Rhizobacterial communities associated with spontaneous plant species in long-term arsenic contaminated soils

Lucia Cavalca, Anna Corsini, Enrica Canzi, Raffaella Zanchi*

Dipartimento Scienze per gli Alimenti, la Nutrizione e l'Ambiente, Università degli Studi di Milano, via Celoria 2, Milano-20133, Italy

*corresponding author: Raffaella Zanchi

e-mail: raffaella.zanchi@unimi.it · tel. +30 02 503 19124 · fax +39 02 503 19238

Abstract

The microbial community composition in three soil fractions (bulk soil, rhizosphere and rhizoplane) of the root-soil system of a thistle, *Cirsium arvense*, and of a tufted hair grass, *Deschampsia caespitosa*, was investigated. The two spontaneous wild plant species were predominant in two Italian lands contaminated since centuries by arsenic and at present show high levels of arsenic (from 215 to 12.500 mg kg^{-1}). In order to better understand how the rhizobacterial ecosystem responds to a long-term arsenic contamination in term of composition and functioning, culture-independent techniques (DAPI counts, fluorescence *in situ* hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) analysis) along with cultivation-based methods were applied.

Microbial community structure was qualitatively similar in the two root-soil systems, but some quantitative differences were observed. Bacteria of the *α*-, *β*-, and *γ*-subclasses of the *Proteobacteria* were dominant in all fractions, while the subdominant groups (Cytophagaceae, gram-positive spore-forming, and filamentous bacteria) were significantly more abundant in the root-soil system of *D. caespitosa*. As regards to arsenic resistant strains, *Firmicutes*, *Actinobacteria*, *Enterobacteria* and *γ-Proteobacteria* were isolated from soil system of both plants. Our results suggest that the response to a high level of arsenic contamination governed the rhizosphere microbial community structure together with the soil structure and the plant host type effects. Data from this study can provide better understanding of complex bacterial communities in metal-polluted soils, as well as useful information of indigenous bacterial strains with potential application to soil remediation.

Keywords

Arsenic; Bacteria**;** Soil; Rhizosphere; Fluorescence in situ hybridization; *Cirsium arvense*; *Deschampsia caespitosa*

Introduction

The occurrence and accumulation of arsenic (As) in the environment, consequent to natural processes and anthropogenic activities, constitutes a diffuse environmental hazard all over the world. Arsenic has been classified in 2001 by the World Health Organization as one of the main problems of public health, due to its characteristics of toxicity and carcinogenicity (WHO 2001).

In the environment, As is present mainly as inorganic forms [arsenate, As(V) and arsenite, As(III)]. As(V) is found primarily in aerobic conditions mainly bounded to minerals in the solid phase and thus is less available by plants. On the contrary, As(III) is most common in the aqueous phase in soils and can be taken up by plants under most environmental conditions.

Plants grown in arsenic-contaminated soils harbour in their rhizospheres unique As-resistant microflora that, through oxidation-reduction and methylation reactions, regulates the immobilization and solubilisation of As in soils (Páez-Espino et al. 2009). Bacteria strongly influence As environmental cycling through changes in pH, redox potential, solubilization of nutrients and minerals, and production of plant growth-promoting (PGP) compounds, thus alleviating metal toxicity or enhancing metal uptake by plants (Kamaludeen and Ramasamy 2008).

In the recent years, most published works on As effects on soil microbiology focused on impact of the microbial metabolisms on the As cycling in soils and plant rhizopshere (Pepi et al. 2007, Corsini et al. 2010, Cavalca et al. 2013) and on screening and isolation of PGP bacteria from rhizopshere of plants grown in As contaminated soils (Cavalca et al. 2010, Wevar Oller et al. 2013, Das et al. 2014). At present, the response in term of composition and functioning of the rhizobacterial communities to As contamination is a less active field of research in soil microbiology (Xiong et al. 2010). As one step towards a better understanding the effects of As long-term contamination on rhizobacterial communities, we examined the root-soil system of two spontaneous plants, a thistle, *Cirsium arvense* (L.) Scopoli and a tufted hair grass, *Deschampsia caespitosa* (L.) Beauv. These grasses were the predominant plant species in the two Italian sites considered in this study and contaminated since centuries by As. *C. arvense* fulfil the criteria of phytoremediation crop (Dhillon and Dhillon 2009): is a sub-cosmopolite, sinanthropus, perennial species that usually grows on river and channel banks, in uncultivated grassland, in landfills and it can become a weed in cultivated fields. *D. caespitosa* is a metal-tolerant graminaceous plant (Cox and Hutchinson, 1980) widely distributed in grasslands and woods over the temperate regions of the world. In the present work, dominant bacterial populations in bulk and rhizospheric soils and in rhizoplane were analysed by culture-dependent and culture-independent techniques.

Materials and methods

Sampling site and plant collection

The research was carried out on rhizospheric soil-systems of two spontaneous plant species collected in springtime from two different sites: Scarlino site (Tuscany, Italy), an intensive agricultural soil used for barley cropping at the time of sampling (May 2009); and Pestarena site (Piedmont, Italy) a forest soil with natural plant succession at the time of sampling (May 2010). Soils characteristics are reported in Table 1 (Cozzolino et al. 2010; Marabottini et al., 2013). ~~As~~ concentration at the two sites was above the law limit of 50 mg kg^{-1} (DGL 152, 03.04.2006, n. 152, S.O. n. 96 Gazzetta Ufficiale 14 aprile 2006, n. 88) and the bioavailable As fractions represented 11.3 and 0.8% of total As at Scarlino and Pestarena sites, respectively. The causes of such a pollution are multiple: natural presence of high concentrations of the metalloid in rock substrates mixed to agricultural soil during centuries at Scarlino, and mining activity of iron and gold extraction being both elements associated to arsenopyrite at Pestarena.

