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Long-term influence of red mud on As mobility and soil physico-chemical and microbial parameters in a polluted sub-acidic soil

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ABSTRACT

In this study we evaluated the efficiency of red muds (RM, a bauxite residue) to immobilize the arsenic present in a polluted sub-acidic soil (UP-soil; total As 2428 mg kg^{-1}) and to influence some chemical, biochemical and microbiological properties after 2 years since RM addition. The RM addition caused a pH increase, a striking decrease of total organic carbon and a significant increase of water-soluble C, N and P. The analysis of As mobility through sequential extraction showed a reduction of the water-soluble arsenic in the RM-soil compared to the UP-soil (3.44% and 5.59% of the total As respectively) and a remarkable increase of the residual (non extractable) arsenic fraction in the RM-soil (>300% compared to UP-soil). RM addition increased significantly the microbial abundance and the activity of selected enzymes (dehydrogenase, urease) with respect to UP-soil while had a major influence on the structure of soil microbial communities as evaluated by the Biolog Community Level Physiological Profile. The reduced As mobility, together with an increase of C, N and P labile-pool (likely originating from a "de-structuring effect" of RM on the soil organic matter) were identified as the key factors affecting the biological activity in the RM-treated soil.

1. Introduction

Arsenic (As) is present in the environment as inorganic [As(III) and As(V)] compounds and in organic forms [1]. As concentration in soils can be substantially higher than background concentration (0.1–40 mg kg^{-1} with an average of 5–6 mg kg^{-1} [2]) due to natural or anthropogenic inputs such as an excessive use of fertilizers, pesticides and/or atmospheric transport-deposition [3]. The total concentration of As in soil does not necessarily provide information about its actual toxicity or environmental impact [4–6] whereas an estimation of the bioavailable fraction can be very informative to predict the hazard posed by As towards soil macro/microorganisms and ecosystem functioning [1]. Several microbial parameters estimating the abundance, diversity and activity of microbial communities have been shown to be very sensitive to high levels of mobile heavy metals or metalloids in soil [7,8] and should be taken into account when assessing the pollution status of a soil or the effectiveness of a remediation treatment.

Many alternatives have been proposed for the remediation of arsenic-polluted soils, including physical, chemical and biological treatments [4]. Amongst chemical treatments, the in situ elemental inactivation/stabilisation is an interesting technique whereby an inorganic amendment is added to a contaminated soil in order to decrease the mobility of pollutants through adsorption and/or co-precipitation reactions [3,9].

In this context, a promising amendment is red mud (RM), a by-product of the alumina industry which derives from the digestion of crushed bauxite with caustic soda. In short-term laboratory and field studies, RM revealed effective at reducing the heavy metal mobility in contaminated soils and stimulate microbial abundance, diversity and activity [10–13]. However, very little is known about its capacity to fix hazardous anions, i.e. arsenate and arsenite, present in polluted soils [14] and influence soil microbial features. Despite this, several studies have shown the RM potentiality to interact with arsenic and remove it from water solutions [15,16].

Therefore, in this study we investigated the ability of a RM to influence the arsenic solubility/bioavailability in a contaminated sub-acidic soil after 2 years since the amendment addition. The RM effects on several soil chemical (water-soluble carbon, nitrogen, phosphorus, phenols, carbohydrates), biochemical (dehydrogenase, β-glucosidase, phosphatase, urease) and microbial parameters (microbial biomass-C, fast-growing culturable bacteria and Biolog Community Level Physiological Profile) have been also investigated.

2. Materials and methods

2.1. Soil characteristics and sample preparation

Approximately 300 kg of soil were collected in the vicinity of the dismissed mining site of Baccu Locci (Cagliari, Italy,

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N39°32′48″, E9°31′36″) where arsenic has been extracted from arsenopyrite for decades. Soil samples (n = 50; 0–30 cm depth) were randomly collected from an area of approximately 2 hectares, bulked together, air dried and sieved to <2 mm. Particle size analysis, carried out as previously described [12], identified the soil as sandy clay loam (USDA classification) with the following composition: 36.62% coarse sand, 22.33% fine sand, 16.63% silt and 24.41% clay. X-ray powder diffraction analysis, performed as previously described [17] identified quartz (45 wt.%), illite (30 wt.%), albite (15 wt.%), hematite (6.5 wt.%) and jarosite (3.5 wt.%) as the soil mineral constituents. Aside from crystalline phases, about 20 wt.% of the soil was made of amorphous phases. The XRD analysis did not show the presence of crystallized arsenic mineral phases.

Different soil sub-samples (n = 3; approx. 50 kg/each) were separately amended with RM (RM-soil) that was applied on a 4% (w/w) basis according to Castaldi et al. [15] while additional sub-samples (n = 3; approx. 50 kg/each) were kept untreated (UP-soil). Unpolluted soil samples (n = 25; 0–30 cm depth; approx. 6 kg/each) were also collected in the vicinity of the mining area from a surface of approximately 1 hectare, treated as previously described, and sub-samples (n = 3; approx. 50 kg/each) used as reference (C-soil).

The RM employed in this study derived from the "Eurallumina" plant located in the industrial area of Portoscuso–Portovesme (Sardinia, Italy). Before the addition to soil, RM was oven dried at 60 °C for 48 h, finely grounded and sieved to <0.02 mm. Its main chemical parameters and phases were previously reported [15,17] and summarized in Table 1. Following the RM addition, RM-soil (but also UP and C-soils) were carefully mixed and moisture content raised up to 40% of their water holding capacity. Substrates were then allowed to equilibrate for 2 years during which they were mixed weekly and kept at approximately 20 °C and 60–70% of relative humidity. Water content was maintained at a level of 40–50%.

