Abbanoa industry, Sassari, Italy) and MSWC (provided by the Secit S.p.A. facility plant of the
145 Consorzio ZIR, Chilivani-Ozieri, Sassari, Italy) were oven dried at 60°C for 48 h and sieved to <2
146 mm.
147 After amendment, treated (and untreated) soils were carefully mixed, brought to 60% of their
148 water holding capacity and equilibrated for 5 months at 20°C. During this period, soils were mixed
149 twice a week and maintained at 60-70% relative humidity.
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151
152 *2.2. Amendments and soil chemical analyses*
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154 Selected physico-chemical characteristics of the treated and untreated soils after equilibration,
155 and of the amendments used, are reported in Tables 1-2. The pH and electric conductivity (EC)
156 were determined using a 1:2.5 soil/amendment:deionised water ratio according to national

157 standard guidelines (Gazzetta Ufficiale, 1999 and 2002). The total organic matter and N were
158 determined according to the Walkley-Black and to the Kjeldhal method respectively (Gazzetta
159 Ufficiale, 1999 and 2002) while total P was determined spectrophotometrically (ascorbic acid
160 method) (Gazzetta Ufficiale, 1999 and 2002). The dissolved organic carbon (DOC) was estimated as
161 described by Brandstetter et al. (1996) while the point of zero charge (pH_{PZC}) has been determined
162 by potentiometric titration as described in Appel et al. (2003). The method reported by Ciavatta et
163 al. (1990) was used to determine the content of humic substances (i.e. humic and fulvic acids)
164 while the specific surface area was determined by applying the BET model to the N_2 adsorption
165 results obtained from a Carlo Erba Sorptomatic instrument (Milan, Italy) (Castaldi et al., 2010).
166 Total and active carbonates were determined by acid dissolution (volumetric calcimeter method)
167 and by reaction of soil with 0.1 M ammonium oxalate respectively (Gazzetta Ufficiale, 1999 and
168 2002). Soil cation exchange capacity (CEC) was determined using the $BaCl_2$ and triethanolamine
169 method (Gazzetta Ufficiale, 1999).
170 Total concentration of metal(loid)s in soils and amendments was determined after digestion with
171 an HNO_3/HCl mixture (1:3 v/v ratio) using a flame atomic absorption spectrometer equipped with
172 a HGA-600 graphite furnace (Perkin Elmer Analyst 600). The NIST-SRM 2710 was used as standard
173 reference material for quality assurance and quality control.
174 The Sb mobility in soil was assessed through the sequential extraction procedures of Wenzel et al.
175 (Wenzel et al., 2001) (originally designed for soil As fractionation) while the protocol of Basta and
176 Gradwohl (Basta and Gradwohl, 2000) was used to assess the mobility of Cd, Zn and Pb. Briefly,
177 labile and surface-bound Sb in soil was assessed after extraction with 0.05 M $(NH_4)_2SO_4$ (Step 1)
178 and 0.05 M $(NH_4)H_2PO_4$ (Step 2) respectively, while the Sb associated to amorphous and crystalline
179 Al- and Fe-oxides was determined after extraction with 0.2 M NH_4-oxalate (Step 3) and 0.2 M NH_4-
180 oxalate solution + 0.1 M ascorbic acid (Step 4) respectively (Wenzel et al., 2001). Exchangeable

and readily soluble Cd, Zn and Pb in soil were determined after extraction with 0.5 M $Ca(NO_3)_2$ while acid-soluble metals and weak surface complexes were determined after extraction with 1 M NaOAc (pH 5) (Basta and Gradwohl, 2000). Surface complexes and metal precipitates were determined after 0.1 M Na_2EDTA extraction. After each step of the respective extraction procedures, the samples were centrifuged at 8000 rpm for 10 min, filtered to completely separate the liquid and solid phases and metal(loid)s in the supernatant determined using a flame atomic absorption spectrometer equipped with a HGA-600 graphite furnace (Perkin Elmer Analyst 600). The residual Sb in soil as well as the very insoluble and/or occluded metals were determined after microwave digestion with an HNO_3/HCl mixture as previously mentioned.

2.3. Heterotrophic microorganisms, Community Level Physiological Profiles and soil enzyme activities

Fast-growing culturable bacteria, fungi and actinomycetes were enumerated in soil microcosms (after equilibration) using the conventional ten-fold serial dilution and spread plate method. Solidified (15 g L^{-1} agar) 1:10 strength TSA (Tryptic Soy Agar, Microbiol, Cagliari, Italy), GYEP at pH 4.5 (Glucose Yeast Extract Peptone medium) and AIA (Actinomycete Isolation Agar, Difco, Milan, Italy) were used as growth media for the enumeration of culturable bacteria, actinomycetes and fungi respectively (Pinna et al., 2012). Microbial counts were conducted after incubation of the plates for 72h at 25°C.

The Community Level Physiological Profile (CLPP) of microbial communities was determined using Biolog Ecoplates (Biolog Inc., Hayward, CA). Microbial communities were extracted from the respective soils and separately inoculated in each of the 96 microtiter wells of a Biolog Ecoplate as

205 previously described (Pinna et al., 2012). The Biolog Ecoplate contains a triplicate set of 31 carbon
206 sources of soil/environmental relevance, each present in a separate well, and three control wells
207 with no carbon. A tetrazolium dye incorporated in the plate wells reveals oxidative catabolism as
208 purple colour formation. This latter was recorded for each well every 15 min for 96 h using a
209 Biolog Omnilog unit (i.e. a charge-coupled-device camera; Biolog, Hayward, CA) to obtain a colour
210 vs. time curve. For each well/substrate, the area under this curve was control-subtracted and the
211 potential catabolic activity of each microbial community was calculated as the average well area
212 over the 31 carbon sources (AWCD; Garau et al., 2007 and Garau et al., 2014). Richness values (i.e.
213 the number of carbon sources metabolised by each microbial community) were determined as the
214 number of wells with (control-subtracted) areas higher than 1,000 (Biondi et al., 2009).
215 Selected enzyme activities, i.e. dehydrogenase (DHG), β-glucosidase (GLU) and urease (URE), were
216 determined (colorimetrically) in duplicate soil samples collected from each microcosm as
217 previously described (Garau et al., 2007). Briefly, DHG activity was quantified by determining the
218 concentration of triphenyl formazan produced after incubation of soil samples (5 g each) with
219 triphenyltetrazolium chloride for 24 h at 30°C. GLU activity was determined as p-nitrophenol
220 released after incubation of soil samples (1 g each) with p-nitrophenyl glucoside for 2 h at 37°C.
221 URE activity was determined as ammonia released after incubation of soil samples (5 g each) with
222 urea for 2 h at 37°C.
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225 *2.4. Molecular analysis of soil bacterial communities and bioinformatics*
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227 Total DNA was extracted from soil samples (~500 mg) of each microcosm using the FastDNA™
228 SPIN Kit and FastPrep® Instrument (MP Biomedicals, Santa Ana, CA) according to the

manufacturer's instructions with the homogenization step performed 3 times at maximum speed and time (3 x 6.5 m sec^{-1} x 60 sec). DNA extracts were quantified using the Qubit® dsDNA HS Assay kit (Invitrogen, Waltham, Massachusetts, USA) with the Qubit® v2.0 Fluorometer (Invitrogen, ThermoFisher-Scientific, Waltham, Massachusetts, USA) and stored at −20°C prior to downstream analyses. The V3-4 hypervariable region of the bacterial small ribosomal subunit coding gene (SSU) was amplified via polymerase chain reaction (PCR) performed on 0.2 ng of the DNA extracts using the 343SF forward primer (5'-TACGGGAGGCAGCAG-3'; modified version of the S-D-Bact-0343-a-S-15 (Vasileiadis et al., 2012)) and a modified version of the 802R reverse primer (5'-TACNVGGGTWTCTAATCC-3'; version of S-D-Bact-0785-b-A-18 (Claesson et al., 2009)) in 20 µl reactions. The PCR reactions were performed in two steps to restrict amplification and high throughput sequencing indexing related biases as described previously (Berry et al., 2011). The primers mentioned above were used in the first step while the indexed primer versions (for multiplexing prior to sequencing) provided in Table S1 were used in the second step. Briefly, 2 µl of DNA extracts diluted to 0.1 ng·μl^{-1} were added as templates to 18 µl mixtures containing 10 µl Phusion Flash High-Fidelity PCR Master mix (Finnzymes, ThermoFisher-Scientific, Waltham, Massachusetts, USA), 1 µl of each primer solution (10 µM), 0.4 µl of bovine serum albumin (BSA – ThermoFisher-Sientific – Waltham, Massachusetts, USA – 20 mg·ml^{-1}) and 5.6 µl PCR grade water. The resulting 20 µl reaction mixtures were subject to the first PCR comprising: an initial step of 3 minutes at 95°C for enzyme activation/DNA-denaturation; followed by 28 cycles of 95°C for 15 seconds for DNA denaturation, 50°C for 15 seconds for primer annealing and 72°C for 15 seconds for DNA elongation; followed by 10 minutes at 72°C for the final elongation step. Two microliters of the resulting amplification products were used as DNA template in the second PCR cycle (same mixture as above; 7 cycles only) to add indexed primers (Table S1) in preparation for sequencing. Equimolar amounts of the PCR products were then multiplexed and sequenced with the Illumina

HiSeq 2500 platform for 300 cycles using the paired-end reads module by Fasteris SA (Geneva, Switzerland).

