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A multi-biological assay approach to assess microbial diversity in arsenic (As) contaminated soils

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Abstract

Microbial genetic, structural and functional diversity was assessed in response to Arsenic (As) pollution in a former gold mine soil. EL-FAME, qPCR, DGGE, enzyme activities and MicroResp techniques were used.

Multivariate analysis showed that As bioavailability in soil was an important driver affecting microbial diversity. Microbial biomass assessed by EL-FAMEs and q-PCR generally decreased under higher bioavailable As, as well as enzyme activities and C substrates utilization. Conversely actinomycetes and fungal biomass increased along with total As content suggesting the selection of more resistant species.

Keywords

arsenic, soil enzymes, EL-FAME, DGGE, MicroResp™

1. Introduction

A considerable body of information has been obtained on the effects of metals and metalloids on soil microorganisms and microbially-mediated soil processes related to nutrient cycling, C sequestration and organic matter decomposition (Tripathy et al., 2014; Liao and Xie, 2007; Giller et al., 2009). Large accumulation of heavy metals may decrease microbial biomass and activity and affect the structure of soil microbial communities (Bååth, 1989; Laskowski et al., 1994; Pennanen et al., 1998). A decrease in total soil microbial biomass under chronic metal stress has been widely observed but it is likely to be preceded by changes in community structure and/or functions. Decreased size of microbial biomass can be partially explained by physiological causes such as a decrease in microbial substrate utilization efficiency and an increased maintenance energy requirement (Giller et al., 2009). The structural diversity of a microbial community has been found to be very sensitive to environmental changes, reacting by shifts in its composition (Kandeler et al., 1996). In particular, high heavy metal loads may reduce genetic/functional diversity of soil microbes (Gans et al., 2005) and these less diverse microbial communities may be less resistant to additional disturbances (Degens et al., 2000). Pollution may thus lead to decrease in microbial diversity in terms of species richness due to the extinction of species lacking sufficient tolerance or to shifts towards more tolerant groups. In fact, Gadd (2013) reports efficient tolerance strategies adopted by fungi in response to heavy metal contamination in soil.

Genetic and structural diversity studies are thus crucial in determining the response of a population to changing conditions. Several indices such as species richness, diversity and evenness are used to describe the structural diversity of a community and to monitor changes in

microbial diversity due to environmental fluctuations, land management practices and pollution (Øvreås, 2000).

Moreover, metals and metalloids in soil may alter microbial metabolism and functions, thereby reducing enzyme activities (Bhattacharayya et al., 2008), respiration (Marabottini et al., 2013), and C utilization efficiency (Liu et al., 2012).

In order to understand the overall response of soil microorganisms to metal stress, Giller et al. (2009) and Waldrop et al. (2000) call for a pressing need to link studies of microbial populations including microbial diversity in terms of function. In particular, Blagodatskaya and Kuziakov (2013) recently claimed for a broad application of dynamic approaches aimed to discriminate within total microbial biomass the active portion of microorganisms and their specific functional activities to understand biogeochemical processes and their biotic drivers.

Arsenic (As) is toxic to almost all soil microorganisms by inhibiting cellular functions linked with energy metabolism; long term effects of As contamination are evident since microbial biomass size and metabolism are strictly related to its bioavailability (Singh et al., 2015; Marabottini et al., 2013; Das et al., 2013; Ghosh et al., 2004). All the above authors reported, in fact, a close relationship between most microbial indicators and water soluble As concentration in soil.

From the above studies and results it is evident that microbial responses to metals and metalloids pollution, As in particular, may range from changes of size and basal metabolism to changes of structural composition, in terms of richness in species and physiological performances. Furthermore, variations in microbial population, activity and diversity could also function as early predictors of changes in soil health level (Nielsen et al., 2002).

Xiong et al. (2010) reported that the response in terms of composition and function of microbial communities to As contamination is an understudied field in soil microbiology. To our knowledge, very few authors reported in the same study an integrated methodological approach aimed to assess microbial diversity in As contaminated soils from the genetic, structural and functional point of view, including a new reliable functional approach such as MicroResp^{TM}; Sheik et al., (2012) reported a notable impact of As and Cr on microbial biomass (genetic and structural diversity) subjected to chronic exposure in Pakistan but could not infer the functionality of the changed community. Liu et al. (2012) published an interesting complete study comprising a different range of microbial responses to a mixture of metals such as Pb, Cd, Zn and Cu but no As.

In a previous study (Marabottini et al., 2013), performed in an As chronically contaminated mine-soil, we reported a significant effect of bioavailable As forms on microbial biomass respiration and metabolic quotient. Therefore it was hypothesized that changes occurred within microbial community in terms of structural/genetic and functional diversity.

The aims of this paper were: i) to assess changes on microbial genetic (quantitative PCR and Denaturating Gradient Gel Electrophoresis analysis), structural (Ester-Linked Fatty Acid Methyl Ester composition) and functional (enzyme activities and MicroResp) diversity induced by As pollution and ii) to compare the results obtained by means of a multi-biological approach.

1. Material and Methods

2.1 Site description, sample collection, storage

The site of the abandoned mining area of Pestarena, located on the Eastern slope of the Monte Rosa Mountain, in Northwestern Italy, is an interesting study area for As pollution over a wide

history of mining activity. The gold mines of the Monte Rosa perimeter have been exploited since ancient times, dating to the Roman civilization; the history of the mining activity at Pestarena was documented throughout the Middle Ages up to the year 1961, when the mine and the ore processing plant were closed (Preite et al., 2007). Gold ores have been exploited for centuries and tailings and wastes, associated with large amounts of arsenopyrite minerals, have often been disposed in open air, causing a high hot spot contamination with arsenic in a wide area around the mines and mineral processing plants. The contamination is a consequence of As release after the oxidation of sulphide minerals contained in ore and tailing dumps, a process inducing soil acidification.

In our study the soil samples were taken from the highly As-contaminated mining site, located along the slope of the left bench of the Anza creek.