C. arvense was the dominant spontaneous species at Scarlino site, while *D. caespitosa* was dominant at Pestarena site. Only those plants that were surrounded by plants of the same species ~~have been~~ sampled. Thus, soil was highly impacted by the roots of the respective plant species. Nine individual plants of each species, chosen randomly at locations across the polluted areas, were collected using shovel and hand trowels. The plants ~~have been~~ excavated in points approximately 100 m apart. The uppermost 20 cm of the plant root system with undisturbed soil around the roots ~~were~~ manually removed, placed into plastic bags to avoid moisture loss, and transported in ice box to the laboratory on the same day. Recovery of soil fractions from roots was performed the following day.

Separation of the rhizospheric soil fractions

From the root system of *C. arvense* and *D. caespitosa* three fractions were separated: bulk and rhizospheric soil~~s~~ and rhizoplane. Bulk soil was obtained by carefully hand shaking the roots to remove not adhering soil. The bulk soils collected from 9 plants were pooled together, homogeneously mixed and then sieved (0.2 mm mesh width). Rhizospheric soil, defined as the root loosely bound soil, was removed by washing the root surface. Roots, separated from the bulk soil fraction, were grouped in three sets of about 10 g (ww), then washed (1/10, w/v) in 90 ml of tetrasodium pyrophosphate (PP) (Sigma-Aldrich Co., St. Louis, USA) 0.2 % (w/v), pH 7.0, and stirred (180 *rev* min^{-1}) for 1 h at 4°C. After removal of the roots, the PP suspensions were centrifuged (10 000 *g*, 10 min, 4°C), and the resulting pellets were considered as the rhizosphere fraction. The PP-washed roots were then suspended in 45 ml of phosphate-buffered saline (PBS, 3 mmol l^{-1} NaH_2PO_4, 7

100 mmol l^{-1} $NaHPO_4$, 130 mmol l^{-1} NaCl, pH 7.2), and sonicated with Ultrasonic Processor UP100H (100W,
101 30kHz) (Hielscher Ultrasonics GmbH, Teltow, Germany) for 30 s at 50% speed for three times. The remaining
102 roots were then removed and suspensions were centrifuged (10 000 *g*, 10 min, 4°C), thus yielding pellets which
103 were considered the rhizoplane fraction. Dry weights of the different fractions were calculated based on the
104 moisture content (at 105 °C until constant weight).
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106 Microscopic analysis
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108 The characterisation of the bacterial community of bulk and rhizospheric soils and of rhizoplane was performed
109 by Fluorescence *in situ* hybridization (FISH) analysis (Bertaux et al. 2007) using 16S or 23S rRNA-targeted
110 oligonucleotide probes, labelled with fluorochrome Cy3 (red signal) to the 5'extremity. Probe details and
111 references are given in Table 2. For total counts of active eubacteria a mix of three EUB probes in equimolar
112 mixture was used to detect most members of the domain Bacteria, including Planctomycetales and
113 Verrucomicrobiales; NONEUB338 was used to determine the nonspecific binding. Aliquots of the three soil-
114 system fractions were separately fixed: six replicates (about 0.2 g wet weight) of each fraction were added to 2
115 ml of 3% (w/v) particle free paraformaldehyde in PBS, mixed up completely, and then stored at 4°C for 3 h in
116 horizontal position. The fixed samples were washed twice with PBS, and stored in PBS/ethanol (1/1, w/w) at
117 −20°C until further processing. In order to detect more accurately Gram positive-cells, six replicates from each
118 sample were fixed directly in 2 ml of 50% (v/v) ethanol-PBS and stored at -20°C. Defrosted samples were
119 washed twice with PBS; each pellet was then added to 2 ml of Na_2EDTA 5 mmol l^{-1} and 7 mg of
120 polyvinylpolypyrrolidone (Sigma), and shaked at 300 *rev* min^{-1} for 1 h. After settlement for 5 min to remove
121 large particles, 1 ml of supernatant was transferred on the top of 1 ml of Nycodenz (density 1.3 g ml^{-1}, Gentaur,
122 SanJose, CA, USA). The Nycodenz-cell suspension gradient was centrifuged at 16 400 *g* for 30 min at 18°C,
123 and the upper 1800 µl of the gradient were collected for the analysis. Two replicate aliquots of each cell
124 suspension were opportunely diluted with PBS, and bacteria were collected by filtration onto 0.2 µm pore-size
125 black polycarbonate filters (diameter, 25 mm; Millipore) mounted in a glass holder (3 cm^2 filtration area;
126 Millipore) applying a vacuum of 30 kPa. The filters were rinsed with 3 ml of filter PBS, air dried, dehydrated
127 by dipping them in 50, 80 and 96% aqueous ethanol subsequently, air dried, and stored in dark at room
128 temperature until further processing. For the *in situ* hybridization 4 small sections of filters were cut out of the
129 whole polycarbonate filters. Each filter section was aligned on silicon coated slides, covered with 96 µl of