Output sequences were analysed with the LotuS v1.44 software (Hildebrand et al., 2014) as follows: reads were de-multiplexed and quality controlled with the LotuS native simple de-multiplexer (smd) v1.26 using the default parameters with the exception of a minimum good quality sequence length of 170 bp; read pairs were then merged with FLASH v1.2.8 (Magoč and Salzberg, 2011); clustering of reads into 97% identity operational taxonomic units (OTUS) was performed with the USEARCH/UPARSE v8.0.1623 algorithm (Edgar, 2013); chimeric OTUs were removed with UCHIME v4.2 (Edgar et al., 2011) and the ribosomal database project (RDP) gold database version; and representative OTU sequences were classified into known taxa using the RDP naïve Bayesian classifier v2.11 (Wang et al., 2007). Selected OTU sequences were further analysed to obtain phylogenetic information according to a maximum likelihood approach, whereby closely related sequences were obtained from the National Center for Biotechnology Information (NCBI) 16S rRNA gene type strain collection using the basic local alignment search tool (BLAST) (Altschul et al., 1990). The best BLAST hits were clustered with Cdhit v4.6 (Li et al., 2001) to reduce sequence redundancy and the cluster representative sequences were aligned with Muscle v3.8.31 (Edgar, 2004). Non-properly aligned and uninformative alignment blocks were removed using Gblocks v0.91b (Talavera and Castresana, 2007) and the remaining concatenated alignment blocks were subjected to maximum likelihood phylogenies with the RAxML software v8.1.24 (Stamatakis, 2014) using the general time-reversible (GTR) model for gamma rate heterogeneity and considering invariable sites using 1000 bootstrap replicates. The sequences obtained were submitted to the NCBI (NCBI - https://www.ncbi.nlm.nih.gov) Sequence Read Archive (SRA - https://www.ncbi.nlm.nih.gov/sra/) and are currently available under the accession numbers SRR4424247-55.

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279 *2.5. Plant growth experiment and plant analysis*

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281 At the end of the 5 months incubation, soils from each microcosm were used to grow *Helichrysum*
282 *italicum* (Roth) G.Don plantlets (approx. height 5 cm) in plastic pots [23 cm (L) x 15 cm (H) x15 cm
283 (D)] filled with ~3 kg of soil. We selected this plant species as it was growing abundantly at the site
284 investigated suggesting an apparent tolerance to the metal(loid)s present and good adaptability to
285 the low fertility conditions which characterised the soil. A total of thirty-six *H. italicum* plantlets
286 were grown on each treated and untreated soil in 6 pots (6 plants x pot; 2 pots x microcosm) for
287 16 weeks under controlled conditions (20°C; 60–70% relative humidity). At harvest, shoots and
288 roots were separated, thoroughly washed with distilled water, and oven-dried at 70°C for 96 h for
289 dry weight determination.
290 Metal(loid)s in shoots and roots were determined, after mineralization of the plant material (EPA
291 Method 3052), using a flame atomic absorption spectrometer equipped with a HGA-600 graphite
292 furnace (Perkin Elmer Analyst 600). Peach leaf was used as standard reference material (NIST-SRM
293 1515).

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296 *2.6. Data analysis*

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298 Chemical (i.e. sequential extraction) and (micro)biological data were subjected to One-way
299 analysis of variance and when significant *P* values were obtained ($P<0.05$) differences between
300 individual means were compared with the post-hoc Tukey Kramer multiple comparison test

301 (P<0.05) using the NCSS software (Keysville, Utah). The 16S rRNA gene sequence data were
302 analysed using the R v3.2.3 software (R Core Team, 2015). Tests performed included: Hellinger
303 transformation of the OTU matrix as suggested by Legendre and Gallagher (Legendre and
304 Gallagher, 2001) followed by hierarchical clustering using the unweighted pair group method with
305 arithmetic mean (UPGMA) and principal component analysis with modelling of the
306 physical/chemical variables with the Vegan package v2.4-0 (Oksanen et al., 2016). Treatment
307 group related OTU differential abundances were assessed with Fisher's exact tests as
308 implemented by the Edge R v3.12.0 package using the Holm correction for multiple hypothesis
309 testing (Robinson et al., 2010).
310
311
312 **3. Results and Discussion**
313
314 *3.1. Influence of the amendments on physico-chemical soil features and metal(loid) mobility in soil*
315
316 In this study we evaluated the combined application of Fe-WTRs and MSWC, at two different rates
317 (i.e. at 1 and 2%), as potential amendments for the remediation of an alkaline (pH 8.0) degraded
318 soil polluted by Sb and co-occurring metals. The soil was characterized by a significant content of
319 sand (~83% coarse and fine sand), a medium cation exchange capacity (CEC, ~17 $cmol_{(+)} \cdot kg^{-1}$ soil)
320 and very low organic matter content (OM, ~0.4%) (Table 2). Moreover, the total concentrations of
321 Sb, Cd, Zn and Pb all exceeded the national (Italian) threshold values for private, residential and
322 public green areas and for commercial and industrial sites (Tables 2 and S2).
323 The combined addition of Fe-WTRs and MSWC clearly improved some fertility characteristics of
324 the soil such as the CEC (>25 and 97% increase at 1 and 2% amendment rates, respectively), OM

(>50 and 90%), and N content (>15 and 45%) (Table 2). DOC also increased significantly after soil amendment (>400 and 700% at 1 and 2% amendment rates, respectively) while pH, EC, total and active carbonate were little affected (Table 2).

The amendment additions did not change the total concentrations of metal(loid)s in the soil (Table 2). On the other hand, the sequential extraction data indicated a relatively low content of readily labile and surface-bound Sb in the untreated polluted soil, i.e. the water-soluble and easily exchangeable Sb extracted in Step 1 and the Sb forming inner-sphere complexes extracted in Step 2 respectively (Fig. 1). This may be encouraging from an environmental point of view since these Sb fractions can be considered to have the greatest impact on soil biota (Wenzel et al., 2001; Garau et al., 2014). Most of the Sb in the polluted soil (~ 85%) was instead associated with amorphous and especially crystalline Al- and/or Fe-oxides as highlighted in Step 3 and 4 respectively (Fig. 1).

The added amendments had no obvious effects on the readily labile Sb but significantly reduced the amount of metalloid extracted in Step 2 (Fig. 1). This is relevant as this Sb fraction can be simply mobilised (i.e. becoming bioavailable) following a pH change (e.g. due to plant and/or microbial activity) or phosphate increase (Wenzel et al., 2001). The Sb fraction associated with amorphous Al- and Fe-oxides (Step 3) increased significantly after soil amendment while the opposite was true for the metalloid fraction associated to crystalline Al- and Fe-oxides (Step 4; Fig. 1). Finally, the residual Sb fraction (i.e. that is strongly retained and not expected to be readily released) increased by approximately 3- and 4-fold in the soils amended at 1 and 2% rate respectively.

Overall, these data show that the combined addition of Fe-WTRs and MSWC induced a significant redistribution of Sb in soil, i.e. a reduction of its more labile and bioavailable forms and an increase of the residual (i.e. not readily bioavailable) forms. This can be the result of different mechanisms

induced by the amendments, likely including Sb mobilisation from soil and its redistribution within the soil/amendment components. In particular, Sb mobilisation in the treated soils could have occurred through competitive exchange phenomena involving organic and inorganic components of both amendments, but also through partial dissolution of Sb-containing Al and Fe-(hydr)oxides (amorphous and crystalline) due to solubilisation of Fe/Al nuclei. As recently highlighted (Chen et al., 2016), fulvic acids within Fe-WTRs and MSWC may have played an important role in this (Table 1). On the other hand, the formation of soluble complexes between the organic matter within Fe-WTRs/MSWC and Sb is not expected to be relevant at the pH value of this soil (Filella and Williams, 2012).

As supported by the sequential extraction data (Step 3; Fig.1), sorption of Sb in the amended soils may have involved existing amorphous Al- and Fe-(hydr)oxides in soil and, above all, the amorphous Al- and Fe-(hydr)oxides added with Fe-WTRs (Table 1). This could explain the significant increase of the Sb detected in Step 3 and the reduction of that extracted in Step 4 (Fig. 1). Moreover, the precipitation of part of the released $Sb(OH)_6^-$ with soluble Ca^{2+} (abundantly present within MSWC; Table 1) may also have contributed to the increase of the residual Sb pool in the treated soils (Johnson et al., 2005 and Conesa et al., 2010).