Three homogeneous plots, 50x50 m, were chosen and were representative of high As contamination (H), medium (M) and low (L) contamination. They were respectively located at an increasing distance from the main dump area, up to 1 km away; uncontaminated soil samples were not found within the pedoenvironmental frame of the study area. All soil samples were collected at 20 cm depth during Spring 2010. Low contaminated soils (L) were taken from an area outside the mining site under herbaceous vegetation (45°57'44"N, 8°1'28"E). Medium contaminated soils (M) were taken a few meters from a mine tailing deposit under herbaceous vegetation (45°57'28"N, 8°0'51"E). High contaminated soils (H) were taken within the mining perimeter from an area where some perennial vegetation (*Abies alba* and other conifers) was present, together with herbaceous understory vegetation (45°57'29"N, 8°0'56"E) (Figure 1). In

each plot, five soil cores (approx. 500g) were taken at a maximum distance of 10 m from each other. All 15 samples were investigated separately.

An aliquot of 50 g was immediately frozen (-20°C) for EL-FAME and qPCR, DGGE analyses. For these last two analyses the five soil cores from each site were pooled together to obtain a representative sample.

Mine tailings were accumulated beside the Anza creek where the leaching of As can have dangerous outcomes. For this reason, the amount of bioavailable As, the labile fraction potentially hazardous for the environment, was evaluated with two soil fractions: water soluble (WS, ultrapure water from a Milli-Q system on an end-over-end shaker at 20°C for 12h) and not-specifically sorbed (NSS, 0.05 M $(NH_4)_2SO_4$, 20°C for 4h). Table 1 shows the physico-chemical properties and As concentration (total and bioavailable) of Pestarena soils (Marabottini et al., 2013).

2.2 EL-FAME analysis

Microbial community structure was characterized by ester linked fatty acid methyl ester (EL-FAME) analysis, which employed a mild alkaline methanolysis method to extract ester-linked (EL) fatty acids but not free fatty acids (Schutter and Dick, 2000). To summarize, 16.6 ml of 0.2 M potassium hydroxide in methanol and 5 µl of internal standard solution (0.023 mg ml^{-1} methylnonadecanoate 19:0) were added into a 50 ml centrifuge tube containing 5 g of freeze-dried soil. The tubes were incubated at 37 °C for 1 hour (h) in orbital shaker. Then, 6.6 ml of 1.0 M acetic acid were added to neutralize the pH of the tube contents. FAMEs were extracted by adding 10 ml of hexane. The mixture was vigorously mixed and centrifuged at 4500 rpm (2037.55 G) for 15 min. The hexane layer was transferred into a clean glass tube, dehydrated

with sodium sulphate, filtered and evaporated under a steam of N_2. Finally, FAMEs were dissolved in 200 µl of dichloremethane and transferred to an amber vial for GCMS quantification analysis. Mass spectra was recorded, as previously described by Crognale et al. (2013), by the use of a QP-5050 (Shimadzu, Japan) spectrometer equipped with an AT 20 capillary column (0.25 mm i.d., 25 m) (Alltech, Deerfield). 14 methylated fatty acids were identified according to their mass spectra and using BAME 24 (47080 U) and 37 FAME Mix (47885-U, Sigma–Aldrich) as chemical standards (Table 2). Data were expressed as a relative amount, calculated as the area of each EL-FAME peak relative to the summed area of all EL-FAMEs peaks. Methylnonadecanoate, C19:0, at known concentrations, was used as internal standard. Three analytical replicates of each sample were extracted. Standard nomenclature is used to describe FAs, which are designated by the total number of carbon atoms/number of double bonds, followed by the position of the double bond from the methyl (aliphatic) end (ω) of the molecule. The prefixes "a" and "i" refer to anteiso- and iso-branched FAs. The prefix "10Me" indicates a methyl group on the tenth carbon atom from the carboxyl end of the molecule and "cy" indicates cyclopropane FAs. Microbial groups were assigned to ester-linked fatty acids (El-FAME) following Fierer et al., (2003) and Frostegård et al., (1993). The ratio of cyclopropyl fatty acid to the monoenoic precursor [(cy17:0)/(16:lω7), abbreviated as cy/pre] and iso/anteiso (iso methyl branched to anteiso methyl branched ratio), were used as indicators of physiological or nutritional stress in bacterial community (Bach et al. 2010; Moore-Kucera and Dick 2008; McKinley et al. 2005).

2.3 DGGE (Denaturating Gradient Gel Electrophoresis) analysis

Whole genomic DNA of each soil sample was extracted using the Power Soil DNA extraction Kit (Mo Bio Laboratories Inc., CA) according to the manufacturer's instructions. The bacterial V3 region of the 16S rRNA gene was amplified using the universal bacterial 341f (5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAG CAG3') and 534r (5′-ATTACCGCGGCTGCTGG-3′) primers with the length of approximately 200 bp (Muyzer et al., 1993). Fungal 18S ribosomal DNA was amplified using the universal fungal primers FUN_NS1 **(**5′GTAGTCATATGCTTGTCTC 3′) and GC fung (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCATTCCCCGTTACCCGTT G-3) (Das et al., 2007). The reaction mixture consisted of 2 µl of template DNA (ca. 20 ng), Tris–HCl (20 mM, pH 8.3), KCl (100 mM), $MgCl_2$ (3 mM), Hot Start Taq-polymerase (0.1 U) (NZYtech, Portugal), primers (0.2 µM of each) and double deionized water (ddH2O) to bring the final volume to 50 µL. For Bacterial amplification, the touchdown PCR program was performed in a Primus PCR thermo cycler (MWG biotech, Germany) according to Muyzer et al., 1993 while for fungal amplification the PCR program was previously reported by Das et al. (2007). All amplicons (5 µl) were analysed on 1 × TAE agarose gel before they were used for DGGE. The INGENY phorU DGGE system (Ingeny, Netherlands) was used for sequence-specific separation of PCR amplified fragments. For PCR amplicons obtained with the 341f-GC and 534r primers, electrophoresis was performed in a polyacrylamide gel (8% (w/v) acrylamide/bis-acrylamide gel 37.5:1), containing a 40–60% urea-formamide denaturing gradient (100% corresponds to 7 M urea and 40% (w/v) formamide). For fungal amplicons the urea-formamide denaturating gradient was from 20 to 35%. After DGGE electrophoresis, gels were stained with

Gel star solution (Lonza, USA) at room temperature for 45 min and photographed under a UV-transillumination table with a digital camera (GelDoc XR, Bio-Rad, USA). The fingerprinting profile obtained from DGGE was investigated using the Quantity one software (Bio-Rad, USA).