hybridization buffer (900 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris-HCl pH 8.0, 0.01% SDS, set to different formamide concentration according to different probes, see Table 2) and 4 ml of the labelled probe (50 ng μl^{-1}) (MWG-Biotech, Ebersberg, Germany). Each slide was placed in a 50ml plastic tube, which was humidified with the surplus of hybridization buffer, as a moisture chamber. Hybridization was performed at 46°C for 5 h. After hybridization each filters was transferred into 2ml of warmed washing buffer (20 mmol l^{-1} Tris-HCl pH 8.0, 5 mmol l^{-1} EDTA, 0.01 % SDS, pH 8.0, with different NaCl concentration to achieve appropriate washing stringency, see Table 2) for 10 min at 48 °C and was then washed into 2 ml refrigerated Milli-Q water for few seconds. Finally, filters were dried at 65°C for 2 min, and stored at -20°C until fluorescence microscopy counting was performed. The filters were mounted on a microscope slide with anti-fading oil (Citifluor Ltd, London, United Kingdom) to prevent a fast bleaching of probe signals, and examined at epifluorescence microscope Axioskope (Zeiss, Oberkochen, Germany) equipped with a 50-W type HBO high-pressure mercury lamp (Osram, Munich, Germany) and the Zeiss 15 filter set. An eyepiece with a calibrated reticule was used for bacterial counting. At least 30 randomly selected microscopic fields with 10 x 10 square units of the reticule, with objectives 100× (Plan-Neofluar, Zeiss, Oberkochen, Germany) were inspected for each filter. Three replicates were used, and at least 300 cells were counted for each sample. When the probes have a very low cell densities, a total of 60 microscopic fields were inspected, which corresponded to 0.5% of the sample filter. Total microbial counts in the three fractions were also estimated by DAPI (4,6-diamidine-2-phenylindole) staining (Kepner and Pratt, 1994), starting from surnatants after Nycodenz gradient centrifugation as above described. Two replicate aliquots of cell suspensions were opportunely diluted with PBS, mixed with the stain at a final concentration of 5 µg ml^{-1} for 15 min at room temperature in the dark. Bacteria were collected by filtration through a 0.2 µm pore-size black polycarbonate filter (diameter 25 mm; Millipore). The filters, air dried, were mounted in citifluor on a microscope slide and counts were determined with the fluorescence microscope (Zeiss 01 filter set).

Denaturing Gradient Gel Electrophoresis (DGGE) analysis

Total DNA was extracted from the bulk and rhizospheric soils in duplicate samples by using Power Soil DNA extraction kit (MO BIO Laboratories, Carlsbad, CA, USA) and subjected to DGGE analysis. Rhizoplane fraction was not analysed due to technical constraints in the DNA recovery. Primer pair V3-GC clamped forward and V3 reverse (Muyzer et al. 1993) was used for PCR amplification of hyper-variable V3 regions of

bacterial 16S rRNA gene. PCR reactions were performed in a final volume of 50 µl containing the following: 10 ng of DNA, 1.5 U of Taq polymerase, 0.3 µmol l^{-1} of each primer, 0.2 mmol l^{-1} of dNTPs, 1.75 mmol l^{-1} $MgCl_2$, and 1X PCR buffer. The samples were first denatured for 3 min at 94°C and then subjected to 19 cycles consisting of 1 min 20 s at 94°C, 1 min 30 s at 65°C, and 1 min 10 s at 72°C; the annealing temperature was reduced by 1°C every second cycle until touchdown at 55°C, at which temperature 7 additional cycles were carried out. The last step included an extension of 5 min at 72°C. V3-GC PCR amplicons were then loaded onto polyacrylamide gels (8%) in a D-Code Universal Mutation Detection System apparatus (Bio-Rad, Hemel Hempsted, UK). The linear denaturing gradient of urea and formamide ranged from 40% (top) to 60% (bottom) where 100% denaturant gels contained 7 M urea and 40% formamide. Electrophoresis was performed on samples (10 µl) at a constant voltage of 70 V for 16 h in ~~Tris acetate EDTA (TAE) 1x~~ (4.84 g l^{-1} of Tris base, 1.14 ml l^{-1} of glacial acetic acid, 2 ml l^{-1} of 0.5 mol l^{-1} EDTA solution of pH 8.0) running buffer at 60°C. After completion of electrophoresis, gels were stained in SYBR Green 1 solution according to manufacturer's instructions (Molecular probes, Eugene, Oregon USA) and documented with the GelDoc System (Bio-Rad). DGGE bands subjected to sequence analysis were excised with a sterile scalpel, suspended in sterile Milli-Q water, and used as template for V3 amplification by primers V3 forward without GC clamp and V3 reverse. All reagents were from Invitrogen (Foster City, CA, USA).