The sequential extraction of Basta and Gradwohl (2000) showed a limited amount of water soluble and exchangeable Cd, Zn and Pb in the contaminated soil (Fig. 2). This was somewhat expected in this alkaline (carbonate-rich) soil where a substantial amount of the metals are likely to be precipitated as metal carbonates and/or as metal (hydr)oxides (Kabata-Pendias, 2011). Accordingly, approx. 40, 50 and 66% of total Zn, Cd and Pb respectively were extracted with EDTA (Fig. 2) suggesting that these relatively immobile (and not readily bioavailable) metal fractions were likely involved in inner-sphere surface complexes and/or were in the form of precipitates (Basta and Gradwohl, 2000). The residual metal fractions in the contaminated soil, i.e. very

373 insoluble and/or occluded metal pools, were approx. 30, 45 and 50% of total Pb, Cd and Zn
374 respectively (Fig. 2). The most important amendment effect was the significant reduction of water
375 soluble and exchangeable Cd, Zn and Pb, i.e. the more mobile and bioavailable fractions. This
376 could be mainly explained with the capacity of the organic and inorganic components of both the
377 amendments to sorb the metals considered as previously highlighted (Castaldi et al., 2015, Silvetti
378 et al., 2015 and Manzano et al., 2016). A reduction of the acid-soluble and/or weakly complexed
379 Pb (extracted with NaOAc) was also observed after amendment addition together with a
380 significant increase of the residual Cd (Fig. 2).
381 Overall, the addition of Fe-WTRs and MSWC to the contaminated soil contributed to lower the
382 environmental impact of the metal(loid)s present by limiting the amount of their soluble and
383 exchangeable forms and in some cases (especially for Sb and Cd) by substantially increasing their
384 residual fraction. As previously pointed out, this is expected to have relevant positive effects on
385 the soil microbial component and its functionality (Garau et al., 2007, Garau et al., 2011 and Garau
386 et al., 2014).
387
388
389 *3.2. Influence of the amendments on the soil microbial abundance, enzyme activities and*
390 *community level physiological profile*
391
392 The combined addition of Fe-WTRs and MSWC induced significant increases in all targeted soil
393 microbial groups. In the case of total culturable heterotrophic bacteria and actinomycetes (but not
394 fungi) the increase in CFUs (colony forming units) g^{-1} soil was also positively correlated with the
395 amendment rate (Table 3). This increase of readily culturable microorganisms in the treated soils
396 was likely due to the presence of additional easily metabolisable carbon sources deriving from the

OM within MSWC and Fe-WTRs (Garau et al., 2011). This was supported by the significant increase of the DOC and OM in the amended soils (Table 2). The reduction of labile metal(loid) fractions in the treated soils (Figs. 1-2) could have also contributed to microbial growth by increasing the metabolic energy dedicated by microorganisms to cell growth (and multiplication) instead of high-energy demanding metal detoxification processes (Garau et al., 2011). The number of readily culturable bacteria and fungi can provide an estimate of the potentially active microbial populations in soil, i.e. microbial populations involved in the prompt degradation of soil organic matter and the release (and cycling) of mineral elements essential for plant growth, e.g. N and P (Timms-Wilson et al., 2006). In this sense, the increase of these microbial populations in the amended soils can be considered as positive as it suggests an improved fertility status while confirming a reduced environmental impact of the contaminants.

Enzyme activity data indicated functional improvements in the treated soils. In particular, higher DHG values were recorded in amended soils compared to the control (approx. 6 and 12-fold higher at 1 and 2% amendment rate). Also URE and GLU significantly increased after 2% amendment (2.17 and 2.9-fold higher respectively compared to controls) (Table 3). All this indicated a higher microbial activity in the amended soils and possibly an improved C and N turnover. Pearson correlation analysis indicated significant (P<0.05) positive correlations between the number of culturable microorganisms and all the enzyme activities (r=0.69-0.94; Table S3). This may support the view that culturable microorganisms may play a role in soil functioning and nutrient turnover. However, it should be noted that the relationship between soil functioning and microbial community is only partially explored by culturable techniques and it cannot be excluded that unculturable microorganisms also play a very important role.

The Biolog Community level physiological profile (CLPP) showed significant differences between untreated and treated soils. In particular, the microbial populations extracted from treated soils

displayed a significantly higher potential carbon source utilisation compared to those extracted from control soil (Fig. 3). This increase also appeared proportional to the amendment rate. The same was recorded for richness values, or the number of carbon sources metabolised by the microbial communities (Fig. 3). As pointed out in earlier studies, fast-growing culturable bacteria are the most relevant contributors of colour development in Biolog Ecoplates (Pinna et al., 2012 and Garau et al., 2014) and in this sense, Biolog data support the higher bacterial counts recorded in the treated soils (Table 3). Moreover, CLPP analysis also indicates that the amendment addition had a positive effect on the catabolic versatility of the microbial communities as indicated by the richness values (Fig. 3). For instance, only the microbial communities of treated soils were able to catabolise i-erythritol, L-serine and D-glucosaminic and 4-hydroxy benzoic acids (other than the substrates catabolised by the control soil microbial community) while glycogen and itaconic acid were exclusively metabolised by the microbial populations extracted from the 2% amended soil. The appearance in the amended soils of microbial communities with higher and more diverse catabolic potentials could be partly the result of reduced environmental pressure (i.e. reduced metal(loid) bioavailability) on microbial taxa that were poorly represented in the contaminated soil (e.g. because they are more sensitive to contaminants). All this combined with the larger availability in the treated soils of easily metabolisable carbon sources could have sustained the growth of these rare taxa and allowed for the appearance of the new phenotypes.

3.3. Amendment impact on soil bacterial diversity

Most often, the selection of an amendment for the in-situ treatment of contaminated soils is primarily based on its ability to reduce the concentration of labile metal(loid)s. By contrast,

445 additional effects such as the impact of the amendment on the soil microbial abundance and soil
446 functionality are often neglected as well as the impact of the amendment on the soil microbial
447 diversity. Nowadays it is widely accepted that soil microbial diversity affects many key ecosystem
448 processes such as the biogeochemical cycle of plant nutrients as well as the evolutionary dynamics
449 in plant communities. Since soil microbial diversity allows for the preservation of such functional
450 traits, even in the face of external perturbations (Fitter et al., 2005), its maintenance and/or
451 improvement following amendment addition should be of primary importance. Accordingly, soil
452 bacterial diversity in the treated soils was evaluated by high throughput partial 16S rRNA gene
453 sequencing.
454 The sequencing effort was suitable for screening the vast majority of the existing bacterial
455 diversity as indicated by the Good's coverage estimate (Good, 1953) with a range of 98.4-99.4% of
456 the soil bacterial SSU diversity being uncovered (Table 4). Both inverse Simpson (1/λ) and Gini-
457 Simpson (1-λ) indexes were significantly higher in the treated soils while the other indexes did not
458 reveal significant differences (Table 4). In this regard, it should be noted that the Simpson's index
459 and its derivatives, also known as 2^{nd} order diversity indexes, have been shown to better capture
460 the diversity of dominant OTUs in environmental samples (Mendes et al., 2008 and Gihring et al.,
461 2011) as opposed to other indexes like the Fisher's α diversity (Fisher et al., 1943), the effective
462 number of OTUs based on a generalised Tsallis entropy (Tsallis, 1988) as implemented by Mendes
463 et al. (2008) and, of course, the observed richness (*S*) or richness estimates (Chao, 1984 and Chao
464 and Lee, 1992). PCA of the Hellinger transformed OTU matrix using OTUs with relative
465 participation of 1% or above (13 OTUs in total), and grouping the rest OTUs as a single group of
466 "rare" OTUs, showed a treatment-wise grouping of samples (Figure 4a). Furthermore, modelling of
467 selected soil physico-chemical parameters showed strong correlations of the community with the
468 pH_{PZC}, DOC and OM, CEC and the concentration of the more labile fractions of Sb, Pb, Cd and Zn

(Fig. 4a). Three OTUs, namely OTU 2, 4 and 8 were dominantly associated with the treated soils as shown in Figure 6b. These were classified as *Bacilli* (OTUs 2 and 4) and *Acidobacteria* (OTU 8) according to the RDP classifier with the maximum likelihood phylogenies showing evolutionary associations with halo-philic/-tolerant or metal resistant *Bacilli* for OTUs 2 and 4. OTU 2 was placed next to the *Bacillus persicus* B48 and *B. luteolus* YiM93174 strains (Figure S1a), previously shown to be able to thrive or tolerate NaCl of up to 10 % (w/v) (Shi et al., 2011 and Didari et al., 2013). OTU 4 was placed next to *B. beringiensis* BR035 (Figure S1b), a psychrotolerant isolate of the Bering Sea (Yu et al., 2011), and species like *B. megaterium* which encompasses several metal resistant isolates (Yu et al., 2011 and Fierros-Romero et al., 2016). Maximum likelihood phylogeny showed relation with a versatile *Acidobacterium* named *Holophaga foetida* TMBS4 (Figure S1c) able to degrade aromatic compounds, which, however, did not show tolerance to high NaCl concentrations (up to 1.5 %) (Anderson et al., 2012). Nevertheless, lack of a sufficient number of isolates and the poor 16S rRNA marker gene evolutionary signal of *Acidobacteria* (as shown by the elimination of most acidobacterial representative sequences via the Cd-hit clustering) suggest that deeper investigations are necessary to gain further insights about the niche of the OTU 8 related strains.