Table 1
Main properties of the RM used in this study.

Chemical parameters	RM
pH_{H_2O}	11.1
EC (mS cm^{-1})	8.70
S_{BET}	19.5
PZC	4.77
Total organic matter (% d.m.)	0.60
Element composition (wt.%)	
C	9.15
O	35.12
Na	5.17
Al	9.65
Si	4.32
Ca	1.04
Fe	30.35
Ti	4.13
Cl	1.07
Crystalline phases (wt.%)	
Cancrinite [$Na_6Ca_{1.5}Al_6Si_6O_{24}(CO_3)_{1.6}$]	4.0
Sodalite [$Na_8(Cl,OH)_2|Al_6Si_6O_{24}$]	20.0
Hematite [Fe_2O_3]	44.0
Boehmite [AlO(OH)]	12.0
Gibbsite [$Al(OH)_3$]	4.0
Anatase [TiO_2]	4.5
Andradite [Ca–Fe–Al–Si oxide]	5.5
Quartz [SiO_2]	6.0

2.2. Chemical analysis of soil samples

After the incubation period, the chemical features of RM, UP and C-soils were determined following standard methods [18].

The analysis of water-soluble compounds was performed on 20 g of dried (65 °C) and sieved (<2 mm) soil samples extracted with 200 ml of distilled water (1:10, w/v ratio) by shaking for 24 h. The extracts were centrifuged and filtered through 0.45 μm pores filter membranes and water-soluble carbon (WSC) [18], nitrogen (WSN) [18], phosphorus (WSP) [18], carbohydrates (WS-Carb), [19] and phenols (WS-Phenols) [20] were determined.

The total concentration of As and selected heavy metals (Pb, Cd and Zn) in RM, UP and C-soils was determined after mineralisation with HNO_3 and HCl mixture (1:3, v/v ratio) using a Microwave Milestone MLS 1200. Arsenic and heavy metal concentrations were measured using a PerkinElmer Analyst 600 flame atomic absorption spectrometer equipped with a HGA-600 graphite furnace.

The mobility of inorganic arsenic in soil samples was determined by the sequential extraction procedure of Wenzel et al. [21] with minor modifications: essentially, we added a "Step 0" to the original procedure (which comprises Steps 1–5) in order to estimate the water-soluble As [15].

Sequential extractions with H_2O, 0.1 N $Ca(NO_3)_2$ and 0.05 N EDTA were carried out to estimate the bioavailability of Pb, Cd and Zn in soils [12].

2.3. Enumeration of culturable heterotrophic bacteria and estimate of microbial biomass-C in soil samples

Total fast-growing heterotrophic bacteria and As(V) and As(III) resistant bacteria and *Pseudomonas* spp. were enumerated in triplicate samples (10 g each) from each RM, UP and C-soils using conventional spread plate method. Solidified TSA (Tryptic Soy Agar, Microbiol, Cagliari, Italy; pH 6.5) was used as the growth medium for heterotrophic bacterial counts while solidified TSA (pH 6.5) amended with 7 mM As(III) and 20 mM As(V) was used to enumerate arsenite and arsenate resistant bacteria respectively. The number of *Pseudomonas* spp. in soil samples was determined using solidified PSA (Pseudomonas Selective Agar, Microbiol, Cagliari, Italy; pH 6.5) as the growth medium. Each soil sample (10 g) was dispersed in 90 ml of a pyrophosphate solution (2 g/l) and shaken at 150 rpm for 30 min. Serial 10-fold dilutions in saline solution (0.89% NaCl) were then prepared for each sample and aliquots (100 μl) of each dilution were used to inoculate a triplicate set of plates containing the respective culture media. Bacterial colonies were counted on respective media after incubation of the plates at 28 °C for 3 days and expressed as average Log CFUs (Colony Forming Units) ± standard deviations per gram of dry soil.

Microbial biomass-C was determined in duplicate samples (35 g each) from each RM, UP and C-soil using the rapid chloroform-fumigation extraction method previously described [22]. Briefly, soil samples (35 g) were weighted into 250-ml glass Schott bottles with screw caps and added of 5 ml of distilled water. Unfumigated control samples were immediately extracted with 140 ml of 0.5 M K_2SO_4 solution, shaken for 60 min at 35 rpm, and filtered using a Whatmann no. 42 filter paper. Soil samples for fumigation were added of 2 ml of ethanol-free chloroform (Sigma–Aldrich) and closed bottles were incubated for 24 h in the dark at 25 °C. After incubation, the chloroform was allowed to evaporate in a fume hood and samples extracted as described above. The determination of organic C in fumigated and unfumigated extracts was carried out using the $K_2Cr_2O_7$ oxidation method [18]. Microbial biomass-C (MBC) was expressed as average values (mg kg^{-1} of dry soil) ± standard deviations and calculated as follows: MBC = (organic carbon extracted from fumigated soil − organic carbon extracted from unfumigated soil)/0.38 [23].