Culturable bacteria counts

Total, As(V)- and As(III)-tolerant aerobic heterotrophic bacteria were determined for bulk and rhizospheric soils. Three replicates of bulk (3 g each) and rhizospheric (0.2 g each) soils were suspended (1/10; w/v) in 0.2% (w/v) PP solution and shaken at 180 *rev* min^{-1} for 1 h. Then, bacterial suspensions were serially 10-fold diluted in saline solution (0.9% NaCl). Total heterotrophic bacteria were determined by plating 1 ml aliquots of the various dilutions, onto double sets of pour plates containing R2A medium (BD Difco, Franklin Lakes, New Jersey, USA). Heterotrophic As-tolerant bacteria were determined by plating 1 ml of the various dilutions onto R2A medium supplemented with 15 mmol l^{-1} of As(V), or 3 mmol l^{-1} of As(III). As(V) and As(III) solutions were prepared from $Na_2HAsO_4 \cdot 7H_2O$ and $NaAsO_2$ salts (Sigma) respectively. Cycloheximide (0.1 g l^{-1}) was added to the media to inhibit fungal growth. Colony forming units (c.f.u.) were counted after incubation at 28 °C for 10 days. The As resistance of the heterotrophs was expressed as percentage growth on R2A without the addition of As.

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191 Isolation and characterization of As-resistant bacteria

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193 Colonies with different morphologies were isolated from plates of As(V)-tolerant bacteria of the bulk soil
194 fractions of *C. arvense* and of *D. caespitosa*. Single colonies were streaked to purity on R2A medium amended
195 with 15 mmol l^{-1} As(V).
196 Resistance levels to As(V) ($Na_2HAsO_4 \cdot 7H_2O$, from 0 to 100 mmol l^{-1}) or As(III) ($NaAsO_2$, from 0 to 50 mmol
197 l^{-1}) were determined by growing the isolates in 20 ml liquid Tris Mineral Medium (TMM) with low phosphate
198 content (Mergeay et al. 1985), supplemented with 0.6% (w/v) gluconate (TMMG) and with increasing
199 concentrations of either As(V) or As(III). Growth was checked after 72 h of incubation at 30°C kept shaken at
200 180 *rev* min^{-1}. Prior to use, the strains were grown to mid-exponential phase in liquid TMMG, at 30°C and kept
201 shaken at 180 *rev* min^{-1}.
202 The ability to oxidize As(III) or to reduce As(V) was tested by inoculating As resistant strains in triplicate vials
203 containing 20 ml of TMMG separately supplemented with 3 mmol l^{-1} As(V) or 1 mmol l^{-1} As(III) each. Three
204 vials without As were inoculated in order to point out possible As toxic effects. Three vials were also prepared
205 without inoculum as controls. At each sampling time, 2 ml of cell suspensions were removed. Cell growth,
206 As(V) and As(III) concentrations were determined spectrophotometrically at OD_{620nm} and OD_{865nm} (Dhar et al.
207 2004), respectively. Arsenic standards were prepared for concentrations ranging from 0–1 mmol l^{-1} for both
208 As(V) and As(III) from $Na_2HAsO_4 \cdot 7H_2O$ and $NaAsO_2$ solutions respectively.
209 Strains were identified by sequence analysis of PCR-amplified 16S rRNA gene, after total DNA extraction
210 conducted by Ultraclean Microbial DNA extraction kit (MO BIO Laboratories). Strains were maintained in
211 glycerol stocks at -70°C.

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213 Arsenic resistance gene amplification

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215 DNA extracted from pure strains were used as template for PCR amplification of As resistance genes for As(V)
216 reductase (*ArsC*), As(III) oxidase (*AioA*) and for different As(III) efflux pumps (*ArsB*, *ACR3(1)* and *ACR3(2)*).
217 Primers P52f and P323r were used to amplify *ArsC* according to Bachate et al. (2009). Primers aoxBM1-2F
218 and aoxBM3-2R were used to amplify *AioA* according to Quéméneur et al. (2008). Primers darsB1F/darsB1R,

dacr5F/dacr4R and dacr1F/dacr4R were used to amplify *ArsB*, *ACR3(1)*, and *ACR3(2)* according to Achour et al. (2007).

Sequence analysis

~~16S rRNA~~, As resistance genes and DGGE DNA bands were sequenced using the Taq Dye-Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) with the respective primers. The forward- and the reverse samples were run on a 310A sequence analyzer (Applied Biosystems, Grand Island, NY). Sequences were compared with the entire GenBank/EMBL nucleotide and amino acid databases using the BlastN and BlastX query programs (http://www.ebi.ac.uk/Tools/blastall/index.html). Nucleotide sequences obtained in the present study are present in GenBank under the accession numbers:

Statistics

Data of bacterial counts, after their logarithmic transformation, are expressed as mean ± SE, unless otherwise indicated. To compare mean values between the two different plant species in the three soil-system fractions, a one-way ANOVA was used. Differences between samples were assessed by Duncan test. Statistical analysis were performed using *STATISTICA* software Package for Windows (verion 10.0, StatSoft Inc., Tulsa, OK, USA).

Results

Bacterial community in the root-soil system

DAPI staining and FISH analysis (Table 3) evidenced some similar characteristics among rhizobacterial communities of the tested plant species. Particularly, in all the three fractions of root-soil system, total bacterial counts determined by DAPI stain were about 1 order of magnitude higher than those obtained by FISH technique, as FISH shows only metabolically active microorganisms. Total bacterial counts both with DAPI or

FISH technique, were about 1 order of magnitude higher in the rhizospheric soil than in the bulk soil, confirming the rhizopshere as an environment of high microbial activity. In bulk fraction of both plant species, total bacterial counts determined with the combined EUB probes were about 1 order of magnitude higher compared to those of culturable heterotrophic bacteria (Table 4). Similar results were obtained in the rhizosphere of *C. arvense*, on the contrary this difference was less evident in the rhizosphere of *D. caespitosa*, whose bacterial counts determined by FISH were similar to the culturable ones (8.65 *vs* 8.45 $\log_{10}$ c.f.u. g dw^{-1} respectively).