Taken together these data highlight a clear impact of the amendments on the bacterial diversity, with amended soils showing significantly higher α-diversity indices (i.e. inverse Simpson and Gini-Simpson indexes; Table 4). At the same time, β-diversity was also affected by soil treatment as pointed out by the significant differences in the bacterial community composition of treated and untreated soils (Fig. 6a,b). This is likely due to a combination of reduced metal(loid) mobility and enhanced availability of C sources in the amended soils which eventually lead to the increase of rare taxa (possibly more sensitive to metal(loid)s in soil). Moreover, this can also explain the

492 appearance of new metabolic potentials (i.e. higher Biolog richness values) in the microbial
493 communities of treated soils (Fig. 3).
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496 *3.4. Influence of the amendments on plant growth and metal(loid) uptake*
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498 In order to obtain a comprehensive overview of the treatment suitability we carried out a pot
499 experiment to evaluate the combined impact of Fe-WTRs and MSWC on the growth and
500 metal(loid)s uptake of *Helichrysum italicum* plants.
501 Adding Fe-WTRs and MSWC to the polluted soil had a positive impact on plant growth. Root dry
502 weight increased by approx. 45 and 73 % after amendment addition at 1 and 2% rate respectively
503 and very similar increases were recorded for shoot dry weight (Fig. 5a). This was apparently due to
504 the improved fertility of the amended soils (Table 1) and also to the reduced metal(loid)
505 bioavailability, as indicated by the sequential extraction data (Figs. 1-2). Plants grown in the
506 amended soils showed approximately 50% reduction in Sb concentration in roots at both the
507 amendment rates while Sb concentration in shoots (i.e. stem + leaves) was reduced by 40 and 60%
508 at 1 and 2% amendment rate respectively (Fig. 5b).
509 Significant reductions in Cd, Pb and Zn uptake were also recorded for amended soils (Fig. 5c).
510 Irrespective of the amendment rate, metal concentrations in roots were consistently reduced (by
511 more than 50% compared to the untreated soil on average) while the addition of the 2%
512 amendment brought the concentration of Cd in plant shoots below the instrumental detection
513 limit (i.e. <0.2 $\mu g \cdot kg^{-1}$; Fig. 5c). Moreover, the amendment addition did not significantly change the
514 translocation factor of Sb (i.e. the roots to shoots metal(loid) concentration ratio) which remained
515 close to 0.1 while decreased significantly that relative to Cd (from 0.50 to 0.14). On the other

hand, an increase of Zn translocation factor was recorded at the 2% amendment rate (from 0.14 to 0.28). Taken together, these data highlight the overall effectiveness of the applied amendments for reducing the mobility and bioavailability of both Sb and selected co-occurring metals and improving soil fertility. Moreover, they also indicated the potential use of *H. italicum* for aided phytostabilisation of metal(loid) contaminated soils.

4. Conclusions

This study showed that Fe-WTRs and MSWC can be used as amendments for the chemical and micro-biological recovery of degraded subalkaline soils contaminated with Sb and co-occurring metals such as Pb, Cd and Zn. The amendments addition had a clear positive impact on the soil fertility attributes and reduced the labile fractions of the metal(loid)s in soil, especially Sb. All this likely had a substantial influence on the microbial community, and functionality, of the amended soils. Culturable microorganisms were more abundant in the treated soils and dehydrogenase, β-glucosidase and urease activities were also significantly enhanced. Moreover, microbial communities extracted from the amended soils showed higher metabolic potential and catabolic versatility as well as greater α-diversity values based on high throughput partial 16S rRNA gene sequencing. Plant growth in the treated soils was also greatly stimulated while metal(loid)s uptake was significantly reduced. These results suggest that Fe-WTRs and MSWC can be effectively used to alleviate the impact of inorganic xenobiotics on soil microbiota and promote a recovery of ecosystem functioning.

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Figure caption

Figure 1. Sequential extraction of Sb from untreated polluted and amended soils.

Figure 2. Sequential extraction of metals from untreated polluted and amended soils.

Figure 3. Average carbon source utilisation and richness values of microbial communities extracted from untreated polluted and amended soils.

Figure 4. Triplot of the PCA scores of the S1-9 soil samples (black characters; percentage of variance explained by each axis is also provided) according to their composition in dominant OTUs using the Hellinger transformed values, the scores of the dominant OTUs (red characters) and the fitting of selected soil physico-chemical parameters (blue characters and arrows; highly significant fits with $P \leq 0.01$) (panel a), and differential abundance of the dominant OTUs (different letters denote significant differences for $\alpha \leq 0.05$ using the Holm correction – panel b). Rare OTUs refers to the group of sequences with relative participation of less than 1%. S1-3, untreated soils; S4-6, soils + 1% amendment; S7-9, soils + 2% amendment ; OM, organic matter; DOC, dissolved organic carbon; CEC, cation exchange capacity; pH_{PZC}, point of zero charge; Sb, Pb, Cd and Zn lab, concentrations of metal(loid) labile fractions (i.e. Sb extracted in Step1+2 and Pb, Cd and Zn extracted with $Ca(NO_3)_2$ + NaOAc).

Figure 5. Biomass (a), Sb (b) and metal uptake (c) of *H. italicum* plants grown in the untreated polluted and amended soils.

Table 1. Selected physico-chemical characteristics of the amendments used in this study (dry matter basis).

Physico-chemical parameters	*Fe-WTR*	*MSWC*
pH(H_2O)	7.88±0.04	7.93±0.06
EC ($mS \cdot cm^{-1}$)	3.01±0.02	3.26±0.03
S_{BET} ($m^2 \cdot g^{-1}$)	35.00	2.78
pH_{PZC}	7.80	4.53
Organic matter ($g \cdot kg^{-1}$)	244.71±2.79	420.19±3.44
Fulvic and Humic acids ($g \cdot kg^{-1}$)	24.34± 1.05	153.24±2.76
DOC ($mg \cdot g^{-1}$)	0.101±0.00	0.573±0.04
Total N ($g \cdot kg^{-1}$)	8.04±0.28	21.84±0.42
Total P ($g \cdot kg^{-1}$)	0.68±0.03	7.16 ±0.41
Total metals ($mg \cdot kg^{-1}$)		
Fe	245,480±1,350	5,587±88
Mn	31,636±764	141±6
Ca	89±2	63,444±130
Mg	69±2	5,403±135
Na	32±1	993±24
K	99±3	1,709±21
Pb	12±0.3	3.7±0.1
Zn	246±7	30±3
Cd	0.2±0.0	n.d.
Cu	29±1	19±2

n.d., not detected

816 Table 2. Physico-chemical characteristics of the treated (amended) and untreated contaminated
817 soils (dry matter basis). Shaded values are exceeding the national (Italian) threshold values for
818 private, residential and public green areas and for commercial and industrial sites (see Table S2 in
819 SI for details).