2.4. Community level physiological profile

The Biolog EcoPlates (Biolog Inc., Hayward, CA) were used to determine the Community Level Physiological Profile (CLPP), or

Table 2
Chemical characteristics of soil samples after equilibration.*

	UP-soil	RM-soil	C-soil
pH_{KCl}	6.22 ± 0.09[a]	7.87 ± 0.05[b]	6.02 ± 0.10[a]
Electric conductivity (mS cm^{-1})	0.41 ± 0.04[a]	0.62 ± 0.09[b]	0.46 ± 0.07[a]
Total organic C (g kg^{-1} d.m.)	34.9 ± 2.9[b]	20.1 ± 1.8[a]	31.9 ± 2.7[b]
Total nitrogen (g kg^{-1} d.m.)	1.60 ± 0.2[a]	1.51 ± 0.2[a]	2.06 ± 0.3[b]
Total phosphorus (g kg^{-1} d.m.)	0.823 ± 0.02[a]	0.863 ± 0.05[a]	1.142 ± 0.08[b]
Water soluble parameters			
WS-carbon (g kg^{-1} d.m.)	0.99 ± 0.06[a]	2.18 ± 0.05[c]	1.18 ± 0.08[b]
WS-nitrogen (g kg^{-1} d.m.)	0.11 ± 0.02[a]	0.21 ± 0.02[c]	0.18 ± 0.03[b]
WS-phosphorus (g kg^{-1} d.m.)	0.0031 ± 0.01[a]	0.0123 ± 0.01[c]	0.0086 ± 0.01[b]
WS-Phenols (g kg^{-1} d.m.)	0.0458 ± 0.05[b]	0.1422 ± 0.08[c]	0.0129 ± 0.02[a]
WS-carbohidrates (g kg^{-1} d.m.)	0.2328 ± 0.08[a]	0.5148 ± 0.09[b]	0.2588 ± 0.08[a]
Concentrations of total As and heavy metals			
Total As (mg kg^{-1} d.m.)	2428.0 ± 43.2[a]	2489.2 ± 58.2[a]	n.d.
Total Pb (mg kg^{-1} d.m.)	558.59 ± 13.2[b]	562.52 ± 16.2[b]	48.22 ± 1.42[a]
Total Cd (mg kg^{-1} d.m.)	19.53 ± 1.02[a]	19.14 ± 0.82[a]	n.d.
Total Zn (mg kg^{-1} d.m.)	438.36 ± 14.6[b]	438.66 ± 11.7[b]	83.55 ± 1.82[a]

* Mean values ± standard deviations followed by the same letter within a row do not differ significantly (Fisher's LSD test, $P < 0.05$).

the carbon source utilization pattern, of soil microbial communities extracted from RM, UP and C-soils. Microbial communities were extracted from 10 g of each RM, UP and C-soils as previously described [24] and 120 μl of the microbial suspension were used to inoculate each well of the EcoPlate. Plates were incubated in the dark at 28 °C for 144 h and the carbon source utilization within each well was quantified by taking absorbance readings at 590 nm (OD_{590}) every 24 h [12]. Raw Biolog data (OD_{590} values) were treated as previously described [12] to determine the Average Well Colour Development (AWCD, a measure of the general potential metabolic activity of the microbial community) and Richness, or the number of the carbon sources utilized by the community. Normalised Biolog data [25] were analysed by Principal Component Analysis (PCA) using the covariance matrix as the interpretation of the PCs is more straightforward as previously shown [26]. All the Biolog-derived parameters reported refer to 120 h incubation since this time-point provided the best discrimination among samples.

2.5. Soil enzyme activities

RM, UP and C-soils were investigated for specific enzyme activities involved in a range of soil processes and reflecting different microbial functions. The dehydrogenase (DHG) activity assay was based on the estimation of the reduction rate of triphenyltetrazolium chloride to triphenyl formazan in soil samples [27]. The assessment of urease activity was based on the colorimetric determination of the ammonia released after incubation of soil samples with urea solution [27]. The detection of β-glucosidase activity was based on the determination of the released p-nitrophenol after the incubation of soil samples with p-nitrophenyl glucoside solution [27]. The activities of the alkaline and neutral phosphatase were based on the determination of the released phenol after the incubation of soil samples with phenyl-phosphate solution [27]. All the enzymatic activities were expressed on an oven-dried soil basis.

2.6. Statistical analysis

All the analyses were performed in triplicate for each RM, UP and C-soil sample unless otherwise stated. One-way analysis of variance (one-way ANOVA) was carried out to compare all the means from different treatments. Where significant *P*-values ($P < 0.05$) were obtained, differences between individual means were compared using the post-hoc Fisher's least significant difference test (LSD, $P < 0.05$). All data were analysed using the NCSS software (Number Cruncher Statistical Systems, Kaysville, UT) for Windows.

3. Results and discussion

3.1. As and heavy metals in soil and RM influence on their mobility

The total concentration of As in the UP-soil was very high (2428 mg kg^{-1}, Table 2). This concentration substantially exceeded 20 mg kg^{-1} which is the guideline limit for the As concentration in agricultural soil proposed by the European Community [28]. However, such a total concentration could not be indicative of the real As toxicity towards soil microorganisms and functionality [6,8,28,29]. This is because arsenic in soil could be present in various forms from easily leachable, mobile and bioavailable, to fixed and unavailable [6,28]. Specific extraction procedures should be employed in order to quantify the different fractions and effectively estimate the biological hazard associated to As in soil. In this study we adopted the procedure of Wenzel et al. [21] with minor modifications to determine the different As fractions in soils and predict its bioavailability.