2.4 Real-time PCR (qPCR) Assay

Bacterial and fungal DNA was quantified using 16S and 18S rRNA gene target sequences respectively, amplified using primers 331 F, 797 R and FR1 F FF 390 R (Vaino and Hantula, 2000). All samples were quantified in triplicates using a LightCycler® 480 System Thermocycler (Roche Applied Science, USA). The quantification was based on the fluorescent dye SYBR-green one, which was bound to double strand DNA during PCR amplification. Each reaction was performed in a 20 µl volume containing 5µl of DNA template, 1 µl of each primer and 10µl of SYBR Green PCR Master mix. Melting curve analysis of the PCR products was conducted following each assay to confirm that the fluorescence signal originated from specific PCR products but not from primer-dimers or other artefacts. A plasmid standard containing the target region was generated for each primer set using total DNA extracted from a sample. The amplified PCR products of the bacterial 16S rRNA gene and the fungal 18S rRNA gene were purified using PCR solution purification kit (Machery-Nagel, Germany), ligated into pGemT-easy cloning vector (Promega, Madison,WI) and cloned into *Escherichia coli* DH5ɖ. Clones containing correct inserts were chosen as the standards for qPCR. Plasmid DNA was isolated using plasmid extraction kit (Sigma, Saint Luis, MO). As the size of the vector and PCR insert were known, the copies of the 16S and 18S rRNA gene were directly calculated from the concentration of extracted plasmid DNA. Standard curves were generated using triplicate 10-fold

dilutions of plasmid DNA ranging from 1.05 $\times 10^2$ to 1.05 $\times 10^{10}$ copies for the bacterial target gene and, 1.14$\times 10^2$ to 1.14$\times 10^{10}$ copies of template for 18S rRNA. High amplification efficiencies of 105% were obtained for the bacterial 16S rRNA gene and 95.3% for fungal 18S rRNA gene quantification.

2.5 Enzymatic analyses

According to Marx et al. (2001), soil enzyme activities were measured using fluorogenic methylumbelliferil (MUF) substrates (SIGMA). The eight enzymes studied are involved in the main biogeochemical cycles: Carbon (Cellulase, β-glucosidase, β-xylosidase,), Nitrogen, (N-acetyl-β-glucosaminidase), Sulphur (Arylsulphatase) and Phosphorus (Acid phosphatase). Furthermore, acetate and butyrate esterase were included as proxies of endocellular activity (Wittman et al., 2004).

An aliquot of 1 g of soil, previously incubated for 12 hrs at 60% water holding capacity, was weighed inside of a sterile jar with 50 ml of water. Soil suspension was obtained by homogenising with Ultra Turrax at 9600 rev x min^{-1} for three minutes, then 100 µl were withdrawn and dispensed into a 96 well microplate. Substrates were prepared with acetate buffer 0.5 M pH 5.5 and an aliquot of 100 µl (1 mM) was added to each well reaching a final concentration of 500 µM. Fluorescence (excitation 360 nm, emission 450 nm) was measured with an automatic fluorimetric plate-reader (Fluoroskan Ascent) and readings were performed after 0, 30, 60, 120 and 180 minutes of incubation at 30° C.

2.6 CLPPs (MicroResp™)

The community level physiological profile (CLPP) was determined using the MicroResp™ soil respiration system (MicroResp^{TM}, Macaulay Scientific Consulting Ltd, Aberdeen, UK) according to Campbell et al. (2003).

MicroResp™ is a technique based on the employment of bulk soil, combining the advantages of Biolog™ and Substrate Induced Respiration (SIR) (Campbell et al., 2003). The C substrates employed were based on their ecological relevance as representative carbon sources inputs metabolised by microbes.

The fifteen substrates consisted of five carbohydrates (D-glucose, N-acetyl-glucosamine, D-galactose, D-fructose, L-arabinose), five aminoacids (L-leucine, L-arginine, glycine, L-aspartic acid and γ-amino-butyric acid), three carboxylic acids (citric acid, oxalic acid and L-ascorbic acid), and two phenolic acids (vanillic and syringic acid). Phenolic acids were selected because they are representative of more recalcitrant organic compounds. Substrates concentration was chosen from values utilised in previous CLPP studies using MicroResp™ (Dalmonech et al., 2010). Soil (30% WHC, water holding capacity) was added to dissolved substrates in deionized water and a water control was present in each deepwell. The emission of CO_2 by the microbial biomass was estimated using a colorimetric method (microplate spectrophotometer) before and after 6 h of incubation at 28° C. The absorbance was read at 595nm. At the end the absorbance was normalised for any difference recorded at time zero and then converted to % CO_2 using the calibration curve y = A+B/(1+ D x Ai). The CO_2% was converted to µg C-CO_2 g^{-1} h^{-1} production rate using gas constant, T° C, headspace volume, soil dry weight (d.w.) and incubation time.

2.7 Diversity indexes

For all methodological approaches Shannon's diversity index ($H' = \sum pi \ln pi$) was used.

For EL-FAME, pi was the proportional amount of each EL-FAME (Zornoza et al., 2009).

For enzymatic rates, pi was the ratio of the activity of a particular enzyme to the sum of all enzymatic activities (Bending et al., 2004) and for MicroResp^{TM} p_i was the respiration rate of each single C-substrate as SIR (Substrate Induced Respiration) (Degens et al., 2000).

To characterize the community composition as obtained by DGGE analyses, Richness (S) and Shannon (H') and Evenness (E) were calculated. In particular S was the number of detected bands. H' index was calculated as above mentioned, where pi was the ratio of the single band intensity to the sum of bands intensity of each lane and Evenness (E) was calculated as H'/lnS (Brons and van Elsas, 2008; García-Delgado et al., 2015).