Physico-chemical parameters	*Untreated soil*	*Amended soils*	
		Fe-WTR+MSWC (1.0 %)	*Fe-WTR+MSWC (2.0 %)*
pH(H_2O)	8.01 ± 0.08	7.97 ± 0.02	7.92 ± 0.03
EC ($mS \cdot cm^{-1}$)	1.43 ± 0.23	1.65± 0.09	1.69± 0.10
Total carbonate ($g \cdot kg^{-1)}$)	174.0 ± 5.0	185.7 ±3.4	199.3 ± 2.9
Active carbonate ($g \cdot kg^{-1}$)	64.0 ± 9.6	79.2 ±3.8	83.3 ± 1.4
CEC ($cmol_{(+)} \cdot kg^{-1}$)	16.92 ± 0.17	21.31 ± 1.24	33.46 ± 2.43
pH_{PCZ}	6.90	7.42	7.49
Organic matter ($g \cdot kg^{-1}$)	3.89±0.03	5.93±0.03	7.48±0.06
DOC ($mg \cdot g^{-1}$)	0.029 ± 0.001	0.123 ± 0.002	0.213 ± 0.006
Total N ($g \cdot kg^{-1}$)	0.70± 0.03	0.81± 0.06	1.02± 0.06
Total metal(loid)s ($mg \cdot kg^{-1}$)			
Fe	40,533 ± 1,197	43,382 ± 470	43,972 ± 698
Mn	1,758 ± 34	1,837 ± 315	2,054 ± 68
Ca	50,132 ± 2,301	49,162 ± 467	50,653 ± 728
K	4,269 ± 362	4,114 ± 394	4,331 ± 321
Sb	108.86 ± 1.74	106.46 ± 0.84	105.85 ± 0.97
As	7.67 ± 0.76	7.44± 0.25	7.42± 0.21
Pb	1,214 ± 31	1,184 ± 4	1,146 ± 25
Zn	5,400 ± 70	5,468 ± 482	5,385 ± 137
Cd	23.17 ± 2.87	22.38 ± 1.24	24.86 ± 4.54
Cu	48.86 ± 2.48	45.65 ± 3.62	46.86 ± 2.62

820
821

Table 3. Counts of heterotrophic microorganisms and selected enzyme activities in the untreated polluted (UP) and amended soils. All values are expressed on a soil dry matter basis. DHG, dehydrogenase; GLU, β-glucosidase; URE, urease. Within each column, values which share a common letter do not differ significantly (Fisher's LSD test, $P<0.05$).

	Microbial counts (log CFU·g^{-1} soil)			*Enzyme activities*		
	Bacteria	*Fungi*	*Actinomycetes*	*DHG (μg TPF·g^{-1} soil)*	*GLU (μg ρ-nitrophenol·g^{-1} soil)*	*URE (μg NH_4-N·g^{-1} soil)*
UP soil	5.81±0.07^{a}	4.79±0.09^{a}	5.57±0.03^{a}	2.93±0.29^{a}	70.67±10.06^{a}	2.37±0.36^{a}
UP soil+1% amend.	6.36±0.07^{b}	4.89±0.03^{a}	5.97±0.01^{b}	18.86±1.72^{b}	95.06±1.79^{a}	3.25±0.06^{a}
UP soil+2% amend.	6.61±0.01^{c}	5.00±0.05^{b}	6.19±0.02^{c}	37.41±6.72^{c}	208.65±16.07^{b}	4.96±0.44^{b}

Table 4. Number of partial 16S rRNA gene sequences investigated and related diversity indexes. For each diversity index different letters denote significant differences between treatment (ANOVA F and P values are reported).

Sample*	Seq# used	Good's Coverage	Gini-Simpson 1-λ	ANOVA: F=118, P=1.51e-05	Inverse Simpson 1/λ	ANOVA: F=35.26, P=4.82e-04	Fisher's α	ANOVA: F=4.156, P=0.073	Effective number of OTUs at q*	ANOVA: F=4.405, P=0.066	Observed S	ANOVA: F=1.864, P=0.235	Chao 1	ANOVA: F=2.401, P=0.171
S1	22889	0.988	0.977		43.8		384		674		1615		1814	
S2	21849	0.992	0.977	a	42.7	a	307	a	602	a	1339	a	1452	a
S3	20865	0.990	0.979		46.6		360		688		1509		1646	
S4	21092	0.990	0.988		82.8		376		790		1561		1693	
S5	17760	0.987	0.989	b	92.5	b	378	a	801	a	1495	a	1664	a
S6	14921	0.984	0.987		75.8		353		704		1359		1508	
S7	22258	0.994	0.991		117.0		293		725		1295		1384	
S8	22520	0.993	0.989	b	93.2	b	329	a	758	a	1422	a	1511	a
S9	18541	0.990	0.990		99.0		303		684		1272		1412	

* S1-S3, untreated soils; S4-S6, soils amended at 1% (Fe-WTR+MSWC) rate; S7-S9, soils amended at 2% (Fe-WTR+MSWC) rate

Figure 1

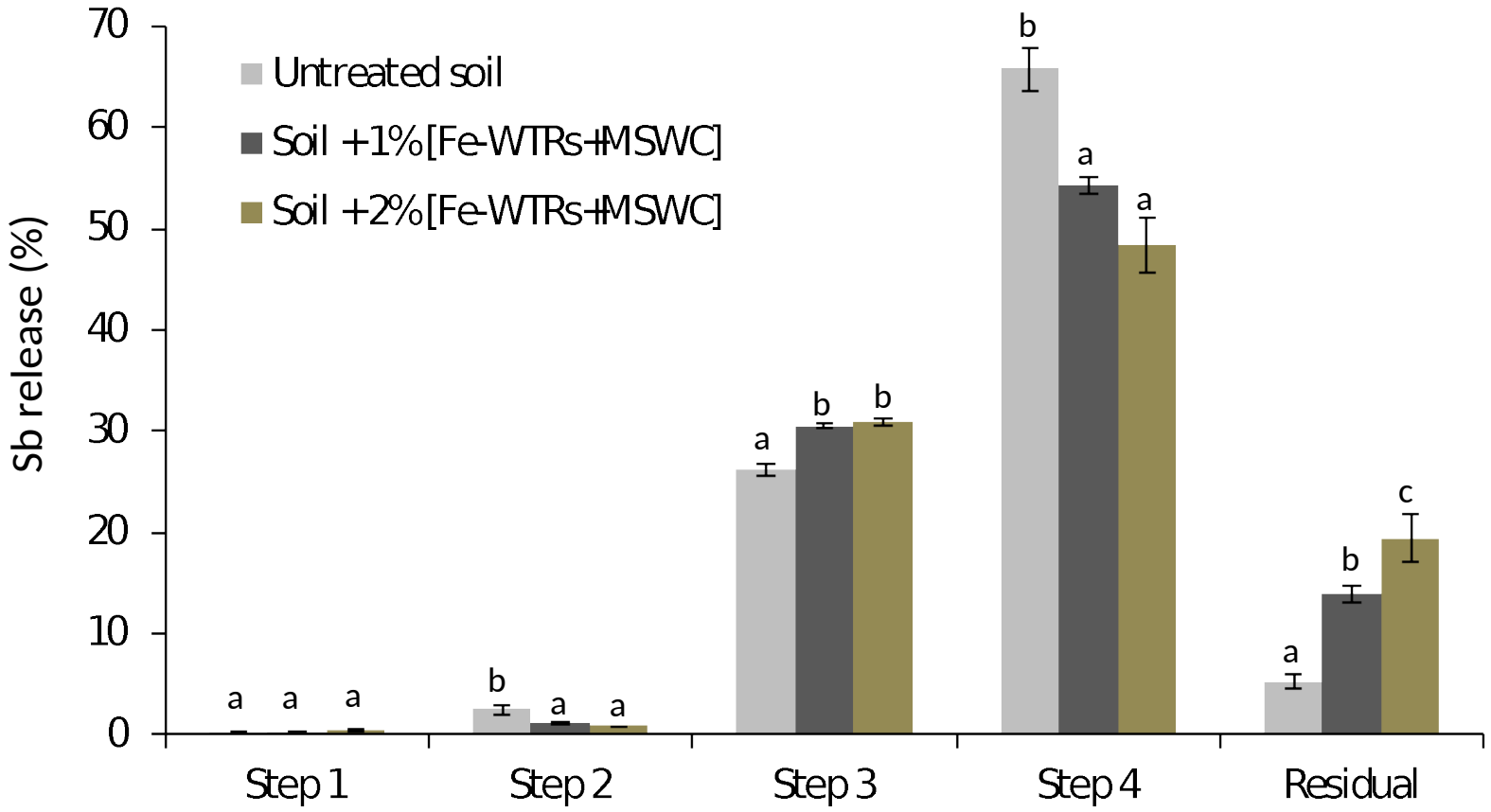

Sb release (%)
70
60
50
40
30
20
10
0
Untreated soil
Soil +1%[Fe-WTRs+MSWC]
Soil +2%[Fe-WTRs+MSWC]
a a a
b a a
a b b
b a a
a b c
Step 1
Step 2
Step 3
Step 4
Residual