FISH analysis evidenced a qualitative profile community, at the level of main phylogenetic studied groups, similar in all the three fractions of both plant species; in particular, Gram-negatives *α*-, *β*-, and *γ*-*Proteobacteria* were dominant, while the counts of Cytophaga-Flavobacterium cluster, Gram-positive filamentous and spore-forming bacteria, were 1-2 orders of magnitude less abundant. These sub-dominant groups accounted individually for 0.1-2 % of total cells detected by the domain-specific EUB338 probe mix. It is to be pointed out that the lowest values are to be considered approximate because around the detection limit of the method.

Despite the similarity in general microbial picture, significant differences in the composition of the bacterial community of the two studied sites were evidenced. Regarding the bulk fraction, the number of bacteria estimated by EUB338 probe mix, was significant higher in *D. caespitosa* than in *C. arvense* samples. Similar results were observed also for *α-Proteobacteria*, *γ-Proteobacteria*, Cytophagaceae and Gram-positive LGC bacteria. The *β-Proteobacteria* group was more represented in the Scarlino bulk soil obtained from *C. arvense*. Regarding the rhizospheric soil, *D. caespitosa* samples presented significantly higher counts of Cytophagaceae and Gram-positive LGC and HGC bacteria, compared to *C. arvense*. Even more marked differences were evidenced by comparison of the rhizoplane fractions: total counts, determined with EUB338 probe mix, as well as the counts of the different phylogenetic groups were significant higher in *D. caespitosa* than in *C. arvense*.

The DGGE analysis evidenced different band patterns of the bacterial communities in the bulk soils, indicating that the reservoir of microbial biodiversity for the two plants was constituted by different bacterial species. Such differences were also present in the DGGE patterns (Fig. 1) of the bulk soil-associated vs. the rizosphere-associated bacterial communities, evidencing that the plant was the driving force in shaping the structure of the rhizobacterial populations. Band richness was higher in rhizosphere soil (30 bands) than in bulk soil (26 bands) of *C. arvense*, whereas the opposite was evidenced in *D. caespitosa* (33 and 16 bands, respectively, for bulk and rhizospheric soils). The composition of bacterial community in the rhizosphere of *C. arvense* and of *D. caespitosa* was assessed by sequence analysis of 16S-V3 DNA bands separated by DGGE (Table 5).

Bacteroidetes (*Flavobacterium* genus) were present in the bulk soils of both plants, whereas different *Pseudomonas* species were retrieved in the rhizopshere of *C*. *arvense* and *Cupriavidus necators* was dominant in the rhizosphere of *D*. *caespitosa*. Although other bands were visible, their sequence analysis did not resulted in any significant identification.

Culturable total, As(V)- and As(III)-tolerant bacteria

Culturable aerobic bacteria counts in bulk and rhizospheric soils of *C*. *arvense* and *D*. *caespitosa* are shown in Table 4. Total heterotrophic, As(V)- and As(III)-tolerant bacteria were generally 1 order of magnitude higher in rhizosphere of both the plants than in bulk soil in accordance with DAPI and FISH analysis, confirming a rhizosphere effect. On the contrary, bacterial counts of the bulk and of the rhizospheric soils were not statistically different between the two plants. A large fraction of the culturable bacteria were tolerant to As(V) in *C*. *arvense* and *D*. *caespitosa* respectively: 42% *vs* 66% in bulk soils, and 86% *vs* 51% in rhizosphere. Lower percentages of bacteria resistant to As(III), considered more toxic than As(V), were observed (10% *vs* 7% in bulk soils and 7% *vs* 4% in rhizosphere of *C*. *arvense* and *D*. *caespitosa*, respectively).

Isolation and characterization of aerobic As-resistant bacteria

Arsenic resistant bacteria were isolated from both sites (Table 6). *Firmicutes* species were isolated from the two soils, whereas *Pseudomonas* and *Buttiauxella* strains were peculiar of *C*. *arvense* and *D*. *caespitosa* respectively. All the isolates had moderate to high resistance levels to As. Particularly, isolates SI-1, SI-2, SI-3, PI-1 and PI-2 showed high As resistance (>300 mmol l^{-1} As(V) and >15 mmol l^{-1} As(III)),whereas the other isolates showed moderate resistance to As(V) (from 120 to 240 mmol l^{-1}) and to As(III) (from 4 to 15 mmol l^{-1}). All the isolates were able to reduce As(V) to As(III) (Table 6). Reduction of As(V) to As(III) was achieved via a detoxification mechanism, as isolates were not able to grow in anaerobic conditions in the presence of As(V) as final electron acceptor (data not shown). None of the isolates was able to oxidize As(III) to As(V). Accordingly, *aioA* gene for As(III) oxidase was absent in the strains, whereas As(V) resistance phenotype and reduction capability were confirmed by the positive amplification of *arsC*, *arsB* or *acr3(1)* genes. Only strain SII-1 lacked this correspondence, possibly due to primer sequence mismatch with strain sequences. None of the strain carried *acr3(2)* type As(III) efflux pump.