2.8 Statistical analysis

All analyses were performed in triplicate for each sample. In order to avoid effects due to differences of soil organic C content, enzymatic activities and MicroResp data were normalized per units of TOC. Comparisons were made using a one-way analysis of variance (ANOVA) with a Tukey post hoc test to reveal significance between groups; differences resulting in $P < 0.05$ were considered statistically significant.

Statistical analysis of results was performed using SigmaStat software v3.5 (Systat Software Inc., San Jose, CA).

A principal component analysis (PCA) was performed using XLStat 2014 with the aim to identify underpinning factors able to synthetize all the variables.

2. Results

The three soil samples had the same texture (sandy loam), acidic to sub-acidic pH, organic carbon (TOC) ranging from 1.9 to 4.5 % and cation exchange capacity (CEC) from 15 to 25 cmol kg^{-1}. The content of total As was 4357 mg kg^{-1} in the highly contaminated soil, 1161 mg kg^{-1} in the medium and 275.6 mg kg^{-1} in the low contaminated soil. Such great As concentrations, even in soils not directly affected by the mining activity, are characteristic of the Anzasca Valley (Caviglia et al., 2015) because of the widespread presence of arsenopyrite in the parent rocks.

The labile As forms (water-soluble and non-specifically adsorbed) represented a greater percentage of the total content in the medium contaminated soil. The pH followed a decreasing trend at increasing As, since the primary As-bearing phases were sulphide minerals, which oxidation involves strong acidifying effects. The percentage of labile As forms was increasing at decreasing CEC, although As is mostly present in soil environments as anionic forms which are not retained by soil cation exchange complex. Differences in As mobility could be linked to the abundance and mineralogy of Fe, Al, Mn oxi-hydroxides, to pH and, possibly, to the concentration and kind of competing anions, such as organic anions, phosphate or silicate (Violante, 2013) (Table 1).

A total of sixteen EL-FAMEs were extracted and 14 types of EL-FAME were identified as shown in Figure 2a; these EL-FAMEs were compounds ranging from C14 to C20. They consisted of the normal saturated EL-FAME, the monounsaturated EL-FAME, the polyunsaturated EL-FAME, the methyl-branched EL-FAME and the cyclopropyl EL-FAME. The dominant EL-FAMEs were 16:0, 18:1w9c, i15:0, and 16:1w7c. As a general trend, the amount of EL-FAMEs ranked as

follows: L>H>M (Fig. 2b). In most cases a lower biomass was determined for M and H soils, for five FAMEs the lowest values were observed for M soils (i14:0, i17:0, a17:0, 16:1w7c and 15:0 as general marker of bacterial biomass). Conversely, for FAMEs related to actinomycetes (10Me16:0) and fungi (18:1w9c and 18:1w9t), a different trend was observed with higher values for M and H respectively. In particular it is meaningful to point out the complete absence of 18:1w9t for fungi in L soils (Figure 2a). Cy/pre and iso/anteiso indexes, calculated from EL-FAME data, are reported in figure 2b. In both cases these stress indexes indicated high stress conditions in M soils. Cy/pre index also showed a significant positive correlation to water soluble As fraction ($r= 0.827$ $p<0.01$, data not shown).

The amount of total biomass, assessed by means of quantitative PCR (qPCR) was not different in L and H soils, while for M it decreased significantly (Figure 3b). This trend was similar if we consider bacterial and fungal biomass singularly; in fact along the different level of As contamination the fungi:bacteria ratio did not change. The genomic diversity of bacterial and fungal communities was assessed by DGGE analysis of PCR-amplified 16S and 18S rRNA gene fragments respectively (Figure 3a and 3b). Bacterial diversity indexes were not affected by the increasing As load, while for fungal community the DGGE profile complexity tended to decrease with the increase of As contamination level. In particular richness dropped significantly in M and H soils while evenness was significantly reduced in H soils. Similar results were obtained from melting curve analysis of the PCR products revealed a constant melting temperature (Tm), along different levels of As, for 16sRNA gene amplicons related to bacterial community while a one-degree shift in Tm was observed for 18sRNA gene amplicons in M and H soils if compared to amplicons in L soil for fungal community (Figure 3b).

Significant positive correlations have been found for water soluble As to EL-FAME and total DNA (r=0.569 and 0.655, p< 0.01 respectively, data not shown) while no correlations were present with total As.

Soil enzyme activities are reported in Figure 4a. All enzymes except cellulase, acid phosphatase and xylosidase decreased in M soils; in particular arylsulphatase, butyrate, chitinase and acetate showed the highest decrease.

The community level physiological profile determined by means of MicroResp^{TM} showed, as an average, a decrease of C sources utilization by 38% for M and by 3% for H soils. In particular, a consistent decrease of most substrates utilization for M soils was evident, being significant for citric acid, arginine, galactose, arabinose, aspartic acid and glucose (Figure 4b).

Shannon diversity index (H') calculated using enzyme activities, CLPP-MicroResp^{TM}, EL-FAME and pPCR-DGGE data showed similar trends for all approaches reporting a lower diversity level for M soils (Figure 5).

Figure 6 reports principal component analysis (PCA) results. Two main components emerged as principal factors resuming all information derived by all variables and explaining together as much as 69.1% of total variance. F1 component is positively related to all microbial variables but negatively correlated to bioavailable As and stress indexes. PCA analysis clearly separated the three sites along the two components.

3. Discussion

This work aimed to assess soil microbial genetic, structural and functional diversity in relation to different levels of As pollution in northern Italy. In a previous study, performed in the same site

(Marabottini et al., 2013), we reported a marked effect of bioavailable As on microbial biomass acitivity hypothesizing shifts in the structural and/or functional diversity of soil microrganisms.

In the present study, therefore, we proposed a quali-quantitative methodological approach aimed to embrace all aspects of microbial diversity (genetic, structural and functional) induced by a historical arsenic pollution.

Total microbial biomass assessed by means of EL-FAME and qPCR indicated that microbial pool dimension was influenced by the effects of an increasing concentration of arsenic associated with mine drainage.

EL-FAME analysis showed a decrease of microbial biomass in M soils, especially for Gram+, being this fraction more sensitive to metals pollution as reported by Chodak et al. (2013). Furthermore this trend is in accordance with the distribution of bioavailable As.