Figure 2

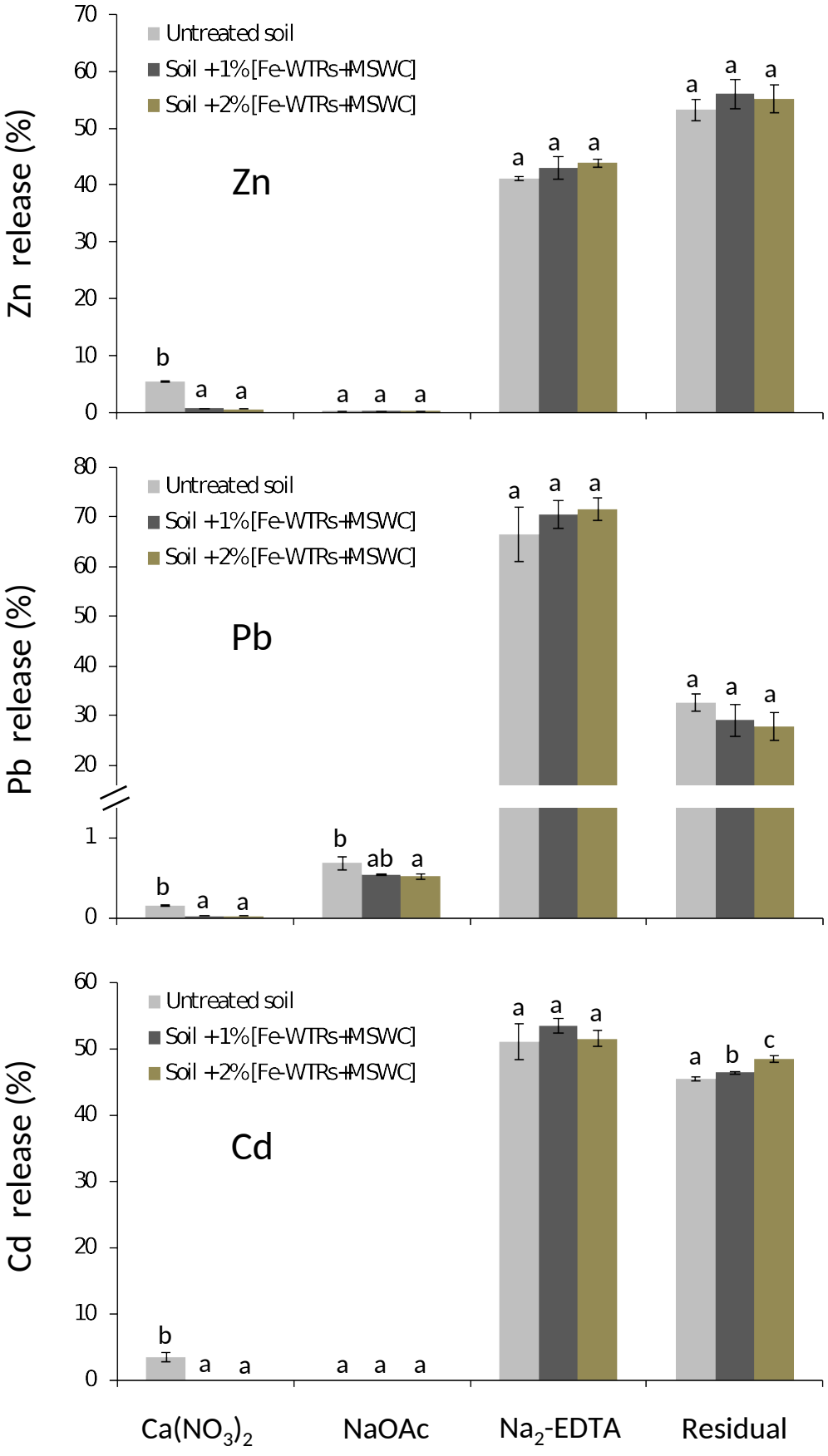

Untreated soil
Soil +1%[Fe-WTRs+MSWC]
Soil +2%[Fe-WTRs+MSWC]
Zn
Zn release (%)
Pb
Pb release (%)
Cd
Cd release (%)
Ca(NO3)2
NaOAc
Na2-EDTA
Residual

Figure 3

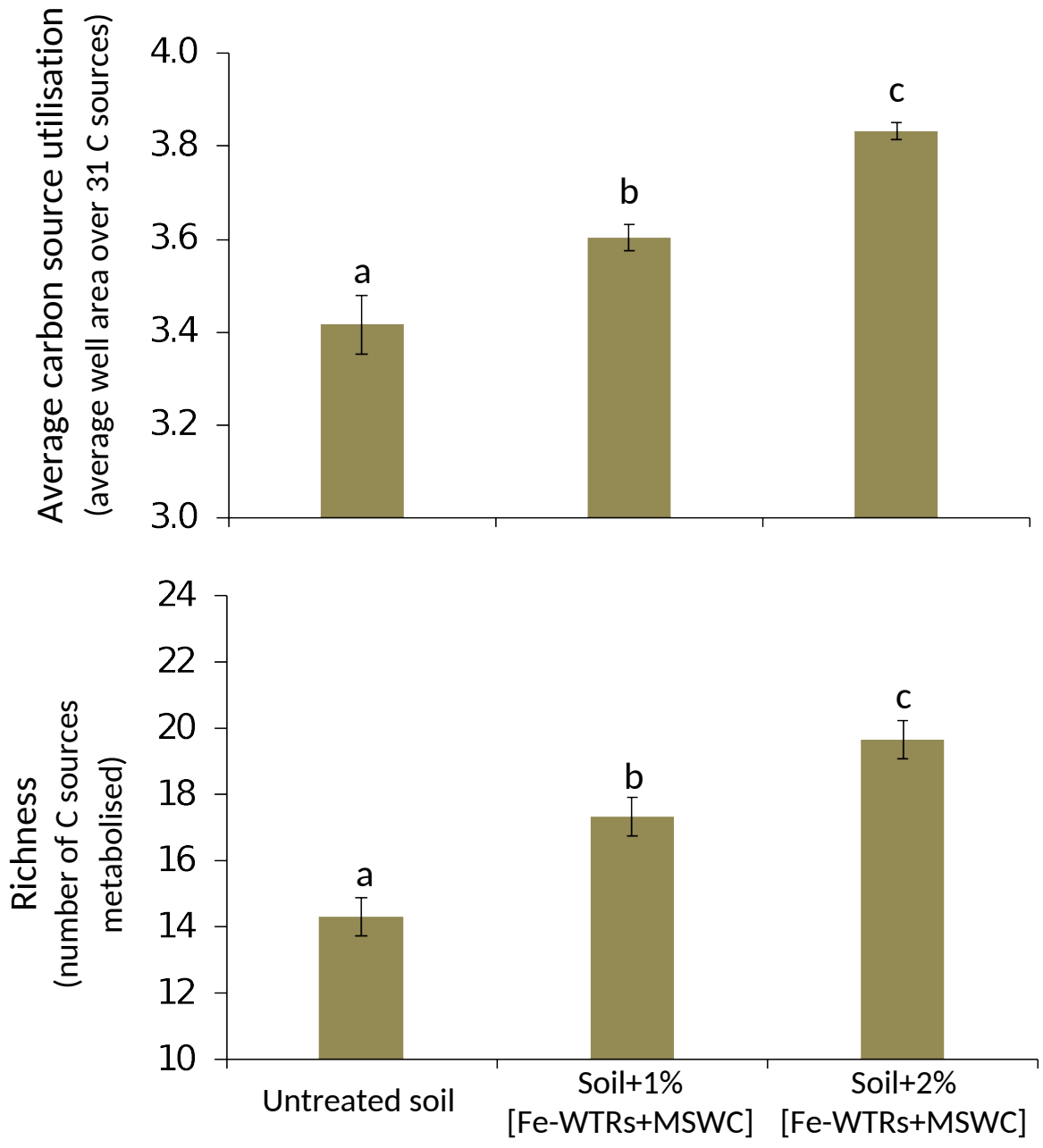
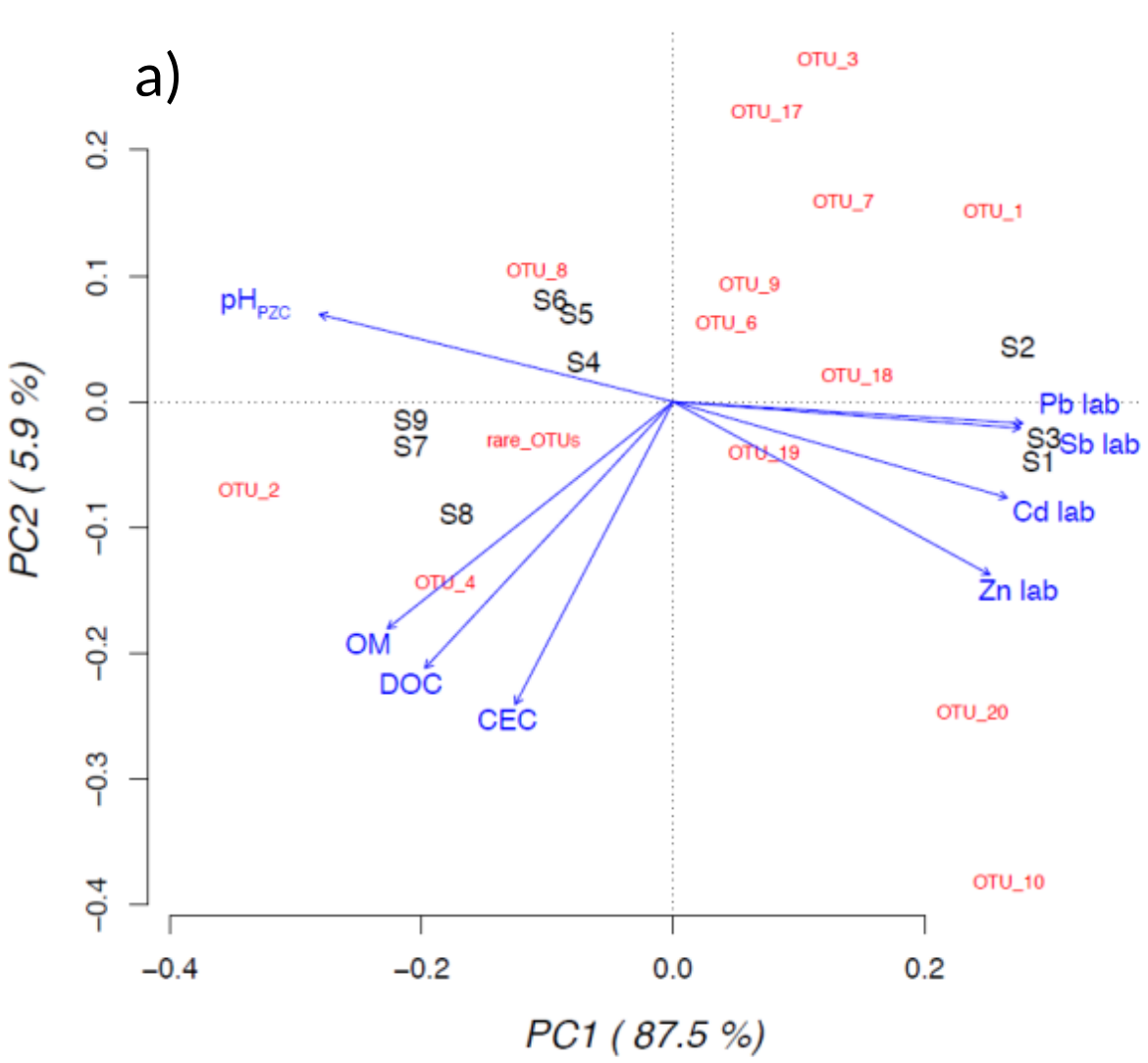