The water-soluble arsenic is considered one of the more labile fractions of the total As-pool [8,29]. Compared to its total concentration, relatively low amounts of water-soluble As were found in the UP-soil (5.59% of the total As) (Step 0; Fig. 1). This is presumably due to the specific chemistry and mineral composition of the

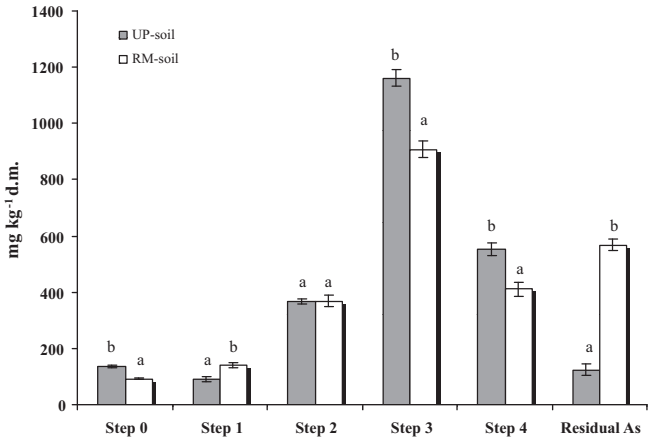

Fig. 1. As fractions extracted with H_2O (Step 0); $(NH_4)_2SO_4$ (Step 1); $NH_4H_2PO_4$ (Step 2); NH_4^+-oxalate (Step 3); NH_4^+-oxalate + ascorbic acid (Step 4), and residual As in UP and RM-soils. Mean values ± standard deviations (error bars) followed by the same letter within the same extraction Step do not differ significantly (Fisher's LSD test, $P < 0.05$).

soil. The RM addition further decreased the water-soluble fraction of arsenic (3.44% of the total As; <32% compared to UP-soil).

The exchangeable As fraction was estimated by $(NH_4)_2SO_4$ extraction [21] (Step 1; Fig. 1). Sulphate allows for the extraction of non-specifically adsorbed arsenic, which can be replaced through anion exchange. Together with the water-soluble arsenic, the sulphate-extracted fraction is considered the most available to soil biota and the most easily leached to groundwater [1]. The RM addition increased the exchangeable arsenic fraction in soil (Fig. 1), despite an increase of soil pH generally causes the desorption of arsenate (and sometimes arsenite) electrostatically sorbed on the surface of soil colloids [9]. It is likely that the increase of soil pH in the RM-soil was counterbalanced by the increased number of exchange sites (particularly of the Fe/Al oxides and oxyhydroxides) present in the RM. This sorbent is a complex mixture of minerals (hematite, gibbsite, boehmite and others), each characterized by a different point of zero surface charge. For example, the pH_{pzc} value of hematite is ~8.1, whereas the pH_{pzc} of gibbsite and boehmite are ~9.4 and 8.6 respectively [15]. The fact that the exchangeable-arsenic fraction increased following RM addition could therefore be due to the formation of electrostatic bond between As(V) and As(III) and the positively charged sites of Fe/Al oxide and oxyhydroxides of RM [15]. An increase of exchangeable As upon alkalinisation was previously reported [30,31].

During Step 2, As(V) and As(III) forming inner-sphere surface complexes with surface groups of soil colloids were replaced by $NH_4H_2PO_4$ through a ligand-exchange mechanism [1,21,29]. In this case, the RM addition did not change significantly the As fraction chemically bounded (Fig. 1).

More than 70% of total arsenic in the polluted soil (UP-soil) was extracted in Steps 3 (extraction with NH_4^+-oxalate) and 4 (extraction with NH_4^+-oxalate + ascorbic acid) [21]. These steps account for the As fractions retained by adsorption at surfaces of amorphous and crystalline Fe/Al oxides and oxy-hydroxides. The RM addition caused a considerable decrease of these fractions, from 70.5% in UP-soil to 49.1% in RM-soil. This decrease could be due to a higher competition for the same adsorption sites on Fe/Al surfaces between As(V)/As(III) and the possible high presence of organic anions of low molecular weight deriving from a likely "RM-driven" dissolution of soil organic matter (see Section 3.2). This competition for adsorption sites on Fe/Al oxides and oxy-hydroxides is well-known and reported by several authors (e.g. [31]). Finally, a significant fraction of the arsenic associated to the Fe/Al oxides and oxy-hydroxides was transferred to the residual fraction in the RM-treated soil (>300% with respect to the UP-soil) (Fig. 1). This suggested that a larger fraction of As in the amended soil was strongly retained (and not expected to be readily released), and most likely bounded to the RM-phases through different mechanisms such as chemical sorption, as previously shown by Castaldi et al. [15]. However, other mechanisms such as diffusion and coprecipitation reactions cannot be excluded *a priori* given the high complexity of the soil system.

To our knowledge this is the first study showing, through a sequential extraction procedure, that RM addition is able to reduce the bioavailable As in a polluted soil and increase significantly its residual fraction.

The total concentrations of Pb, Zn and Cd in the UP-soil were all exceeding the common background soil concentrations (Table 2) [32]. Despite this, Pb and Cd were not recovered in the H_2O and $Ca(NO_3)_2$ extracts (Fig. 2). After 2 years of incubation the RM addition did not increase the total concentration of Pb, Cd, Zn, while they reduced the negligible amounts of water-soluble and exchangeable Zn and increased its residual fraction (Fig. 2). This supports the results of previous studies carried out in our laboratory (not presented here) which showed that most of the heavy metals contained in the red mud (e.g. Pb, Cd, Zn, Cu, Ni and others) are not