An opposite trend was observed for actinomycetes and fungi showing higher biomass with increasing As content. As for the actinomycetes, the observed trend was in accordance with previous studies reporting *Actinobacteria* as the dominant (Berg et al., 2012), highly resistant (Chen and Shao 2009; Větrovský and Baldrian 2015) and metabolically active (Gremion et al., 2003) component of the bacterial community in heavy metal contaminated environments (Lejon et al., 2008), especially those with high As content. In particular, Cavalca et al. (2015), in a parallel study performed using different methodologies at Pestarena, found that actinomycetes were more abundant in the rhizosphere of *D. caespitosa* growing in the more polluted site.

Our results agree with those reported earlier by Turpeinen et al. (2004), where a proportional increase of fatty acid indicators for fungi in presence of increasing metal contaminations was found. Since in this study a variation of bacteria/fungi ratio was not observed with the As

concentration, it might be hypothesized that the 18:1wc and 18:1 9wt are representative of the more resistant species that became dominant with the increasing As concentration. Krishnamoorthy et al., (2015) suggest that heavy metals, deposited on the soils surrounding the smelter area of a former mining site for more than 50 years, led AMF (arbuscular micorrhizal fungi) species to develop adaptation mechanisms against HM (heavy metals) stress.

Microbial DNA extracted from Pestarena soils was significantly lower in M soils although the fungal content was always lower (two order of magnitude) than the bacterial one (Figure 3). Similar fungal to bacterial ratios were also reported by Liu et al. (2012) in metal contaminated soils (Fungi/Bacteria: 0.5 - 3.5 x 100).

Although both methodologies (qPCR and EL-FAME) provide quantitative information, these approaches target soil microbes in diverse cell components: DNA and membrane phospholipids. The different quantitative trends reported in response to As concentration may, thus, account for differences in the vegetative status and/or quiescent conditions that may influence membrane activity (Pepi et al., 2008) but leave DNA patterns unaltered. Positive interrelationships found for water soluble As to EL-FAME and DNA suggest the dependence of microbial biomass on the bioavailable fraction of As and not to the total soil content confirming the results obtained by Marabottini et al. (2013).

As far as microbial genetic diversity is concerned, interesting differences arise when analysing bacterial and fungal communities separately by means of DGGE.

Bacterial community showed no relevant changes of S (richness) and E (evenness) due to As level. This can be explained by the fact that any disturbance of soil bacteria will lead to a rapid initial decrease of the diversity, followed by an increase of the biodiversity due to the

achievement of a new dynamic equilibrium (Van Bruggen and Semenov, 2000). The ability of bacteria to develop metal tolerance without affecting overall community structure and richness has been already reported for Cu long-term disturbance exposure (Lejon et al., 2010; Berg et al., 2012).

Therefore, in this historically contaminated site we observed a long-term effect resulting from a strong selective previous pressure, that induced the bacterial community to adopt possible multiple resistance mechanisms able to cope with As toxicity (Cai et al., 2009; Ryan et al., 2005). The rapid growth rate and genetic plasticity, indicating the possibility to horizontally transfer arsenite transporter and arsenate reductase genes, may have allowed bacteria to survive under As-stressed conditions (Smith et al., 2008; Cai et al., 2009).

In our experimental site the fungal community was more responsive in terms of diversity to As contamination than bacteria community, as also reported by Das et al. (2013) and Liu et al. (2012). On the basis of our results, it seems that fungal biomass was more influenced by As. The increase in Tm of 18s RNA gene amplicons in M and H soils could suggest a shift towards more resistant species. An increase in GC content, coupled to an increase in Tm, has been pointed as an indicator of the diversity and shifting of microbial community (Marzorati et al., 2008). In complex habitats, the relative abundance of the nucleotides guanine and cytosine in a community is influenced mainly by external environmental factors that positively select a GC-rich community (Foerstner et al., 2005). Considering the observed increasing presence of Actinobacteria, characterized by high content of GC, we may suppose that among the various environmental stresses, also As pollution may cause a positive selection toward those strains having a genomic DNA rich in GC.

In this study, similar changes within fungal community have also been confirmed by the different trend of EL-FAME fungal markers; indeed, fungi population showed a shift towards 18:1ω9 (both cis and trans) fatty acids at increasing As concentration; in particular, 18:1ω9t is completely absent in L soils. In a similar study, Krishnamoorthy et al., (2015) reported abundance of uncultured Glomeromycota and Gigaspora rosea in a highly heavy metal contaminated former mining site while these ribotypes were completely absent in moderately and low polluted soils in the same area. From the above evidences, and from the cited literature, it arised that fungal community seems to be more sensitive to increasing As pollution which provoked a selection towards more tolerant species, thus modifying genetic diversity patterns. These results suggest that fungal biomass may act as sensitive and reliable bioindicator of environmental contamination (Rajapaksha et al., 2004).

Marabottini et al. (2013) reported that, although the total concentration of As was extremely elevated in H soils, the most important effects on soil microbial biomass activity were found in M soils where a higher content of bioavailable As was found. Enzyme activities and MicroResp data pointed to an overall decrease of microbial functions in M soils confirming a strict relationship between As bioavailable forms and microbial functional diversity as also reported by Das et al. (2013), Bhattacharyya et al., (2008) and Ghosh et al. (2004) (Figures 4a and 4b). Elevated heavy-metal concentrations may cause soil enzymes to decrease by direct inhibition of the catalytic activity (interaction of heavy metals and the substrate, the enzyme–substrate complex) or an indirect action decreasing or suppressing microbial metabolism and thus enzyme synthesis. Hydrolysing enzymes were reported to be inversely correlated to As labile forms (water soluble and exchangeable) (Bhattacharayya et al., 2008) as ions, in particular, can

inactivate enzymes by reacting with sulfhydryl groups or interacting with the enzyme-substrate complex (Nannipieri et al., 2012). The lack of effects of As on acid phosphatase activity have also been reported by Lyubun et al. (2013) hypothesizing similarities in the chemical behaviour of arsenate and phosphate ions in soil, therefore preventing this enzyme to be a reliable indicator in As polluted soils.