Average carbon source utilisation
(average well area over 31 C sources)
4.0
3.8
3.6
3.4
3.2
3.0
a
b
c
Richness
(number of C sources
metabolised)
24
22
20
18
16
14
12
10
a
b
c
Untreated soil
Soil+1%
[Fe-WTRs+MSWC]
Soil+2%
[Fe-WTRs+MSWC]

Figure 4


a)
OTU_3
OTU_17
OTU_7
OTU_1
OTU_8
OTU_9
pH_PZC
S6
S5
OTU_6
S2
S4
OTU_18
Pb lab
S9
S7
rare_OTUs
OTU_19
S3
Sb lab
S1
OTU_2
S8
Cd lab
OTU_4
Zn lab
OM
DOC
CEC
OTU_20
OTU_10
PC2 (5.9 %)
PC1 (87.5 %)
0.2
0.1
0.0
−0.1
−0.2
−0.3
−0.4
−0.4
−0.2
0.0
0.2

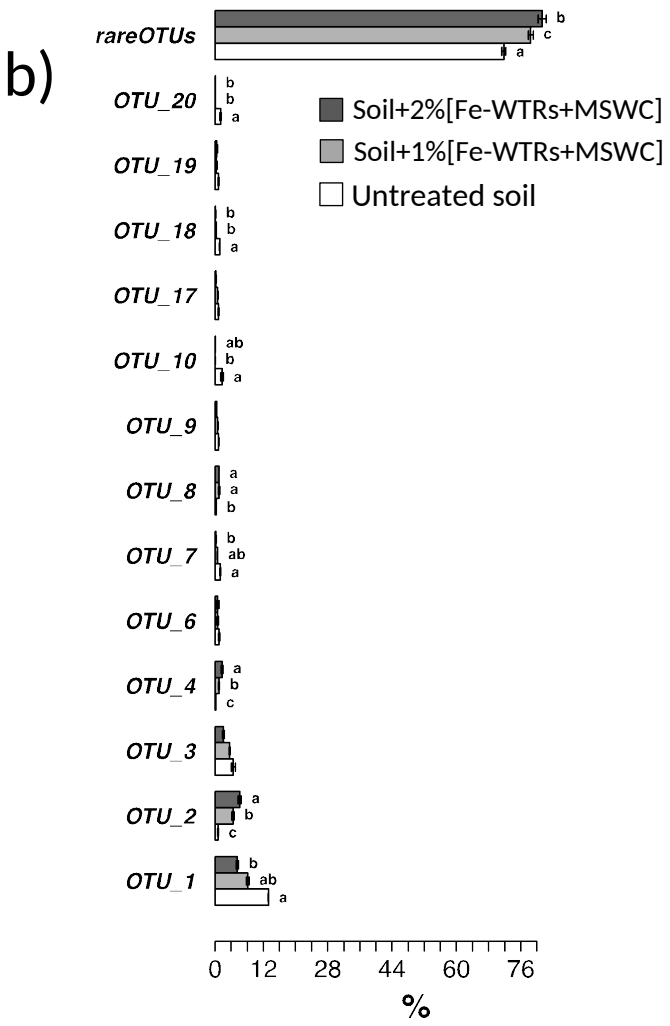
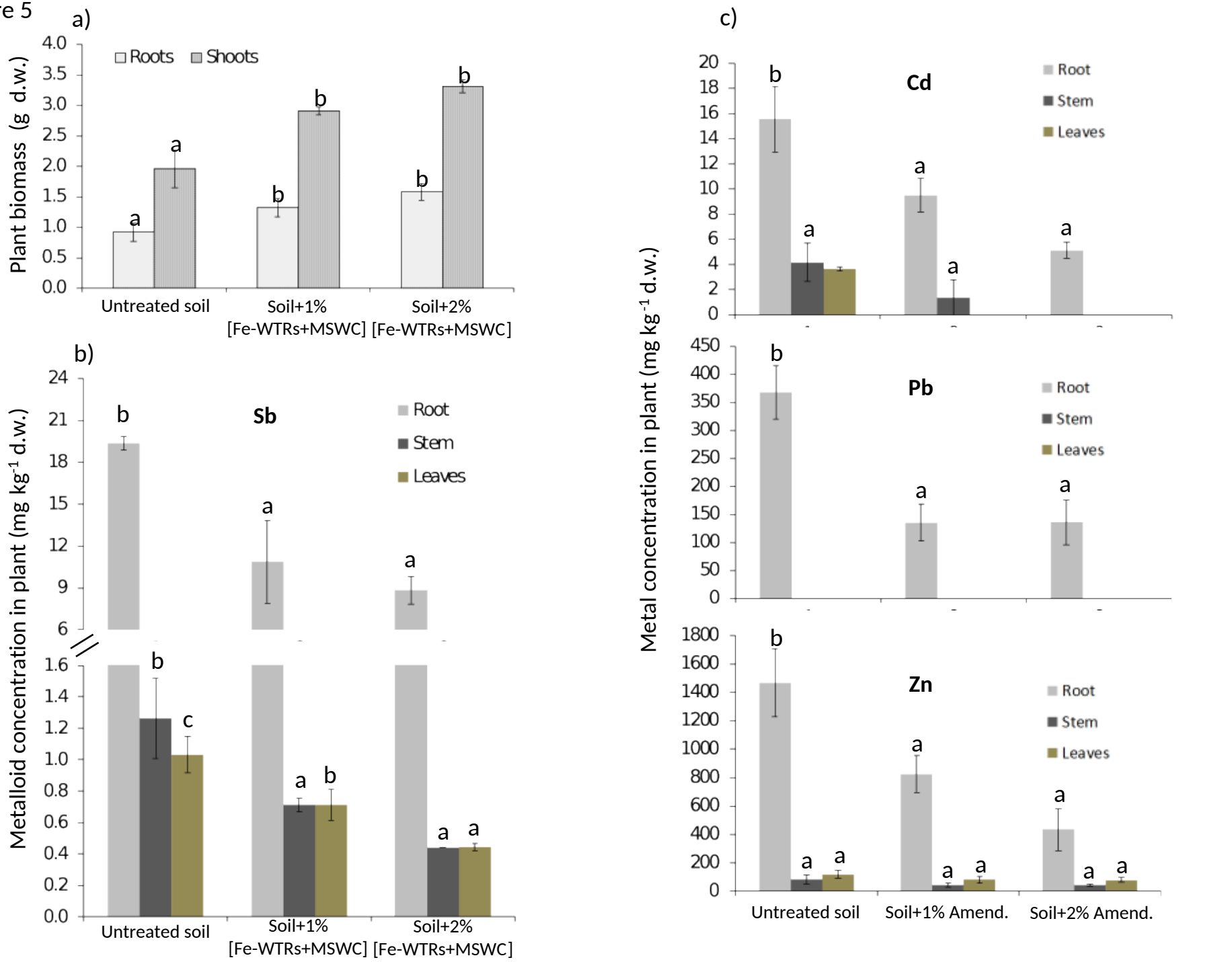

b)
rareOTUs
OTU_20
OTU_19
OTU_18
OTU_17
OTU_10
OTU_9
OTU_8
OTU_7
OTU_6
OTU_4
OTU_3
OTU_2
OTU_1
Soil+2%[Fe-WTRs+MSWC]
Soil+1%[Fe-WTRs+MSWC]
Untreated soil
0
12
28
44
60
76
%