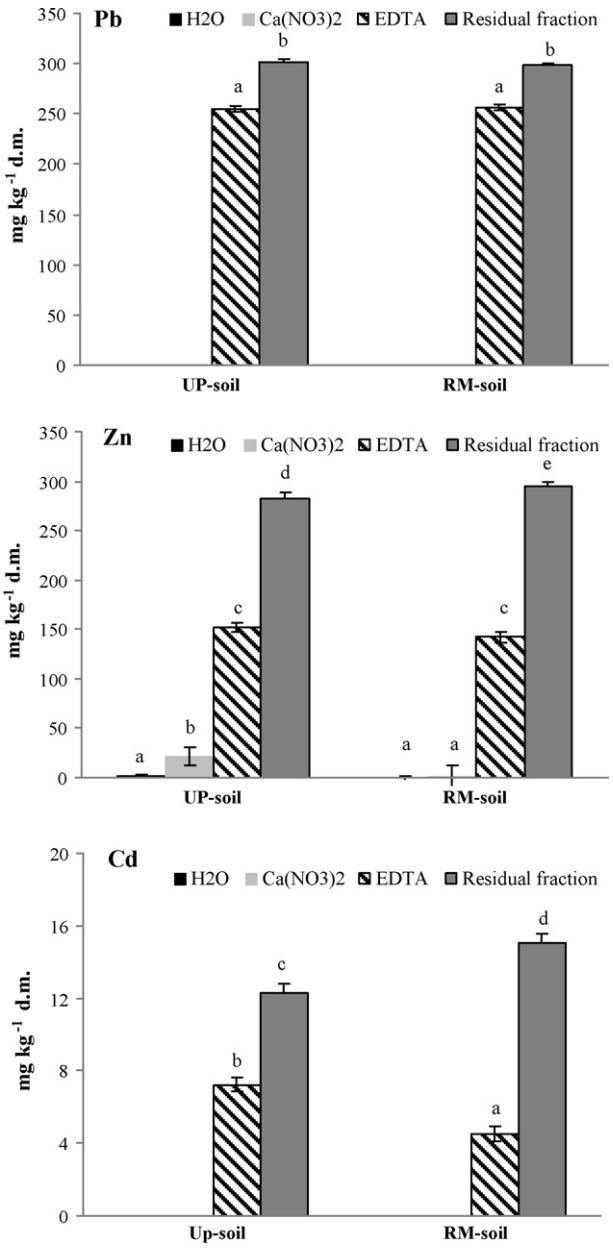

Fig. 2. Heavy metal fractions sequentially extracted from UP and RM-soils. For each metal mean values ± standard deviations (error bars) followed by the same letter do not differ significantly (Fisher's LSD test, $P < 0.05$).

mobile, hence they should not pose an additional environmental threat. Since Pb, Cd and Zn bioavailable fractions in UP-soil were negligible (when detectable) we assumed that most of the toxicity effects towards soil microorganisms and activity could be attributed to the bioavailable fractions of As.

3.2. RM influence on soil physico-chemical properties

After a 2-year equilibration period, the RM addition to the polluted soil resulted in an increase of soil pH and a remarkable loss of soil organic carbon (SOC; ~40% less in RM-soil with respect to UP-and C-soils) (Table 2). Such a striking SOC decrease after RM addition was never reported before and could be due to a massive priming effect initiated by the sorbent. The combined effect of the RM alkalinity and its high sodium content (Table 1) could have initially enhanced the release of WSC and WSN from the stabilised soil organic matter (SOM). This in turn could have stimulated (together

with a reduced mobility of As) the microbial growth and activity, eventually leading to an enhanced/accelerated SOM degradation as a result of co-metabolism and higher enzyme activity [33]. The remarkable increase of all the water-soluble parameters in the RM-soil (Table 2), together with a significant SOC loss, seem to support this view. In particular, the WSC, WSN, WSP, WS-Phenols and WS-Carb increased in the RM-soil of 120%, 90%, 295%, 210% and 121% respectively with respect to the UP-soil. An increase of the dissolved organic carbon (DOC) was previously observed by Lombi et al. [10] after plant cultivation on two RM-treated soils polluted with heavy metals. However, the DOC increase in such soils could not be clearly attributed to the sole addition of RM because of the confounding effect of plants, and unfortunately, SOM content in the amended and control soils was not reported.

The long equilibration period between RM and polluted soil, adopted in the present study, seems a crucial factor governing this hypothetical RM-driven priming effect. In this regard, we could not assess any significant difference between the total organic carbon of RM-treated soils, equilibrated for about 6 months, and the respective control soils [12].

3.3. RM influence on the microbial abundance and potential functional diversity of soil microbial communities

In order to evaluate the RM efficacy at improving the global health *status* of a polluted soil, chemical data may be insufficient and a careful evaluation of the soil microbial content, activity and diversity may be helpful to complement the chemical profile and allow for a more comprehensive evaluation of the amendment [7,11,13]. Accordingly, in this study we evaluated the RM influence on soil microbial biomass, *Pseudomonas* spp., fast-growing culturable heterotrophic bacteria (total and As(V) and As(III)-resistant), and microbial community structure (based on the analysis of the Biolog CLPP).

After the equilibration period, the number of culturable bacteria in the RM-soil was more than 10- and 2.5-fold higher with respect to UP and C-soils respectively (Fig. 3). This burst in microbial growth confirmed the RM efficacy at limiting the As availability for microorganisms (and also the bioavailability of the negligible amounts of Zn). This likely resulted in a higher substrate utilisation efficiency and more metabolic energy diverted towards bacterial growth rather than cell functioning maintenance and/or cell detoxification in the polluted soil [34]. On the other hand, the very large increase of culturable heterotrophic bacteria also suggested a larger availability of readily-usable carbon in the RM-soil that was indeed confirmed by the analysis of water-soluble parameters (Table 2).