ArsB and *acr3(1)* genes of distinct types of As(III) efflux pump were simultaneously present in isolates SI-1 and SI-3, while in the other isolates only *arsB* gene was amplified. The phylogenetic analysis of deduced amino acid sequences (Fig. 2) clustered ACR3(1) of SI-1 and SI-3 with those of members of *Pseudomonas* genus: the fragments were highly homologous (98% identity) to an As(III) efflux pump found in *Pseudomonas* sp. (CAY64635). The phylogenetic analysis of deduced amino acid sequences of ArsB clearly clustered the isolates in two distinct groups: strains SI-2 and PI-1 grouped with members of Firmicutes, whereas strains SI-1,SII-2 and PI-3 grouped with Proteobacteria members of *Enterobacteraceae* family and *Pseudomonas* genus. Particularly, SI-2 and PI-1 possessed ArsB fragments from moderate to highly homologous to As(III) efflux pumps of *Bacillus* spp. (81-92% identical positions to CAK55207 and EWG11173 respectively). Isolates SI-1, SI-3 and SII-2 carried an ArsB-type highly homologous to the As(III) efflux pump retrieved in *Pseudomonas vranovensis* (WP_028945840), while in isolate PI-3 it was present an ArsB 92% homologous to As(III) efflux pump of *Buttiauxella aegrestis* ATTC 33320 (KFC81390).

Discussion

Microbial communities associated to the root-soil system of *C. arvense* and *D. caespitosa*, growing in soils with long history of high levels of As content, were investigated with the aim to better understand how a rhizobacterial community ecosystem responds to a long term As contamination. Culture independent techniques were used to analyze the structure of the total and of the metabolically active bacterial community present in the root-soil system of the plants, and cultivation-based methods were applied to characterize As-resistant bacteria.

Comparative analysis of the values of total bacterial counts, obtained with DAPI and FISH techniques and cultivation-based methods, evidenced that most root-system associated bacteria are viable but non culturable, and that the rhizosphere harbours the highest number of microorganisms compared to bulk soil and rhizoplane, irrespective of As-contamination level (142.3 mg kg^{-1} *vs* 31.1 mg kg^{-1} of bioavailable As in Pestarena and Scarlino sites respectively). The percentage of metabolically active microbial cells (total bacterial counts with combined EUB probes) varied depending on the plant species and on the considered soil fraction, ranging from 3 to 20% of the DAPI counts. In particular, higher percentages were observed in Pestarena site compared to Scarlino site both in bulk (10 *vs* 3% respectively) and in rhizoplane (20 *vs* 9% respectively) fractions. Thereby,

it seems that the bioavailable As content didn't adversely affect the size of metabolically active microbial community.

Total and As-tolerant bacterial counts were similar in the bulk soils of Scarlino and Pestarena, and in the rhizosphere of the two plants indicating that bioavailable As content and plant species did not exerted a significant effect within the two soil. Nevertheless, the higher bacterial counts retrieved in the rhizospheres than in bulk soils envisaged a rhizosphere positive effect for both plants.

FISH analysis showed a similar general picture at the level of main phylogenetic microbial groups in both soil-plant system community: *α*-, *β*-, and *γ*-*Proteobacteria* were the dominant groups, whereas *Cytophagaceae*, *Actinomycetales* and spore-forming bacteria were 1-2 order of magnitude lower. These results were in agreements with literature data, obtained by culture-independent methods, regarding the composition of the microbial rizospheric community from different plant species (Stein et al. 2005; Kielak et al. 2008). However, total percentages of recovery, by group-specific probes all together compared to mix EUB probes were very different, ranging from 33% to 98%. In particular, they resulted higher in bulk soil of Scarlino site (50 %) compared to Pestarena site (32%) and in rhizoplane fraction of *C*. *arvense* (96%) than *D*. *caespitosa* (53%). It could be hypothesized that the selective pressure by As pollution, together with the plant-driven microbial selection, increased the growth of microbial populations belonging to not investigated phylogenic groups.

Differences in the relative abundance of members of the phylum *Proteobacteria* were observed. In particular, the rhizoplane fraction of *C*. *arvense* and *D*. *caespitosa* contained higher percentage of *α*-*Proteobacteria* (13 – 11 % respectively) compared to bulk soil (2 - 4 % respectively). Our data indicated that Gram-negative bacteria were not only relatively more numerous but also active. Soil *Proteobacteria* appeared to be tolerant towards As, while other groups of bacteria (*Actinobacteria* and *Firmicutes*) appeared to be more sensitive by As (Lorenz et al. 2006). Active ~~A~~ctinomycetes were more abundant in the rhizosphere of *D*. *caespitosa* that grows in the more polluted site, than that of *C*. *arvense*. These findings were in accordance with Gremion et al. (2003) which found ~~*Actinomycetes*~~ in the metabolically active bacteria present in heavy metals contaminated soils.

DGGE analysis indicated intra and inter-samples related variations of bacterial community. Intra-samples variations between rhizosphere and bulk soil fractions could be explained by the effect exerted on soil physico-chemical characteristics by the root system, providing evidence of the plant-driven microbial selection. This plant-driven selection, which preferentially stimulated microbial populations that were presumably well adapted to the associated rhizosphere, in turn resulted in decreased microbial diversity in this zone (Smalla et al. 2001; Marschner et al. 2004). This may also indicate the effect of different root exudates which promote metal

bioavailability, thus exerting higher toxicity toward microorganisms in the rhizosphere (Gremion et al., 2004). The observed inter-sample variations confirmed that the type of plant metabolism exerted the major influence in shaping its associative bacterial community.