Figure 5


a)
Plant biomass (g d.w.)
Roots
Shoots
Untreated soil
Soil+1% [Fe-WTRs+MSWC]
Soil+2% [Fe-WTRs+MSWC]
b)
Metalloid concentration in plant (mg kg-1 d.w.)
Sb
Root
Stem
Leaves
c)
Metal concentration in plant (mg kg-1 d.w.)
Cd
Pb
Zn
Root
Stem
Leaves
Untreated soil
Soil+1% Amend.
Soil+2% Amend.

Use of municipal solid wastes for chemical and microbiological recovery of soils contaminated with metal(loid)s

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Highlights

- FeWTRs and MSWC were used as amendments to recover a metal(loid)s-contaminated soil
- FeWTRs and MSWC reduced the labile fractions of Sb, Pb, Cd and Zn in soil
- Treated soils showed higher abundance of culturable heterotrophic microorganisms
- Treated soils showed improved enzyme activity, metabolic potential and α-diversity
- Plant growth in the treated soils was greatly stimulated

Supplementary Information – Tables S1-S3

Table S1. Index sequences, linker (TA) primer (TACGGRAGGCAGCAG) sequences and complete construct of the indexed primers used for the PCR based sequencing bacterial diversity analysis of the V3-V4 hypervariable 16S rRNA gene region together with the reverse primer TACNVGGGTWTCTAATCC as described in the materials and methods.

#Sample ID*	Index Sequence	Linker-Primer Sequence	Index-Linker-Primer Sequence
S1	AACATGC	TATACGGRAGGCAGCAG	AACATGCTATACGGRAGGCAGCAG
S2	AACCGAT	TATACGGRAGGCAGCAG	AACCGATTATACGGRAGGCAGCAG
S3	AACCTGT	TATACGGRAGGCAGCAG	AACCTGTTATACGGRAGGCAGCAG
S4	AACGAGA	TATACGGRAGGCAGCAG	AACGAGATATACGGRAGGCAGCAG
S5	AACGCAA	TATACGGRAGGCAGCAG	AACGCAATATACGGRAGGCAGCAG
S6	AACGCTT	TATACGGRAGGCAGCAG	AACGCTTTATACGGRAGGCAGCAG
S7	AACGTAC	TATACGGRAGGCAGCAG	AACGTACTATACGGRAGGCAGCAG
S8	AACTAGC	TATACGGRAGGCAGCAG	AACTAGCTATACGGRAGGCAGCAG
S9	AACTGAC	TATACGGRAGGCAGCAG	AACTGACTATACGGRAGGCAGCAG

* S1-S3, untreated soils; S4-S6, soils amended at 1% (Fe-WTR+MSWC) rate; S7-S9, soils amended at 2% (Fe-WTR+MSWC) rate

Table S2. Concentration thresholds of selected metal(loid)s in soil as related to the soil specific use*.

Metal(oid)s	Private, residential and public green areas ($mg \cdot Kg^{-1}$)	Commercial and industrial sites ($mg \cdot Kg^{-1}$)
Sb	10	30
Cd	2	15
Zn	150	1,500
Pb	100	1,000

*From: Gazzetta Ufficiale della Repubblica Italiana. Decreto Legislativo 3 aprile 2006, No. 152 — Norme in materia ambientale. Supplemento Gazzetta Ufficiale No. 88 del 14 aprile 2006. Roma, Italy: Istituto Poligrafico dello Stato; 2006. [in Italian].

Table S3. Pearson correlation values/coefficients (r) between microbial counts of fast-growing culturable bacteria, fungi and actinomycetes in amended and unamended soils and DHG, GLU and URE activities. *P* values are also reported.

	Microbial counts		
	Bacteria	Fungi	Actinomycetes
DHG	0.924565	0.853516	0.944112
P	0.00036	0.003422	0.000129
GLU	0.816829	0.696705	0.860052
P	0.007204	0.037031	0.002936
URE	0.892011	0.83243	0.919447
P	0.001224	0.005361	0.000451

Figure S1

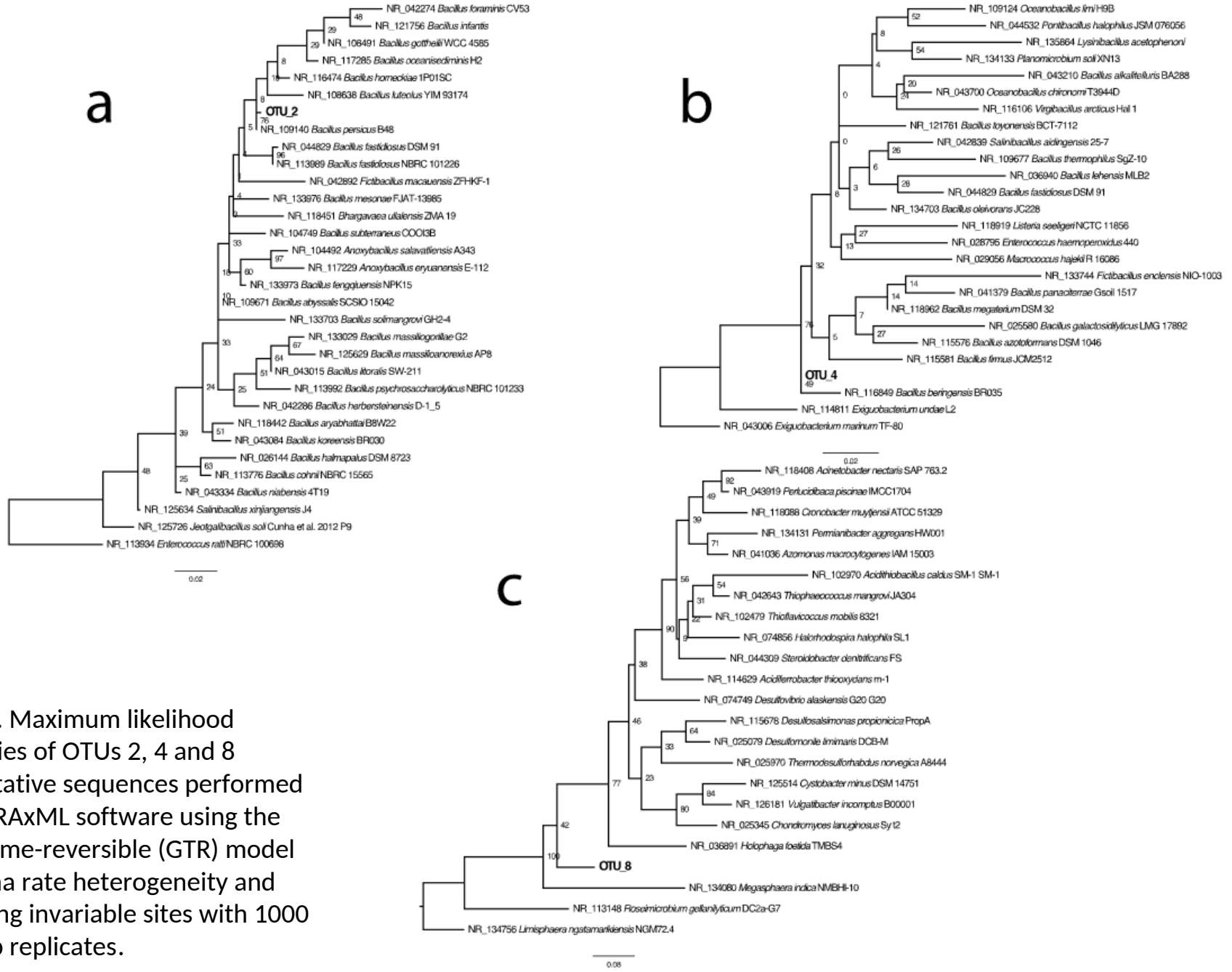

Figure S1. Maximum likelihood phylogenies of OTUs 2, 4 and 8 representative sequences performed with the RAxML software using the general time-reversible (GTR) model for gamma rate heterogeneity and considering invariable sites with 1000 bootstrap replicates.