The RM addition to the polluted soil had a similar effect on the number of culturable fast-growing As(V) and As(III) resistant bacteria (Fig. 3). Despite the bioavailable fraction of As was reduced by the addition of RM, the number of As resistant bacteria in the RM-soil was still significantly higher than that of UP and C-soil. However, when we normalised the data (e.g. calculating the ratio of As resistant to total culturable bacteria), to allow for a more proper comparison of the microbial populations in the different soils, the resulting outcomes (Fig. 3) were fully in agreement with the As availability data (i.e. the % of As resistant bacteria followed the order UP > RM > C-soil). This suggests that the proportion of As resistant bacteria can be a useful indicator of the degree of environmental pollution and/or restoration of As-polluted soils.

The microbial biomass-C (MBC) followed a similar trend. MBC was significantly higher in the RM-soil compared to UP-soil but the former was not statistically dissimilar with respect to C-soil (Fig. 4). Our values for the UP-soil were in substantial agreement with those obtained by other authors (e.g. [35]) for soils with a comparable pollution level of As, Pb and Zn. The RM addition improved the soil MBC content (~30% with respect to UP-soil) approaching the values recorded for the C-soil. Taken together, the data of MBC and total culturable heterotrophic bacteria indicate a clear positive effect of RM on the microbial abundance.

Different cultivation-dependent and independent approaches have been used to resolve the impact of As and heavy metals on soil microbial community structure (e.g. [36]) and to assess the suitability of different amendments at promoting the recovery of the microbial features in polluted soils [12,13,37]. Among culture-dependent approaches, the suppression/absence of *Pseudomonas* isolates on selective media (e.g. PSA medium) revealed as an interesting and macroscopic indicator of changes occurred to the soil microbial community after chronic exposure to metals [36,38]. In the current study we obtained similar findings: while the number of *Pseudomonas* spp. in the undisturbed C-soil was around 4 log CFUs/g dry soil, they were undetectable in UP and RM-soils, even after 2 weeks incubation of the plates.

Among culture-dependent approaches, the Biolog CLPP is a fast and reliable way to differentiate between microbial communities based on their potential functional diversity [25]. This approach, originally developed by Garland and Mills [39], is able to provide a metabolic fingerprinting of soil microbial communities based on their potential utilisation of a range of sole carbon sources. The

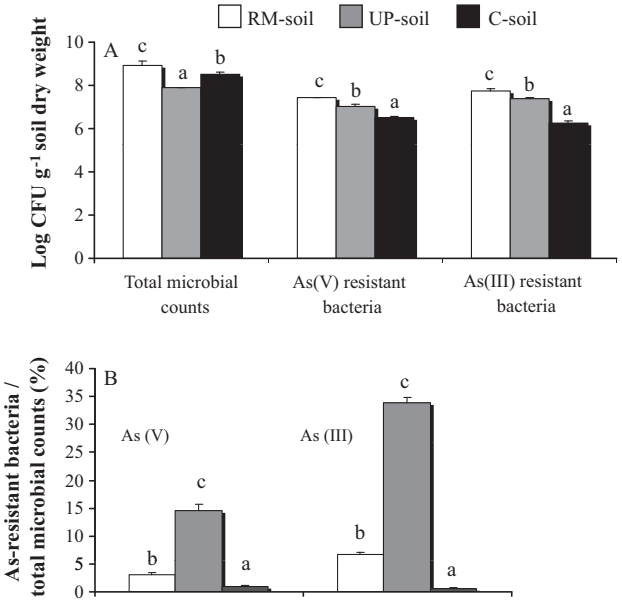

Fig. 3. Fast-growing heterotrophic bacteria in UP, RM and C-soils (A) and ratio of As-resistant bacteria to total bacterial counts (B). Mean values ± standard deviations (error bars) followed by the same letter within the same microbial group do not differ significantly (Fisher's LSD test, $P < 0.05$).

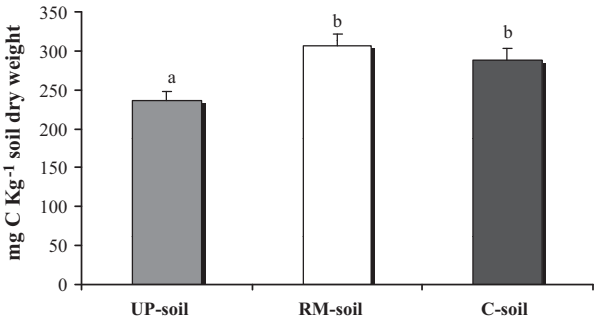

Fig. 4. Soil microbial biomass-C in UP, RM and C-soils. Mean values ± standard deviations (error bars) followed by the same letter do not differ significantly (Fisher's LSD test, $P < 0.05$).

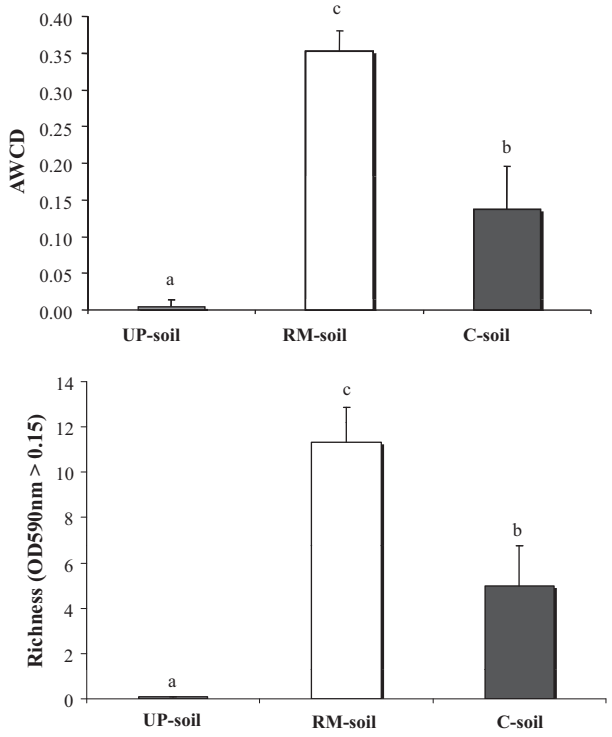

Fig. 5. AWCD and richness (OD_{590} > 0.15) values of microbial communities from the UP, RM and C-soils. Mean values ± standard deviations (error bars) followed by the same letter do not differ significantly (Fisher's LSD test, $P < 0.05$).