In this study, enzyme activities were negatively affected by As pollution, namely in M soils. In particular, it is worthwhile to note that arylsulphatase and chitinase, decreased by 45% and 48% in M and H soils respectively. Arylsulphatase catalyses the hydrolysis of sulphate aromatic esters which are constituents of fungal cell walls while chitinase is one of the three enzymes involved in the degradation of chitin a major structural component of fungi; for this reason both enzymes are generally assumed to be indirect indicators of soil fungal biomass (Moscatelli et al., 2005). The reduction of both enzymes in presence of bioavailable As can thus be ascribed to altered metabolic patterns and/or to fungal sensitivity to the metalloid.

Systematic changes in substrate utilization pattern associated with different heavy metal contents have been found in reclaimed mining soils (Liao and Xie, 2007).

MicroResp™ allows assessment of change in catabolic diversity in microbial community. MicroResp™ has been recently used in a PICT-bioassay (pollution-induced community tolerance) to assess the effect of Cadmium (Cd) and Nickel (Ni) contamination on soil microbial biomass (Bérard et al., 2014). Tlili et al. (2011) reported the reliable use of this technique to assess PICT to metals in acquatic ecosystems. A decrease of microbial metabolic diversity, measured by means of another CLPP approach, such as Biolog, was found by Xiong et al. (2010)

under arsenic contamination. However, to our knowledge, this is the first paper using MicroResp technique in an arsenic contaminated soil.

In our study, in M and H soils, a decrease of C substrates utilization patterns was found: in particular, more recalcitrant substrates decomposition seemed to be less affected by As pollution than easily degradable ones. Similar results were observed by Kenarova et al. (2014), in uranium mining impacted sites, suggesting a shift in the functional capacities of soil microbial biomass which caused a decline of broad spectrum C degraders in favour of specialized groups able to cope with complex polymers.

Shannon diversity index calculated using both enzyme activities and MicroResp data was always lower in M soils according to microbial diversity calculated by means of EL-FAME; a change in microbial diversity which could suggest a different level of tolerance to As was also confirmed by the increase of the indexes cy/pre and iso/anteiso (calculated with EL-FAME) in M soils.

The multivariate analysis proved to be a useful tool to integrate the results obtained from this multi-technique approach; it clearly discriminated the three sites with respect to Arsenic contamination level. The responses of microbial biomass in terms of genetic, structural and functional diversity, independently on the technique used, were coherent at pointing that the different level of contamination in the three sites was an important driver of microbial diversity.

5. Conclusions

Different aspects of microbial diversity have been investigated using a multiple biological assay to evaluate effects induced by increasing As concentration. A chronically contaminated site has been chosen to study the effect of long-term arsenic exposure on activities and genomic structure

of fungal and bacterial community. Bioavailable As influenced the functionality of the microbiome of the polluted site, mostly affecting physiologically active microorganisms. Conversely, total arsenic had major effect on the genomic structure of microbial community. In general, fungi and actinobacteria seemed to be more sensitive than bacteria to total arsenic concentration; this induced us to hypothesize different adaptation mechanisms to cope with heavy metal stress. Further investigations are needed to clarify which different adaptation strategies have been possibly evolved in fungi under arsenic disturbance.

Acknowledgments

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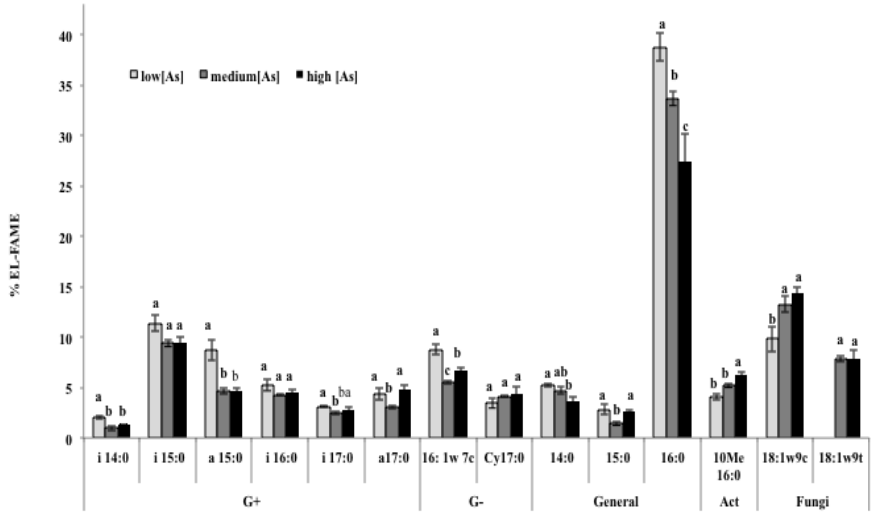
Table 1: Physico-chemical properties of Pestarena soils. As labile forms (water soluble and not specifically adsorbed) are expressed as percentage of total soil As. CEC= cation exchange capacity. Different letters within a column indicate significant difference, ($P<0.05$, Tukey test)(All data were reported by Marabottini et al., 2013.

Pestarena soils	Soil texture	Granulometric fractions	Ph H_2O	pH KCl	Organic C	C.E.C.	Total As	Water-soluble As	Non-specifically adsorbed As
		(%)			(%)	(cmol/kg)	(mg kg^{-1})	(% of total)	
Low As	Sandy loam	Sand 77 Silt 16 Clay 7	6.2 ± 0.3^{a}	5.5 ± 0.4^{a}	4.5 ± 1.0^{a}	20.6 ± 0.46^{a}	275.6 ± 9.7^{c}	1.84 ± 0.15^{b}	4.03 ± 0.14^{c}
Medium As	Sandy loam	Sand 80 Silt 10 Clay 10	5.5 ± 0.3^{a}	4.3 ± 0.4^{a}	3.0 ± 0.5^{a}	15.6 ± 3.25^{b}	1161 ± 230^{b}	4.82 ± 0.14^{a}	6.34 ± 0.41^{a}
High As	Sandy loam	Sand 79 Silt 11 Clay 10	5.3 ± 0.1^{b}	4.1 ± 0.1^{b}	1.9 ± 0.1^{b}	25.1 ± 2.3^{a}	4357 ± 269^{a}	0.88 ± 0.06^{c}	2.39 ± 0.08^{b}