The number of As(V) and As(III) tolerant culturable bacteria represented more than 50% of the heterotrophic cultivable population. In line with these findings, As(V)-resistant strains were isolated from both sites. They belonged to *Firmicutes*, *Enterobacteria* and *γ-Proteobacteria*, showing that As resistance was a common trait shared by different classes of soil Bacteria. Among these, isolate PI-3 was closely related to a species belonged to *Buttiauxella* genus, which has never been described in terms of As resistance. The strains varied in their As resistance pattern. The phylogenetic analysis of the As(III) efflux pumps clearly separated ACR3(1) and arsB in two different clusters, confirming that they are two distinct groups of efflux pumps. The isolates showing presence of two different genes for As(III) efflux pump also showed the highest As(V) reduction activity, suggesting that both genes coding for As(III) efflux pump could be functional in these isolates. The presence of more than one type of As(III) efflux pump in highly As resistant strains has been previously observed, suggesting that presence of more than one set of *ars* genes is common in the bacteria thriving in environments containing high As levels (Achour et al. 2007; Bachate et al. 2009; Cai et al. 2009). *Ochrobactrum tritici* SCII24 strain having two *ars* operon, one with *arsB* gene and other with *ACR3* gene has been reported (Branco et al. 2008). This strain was resistant up to 200 mmol l^{-1} of As(V) and up to 50 mmol l^{-1} of As(III). Achour et al. (2007) reported a *Pseudomonas* sp. which has two genes coding for As(III) efflux pump, one *arsB* and other *ACR3(2)*.

The isolates were able to reduce As(V) to As(III) at different extent, whereas none of them was able to oxidise As(III) to As(V) nor presented *aioA* gene for As(III) oxidase. Reduction of As(V) to As(III) was achieved via a detoxification mechanism, as isolates did not appeared to gain energy from the reaction. *Bacillus firums* strain SI-2 showed highest As(V) resistance. In a similar study Pepi et al. (2007) isolated four *Bacillus* species which showed similar resistance level to AS(III) and As(V). The levels of As resistance were comparable with some of the highly As resistant bacterial strains, i.e. *Corynebacterium glutamicum* which shows resistance to 12 mmol l^{-1} of As(III) and up to 400 mmol l^{-1} of As(V). This strain has been used to remove As from contaminated water (Mateos et al. 2006). Taking into account the resistance level of our isolates and the identification of functional biomarkers for As resistance, they can be considered for possible bioremediation strategies of As contaminated environments. Here they could cope up with high As level and could also be monitored by means of molecular probes.

Our results suggested that the response to a high level of As contamination governed the rhizosphere microbial community structure together with the soil structure and the plant host type effects. Data from this study can provide better understanding of complex bacterial communities in metal-polluted soils, as well as useful information of indigenous populations with potential application to soil remediation.

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517

518 **Figure legends**

519 **Fig. 1** DGGE profiles of 16S rRNA gene fragments of the bacterial community in bulk and rhizosphere soil
520 fractions of *C*. *arvense* (Scarlino site) and of *D*. *caespitosa* (Pestarena site) in duplicate samples. Excised bands
521 are numbered according to sequence data present in table 5.

522

523 **Fig. 2** Phylogenetic relationships of deduced amino acid sequences of arsenite efflux pump genes *ACR3(1)* and
524 *ArsB* retrieved from isolates from rhizopshere of *C*. *arvense* (Scarlino site) and *D*. *caespitosa* (Pestarena site)
525 (in bold). The evolutionary history was inferred using the Neighbor-Joining method.

526

Figure
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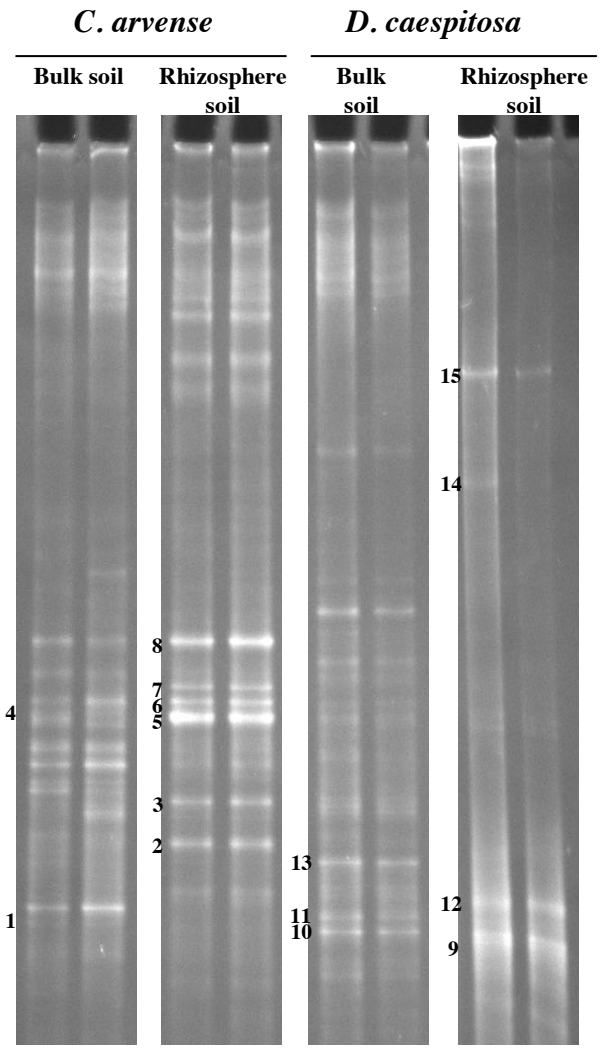
Fig. 1