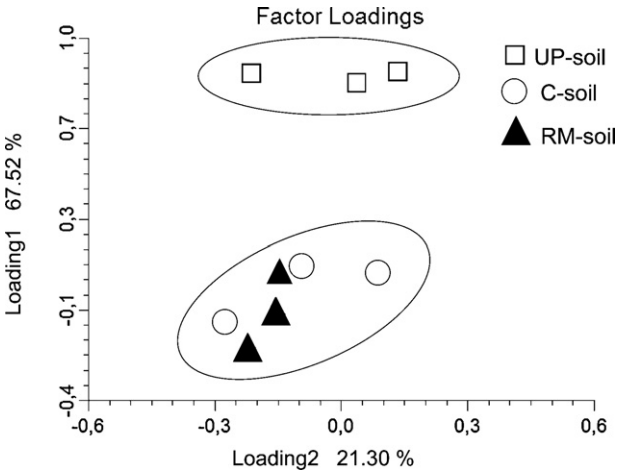

Fig. 6. Principal component analysis (PCA) applied to normalised Biolog data (carbon source utilisation after 120 h incubation) relative to the different soil microbial communities.

Biolog CLPP has been widely applied to investigate the impact of heavy metals on soil microbial communities [36,38,40] and to evaluate possible changes induced on the community by different remediation treatments [7,12,13]. The CLPP data presented in this study, AWCD and Richness in particular, confirmed that metals and metalloids have a strong detrimental effect on the potential functional diversity of soil microbial populations [7]. The lowest AWCD (which is indicating the potential average metabolic activity of the microbial community) was detected in the UP-soil and in general followed the order: UP < C < RM-soil (Fig. 5). Consistently, the Richness values (the number of sole carbon sources catabolised by the microbial community) followed the same order (UP < C < RM-soils) indicating that the recovery of a certain general metabolic capability induced by the RM (higher AWCD values in the RM-soil) was also accompanied by an improved catabolic competence (higher Richness) of the microbial community. Interestingly, the AWCD and Richness values of the C-soil were significantly lower than those recorded for the RM-soil (Fig. 5). Taken together these results suggest the occurrence of marked differences in the structure of the microbial communities inhabiting the three soils. However, when we applied the PCA analysis to normalised carbon source utilisation data we noticed that C and RM-soils were clustered together whereas the UP-soil was clustered apart (Fig. 6). This is possibly indicating a similarity in the patterns of carbon source utilisation between RM and C-soils communities and a substantial difference between these latter and the UP-soil. If this is the case, this also implies that the differences in the AWCD and Richness values, between RM and C-soil, were likely due to the different inoculum density as previously stressed [25].

3.4. Soil enzyme activities

In order to draw a more clear picture of the RM influence on the activity of the soil microbial populations a range of hydrolase activities were determined in soils. It is well known that As and metals in soil can reduce enzyme activity by interacting with the enzyme-substrate complex, by denaturing the enzyme, or by interacting with the protein active groups [6,28]. Therefore soil enzyme activities can be believed as sensitive indicators of the biological effects of RM on polluted soils.

The DHG activity (reflecting the functionality of a group of intracellular enzymes that oxidize the organic compounds) gives a measure of the total oxidative activity of the soil microflora and can provide useful information on the stress level faced by the soil microbial communities [8,28,29]. The UP-soil showed DHG values much lower than the unpolluted control soil, confirming that arsenic and metal contamination can significantly reduce soil microbiological activity [8,28,29]. On the other hand, the RM addition favoured a significant increase of the DHG activity (>43% with respect to the UP-soil) suggesting that a substantial metabolic recovery occurred in the treated soil. Also the specific DHG activity values (i.e. the ratio between DHG activity and microbial biomass-C) of each community (0.0170 for UP-soil, 0.0184 for RM-soil and 0.0244 for C-soil; $P < 0.05$) confirmed that RM had a significant effect on the increase of the metabolic activity of the soil microbial community.

The activity of the alkaline phosphomonoesterases, which catalyse the hydrolysis of organic phosphomonoester to inorganic phosphorus, was higher in the UP-soil with respect to RM and C-soils. Contrasting with previous findings [41,42] this enzyme activity was not inhibited by the arsenic or metals (Fig. 7). However, it is well known that this enzymatic activity is inhibited by orthophosphate in soil [27] and, therefore, the higher content of alkaline phosphatase in the UP-soil could be reasonably due to its lower content of water-soluble phosphorus compared to RM and C-soils (Table 2). Neutral phosphatase activity was very low in all the soil samples and apparently was insensible to pollutants and RM addition.

Urease was strongly affected by soil pollution. In the UP-soil the urease activities was respectively 11.7- and 8.9-fold lower with respect to RM and C-soils. The effect of arsenic/metals contamination on urease activity is rather controversial, and both inhibitory [6] and stimulating or no effects [41] have been reported. Evident positive effects of RM on urease activity were detected in this study and could be due to the reduced bioavailable As fractions in the RM-soil [6].