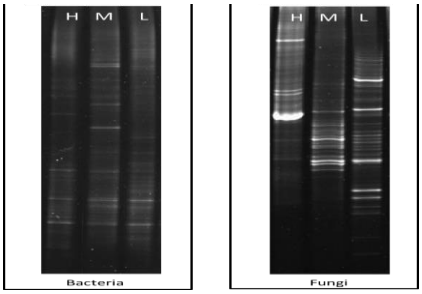
Figure 1: Study area. Pestarena abandoned mining plant and soil sampling areas: H (high As contamination), M (medium As contamination), L (low As contamination).

a)



b)	low[As]	medium[As]	high [As]
Sum G(+)	35±3.30[a]	25±1.34[b]	27±2.04[ab]
Sum G(-)	12±1.07[a]	10±0.22[a]	11±1.01[a]
cy/pre	0.39±0.02[b]	0.75±0.07[a]	0.65±0.07[a]
iso/anteiso	1.11±0.07[c]	1.53±0.09[a]	1.29±0.07[b]

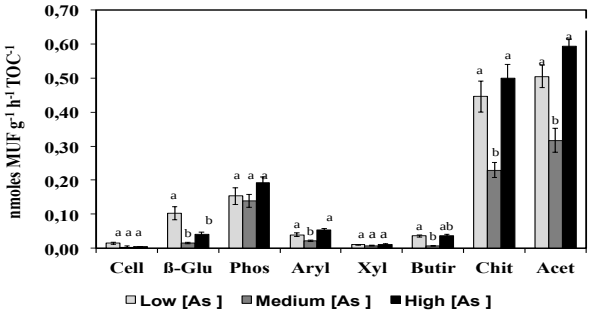
Figure 2: **a)** Total EL-FAME in Low, Medium and High As soils. The concentration of each composite sample was determined by internal standard (19:0). **b)** Sum of EL-FAME related to Gram positive (G+) and Gram negative (G-) and stress indexes assessed by EL-FAME in Low, Medium and High As soils. Stress index unit is a ratio. Bars indicate standard errors. Different letters mean significant differences ($P<0.05$).



b)	Bacterial community(16S rRNA)					Fungal community18S rRNA			
	DGGE		q-PCR			DGGE		q-PCR	
	S	E	(copy/g)×10^9	Tm (°C)		S	E	(copy/g)×10^7	Tm (°C)
Low As	19±1[a]	0.92±0.02[a]	1.25±0.35[a]	87.7±0.7[a]		32±1[a]	0.85±0.03[a]	7.21±0.51[a]	84.6±0.1[b]
Medium As	18±2[a]	0.90±0.04[a]	0.75±0.24[b]	87.4±0.2[a]		26±1[b]	0.82±0.01[ab]	2.56±0.22[b]	85.8±0.2[a]
High As	17±1[a]	0.88±0.02[a]	1.09±0.36[a]	87.6±0.3[a]		27±1[b]	0.78±0.01[b]	5.60±0.43[a]	85.8±0.2[a]

Figure 3: **a)** DGGE profiles obtained for bacterial and fungal communities from high (H), medium (M) and low (L) arsenic contamination **b)** Genomic analysis of fungal and bacterial community by DGGE and q-PCR. S: richness, E: evenness, Tm: melting temperature. Different letters mean significant differences ($P<0.05$)

a)



b)

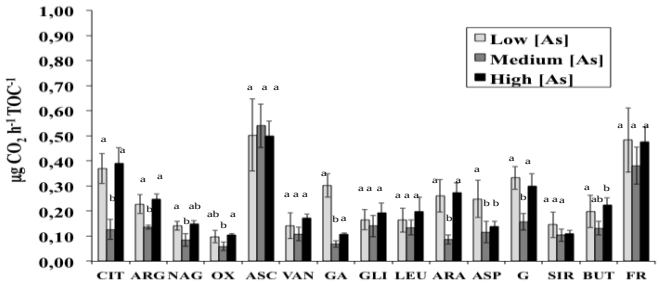


Figure 4: **a)** Enzymes specific activities measured in Low, Medium and High As soils. Cell: cellulose, □-gluc: □-glucosidase, Phos: Ac. Phosphatase, Aryl: Arylsulphatase, Xyl: Xylosidase, Butir: Butirate esterase, Chit: Chitinase, Acet: Acetate esterase. **b)** CLPP (MicroResp) measured in Low, Medium and High As soils. Cit: citric acid, Arg: L-arginine, Nag: N-acetyl-glucosamine, Ox: oxalic acid, Asc: L-ascorbic acid, Van: vanillic acid, Ga: D-galactose, Gli: glycine, Leu: L-leucine, Ara: L-arabinose, Asp: L-aspartic acid, G: D-glucose, Sir: syringic acid, But: γ-amino-butyric acid and Fr: D-fructose. Bars represent standard errors (n=5). Different letters, within each enzyme/substrate, mean significant differences ($P<0.05$).