By contrast β-glucosidase was unaffected by RM addition. The apparent insensitivity of this activity to RM addition is unexplained,

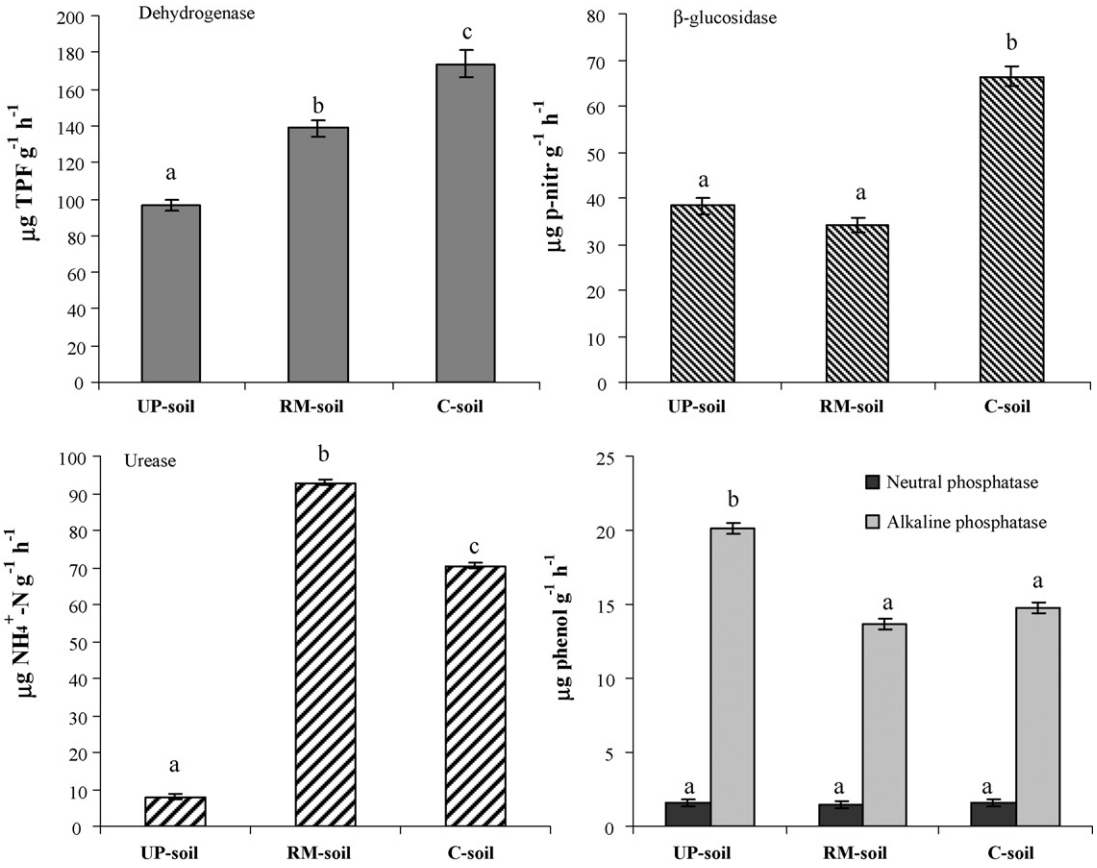

Fig. 7. Enzymatic activities detected in UP, RM and C-soils. Mean values ± standard deviations (error bars) followed by the same letter within the same enzymatic activities do not differ significantly (Fisher's LSD test, $P < 0.05$).

but several authors showed that this enzymatic activity is not a good indicator of heavy metals pollution [12,13].

4. Conclusions

In this study we evaluated the influence of RM on the mobility of arsenic in a contaminated soil. The assessment of the metalloid mobility was accompanied by the monitoring of a number of other soil chemical, biochemical and microbiological properties with the aim to provide a more comprehensive evaluation of the amendment efficacy at promoting the restoration of the disturbed soil.

All the soil features were examined after 2 years since the RM addition and, in this sense, the results presented can be useful to understand and foresee the possible long-term effects of RM towards soil chemical and microbiological parameters in As-polluted soils.

The sequential extraction used in this study showed for the first time that RM addition can favour an increase of the residual soil arsenic, and limit its bioavailable fraction. This could be due to the higher retention of As in the RM-treated soil. The ratio of As(V) and As(III) resistant bacteria to the total heterotrophic microbial counts confirmed that RM was effective at reducing the As availability for soil microorganisms and supported the chemical mobility data.

The results presented showed significant long-term effects of RM on several soil physico-chemical properties: a massive SOC reduction was observed in RM-treated soil together with a substantial increase of the water-soluble carbon, nitrogen, phosphorous, phenols and carbohydrates. This "de-structurating" effect of RM on the soil organic matter was never reported before and could be due to a priming effect initiated by the amendment. The significant increase of the readily culturable heterotrophic bacteria, soil microbial biomass-C, dehydrogenase and urease activity in the RM-soil seem to support this hypothesis.

The analysis of the Biolog CLPP revealed that RM addition can have a significant influence on the potential functional diversity of the soil microbial community despite it was not able to promote a full recovery of the original microbial biodiversity as highlighted by the absence of culturable *Pseudomonas* in the RM-soil.

Despite the results presented clearly show that RM can alleviate the As (and metal) toxicity towards soil microorganisms, and stimulate the recovery of the microbial abundance and activity in polluted soils, further studies are needed to better understand the long-term influence of RM on the SOM depletion and the possible implications on soil fertility and plant growth. These aspects are currently being investigated in our laboratory.

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