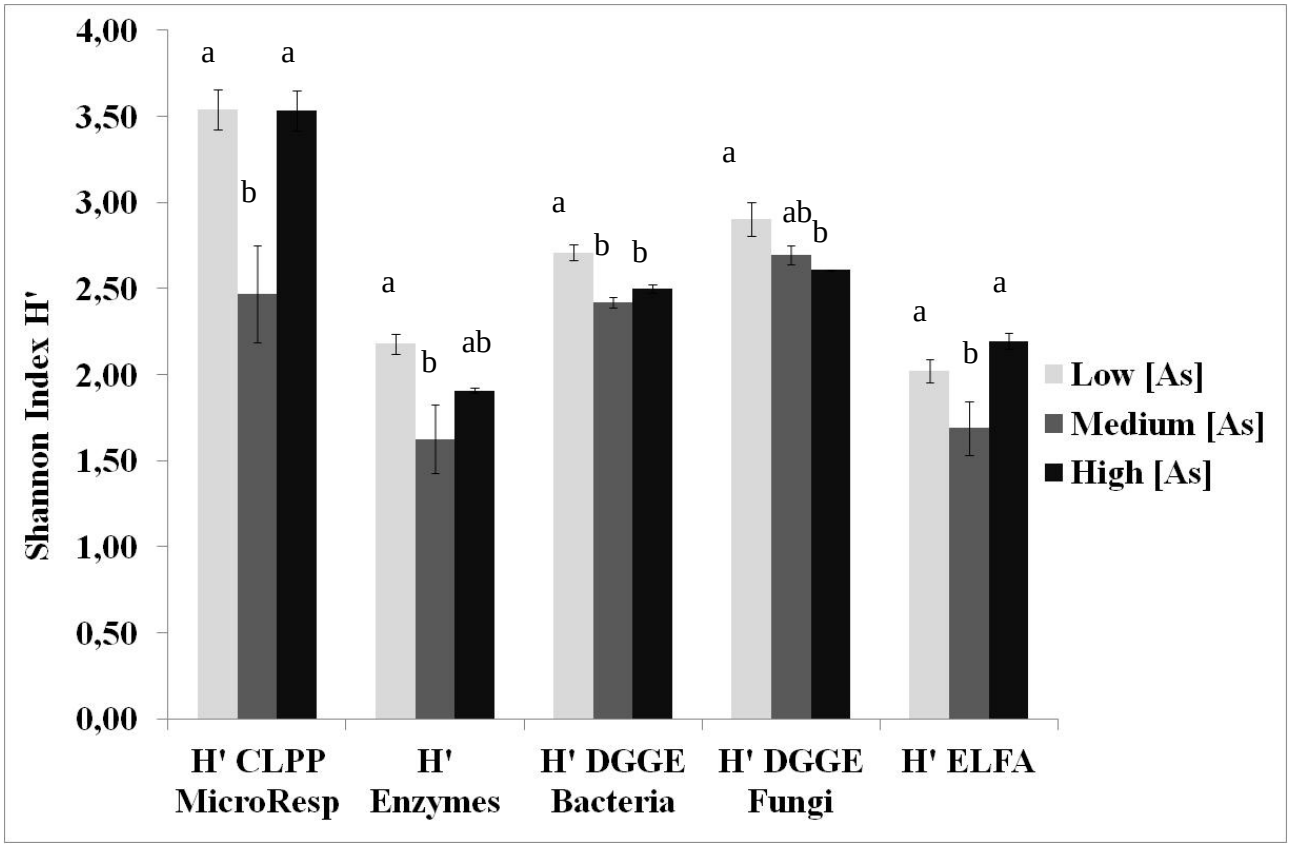


Figure 5: Shannon diversity index (H') calculated using CLPP-MicroResp, enzyme activities, qPCR/DGGE and EL-FAME. Bars represent standard errors (n=5). Different letters mean significant differences ($P<0.05$)

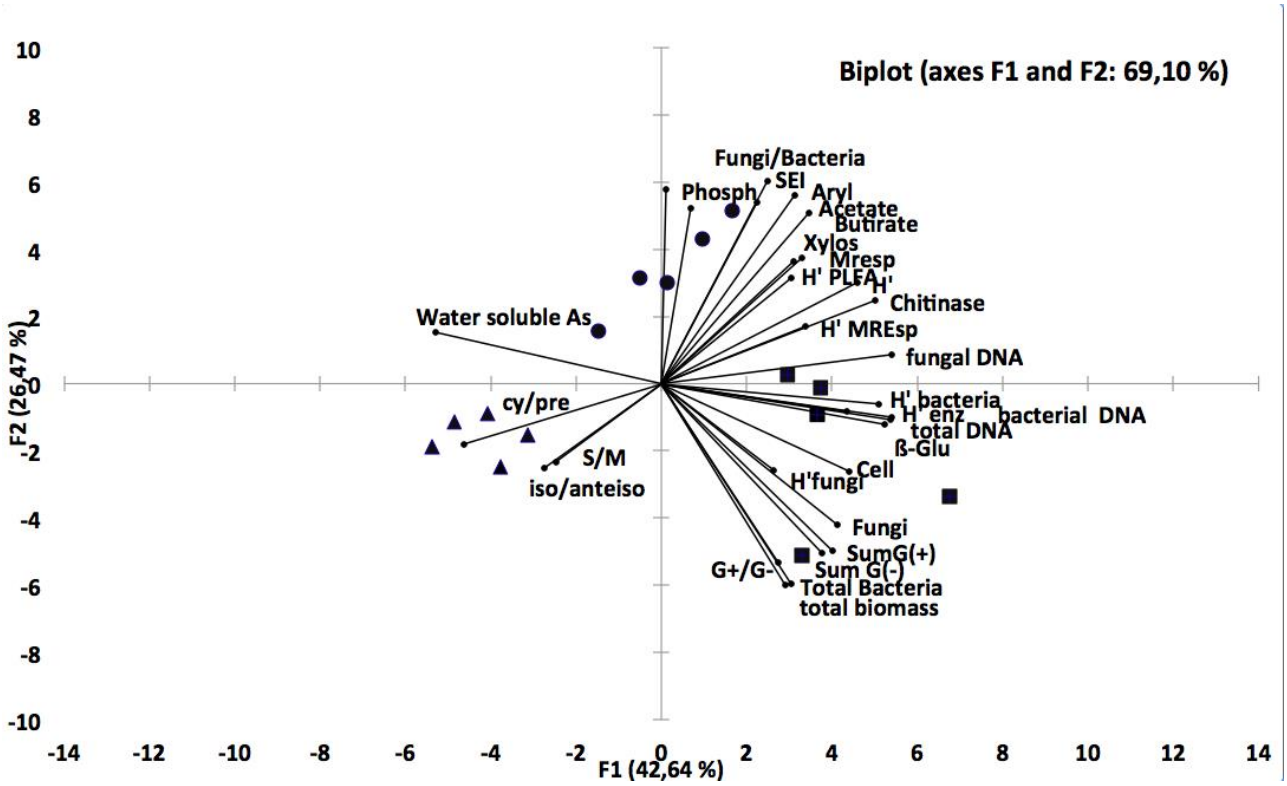


Figure 6: PCA biplot displaying all variables and soil samples along the two main components. Triangles: Medium As, Circles: High As, Squares: Low As.