Figure
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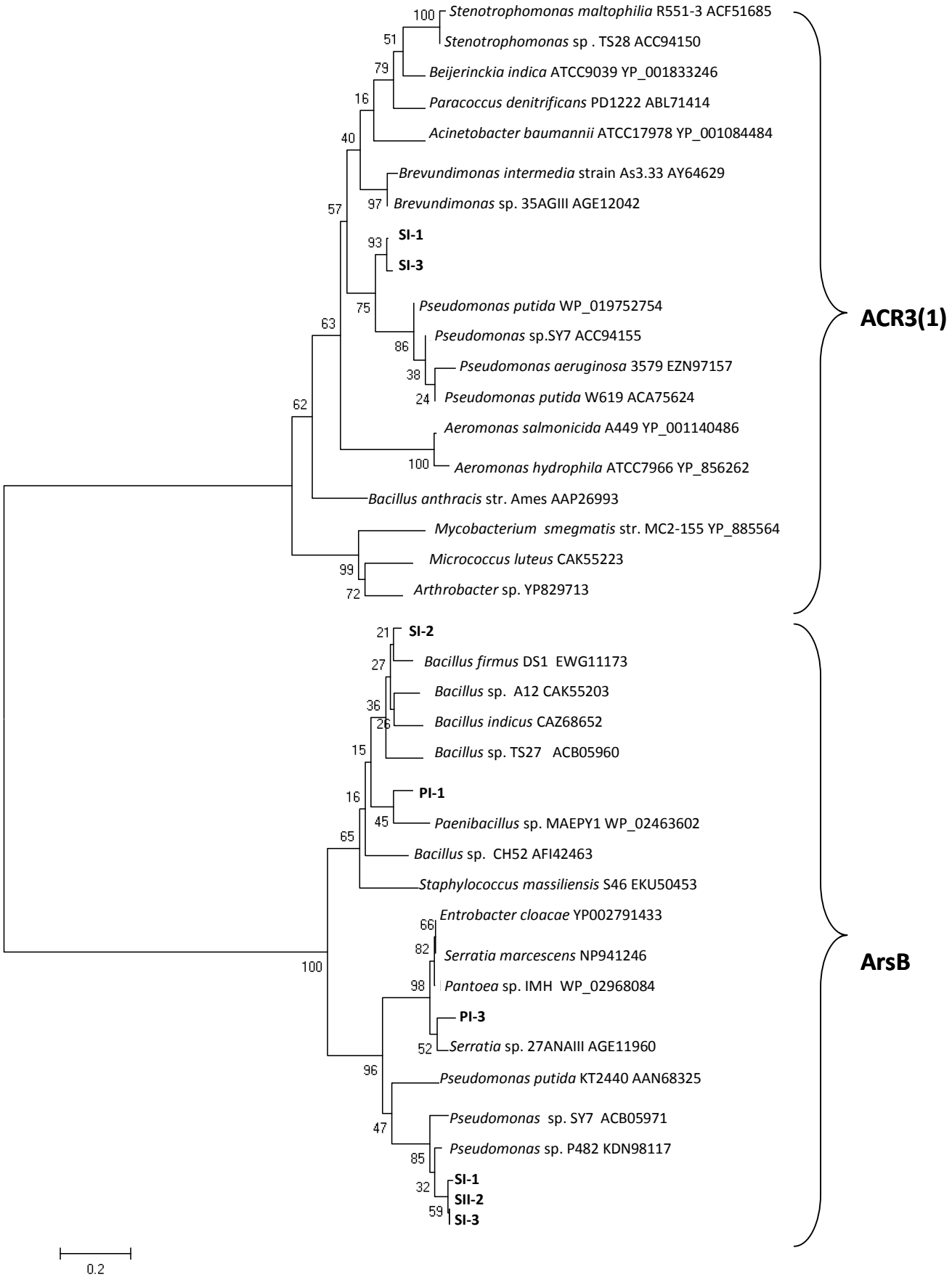
Fig.2



Table
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Table 1 Selected physical and chemical properties of the soils.

Site Name	Sampled plant species (L.)	Texture	pH	Water Content (%)	Total Arsenic (mg kg^{-1} of soil dw)	Bioavailable Arsenic (mg kg^{-1} of soil dw)
Scarlino*	*Cirsium arvensae*	clay-loam	8.0	19.0	304	31.1
Pestarena**	*Deschampsia caespitosa*	sandy-clay-loam	4.8	17.6	4358	142.3

*, data from Cozzolino et al. (2010); **, data from Marabottini et al. (2013).

Table 2 ~~Probes used in FISH analysis performed on bulk, rhizosphere soils and on rhizoplane and target bacterial groups.~~

Probes	**Sequence (5'-3')**	**References**	**Target organisms**	**FA (%)**	**NaCl (mM)**
EUB338	GCTGCCTCCCGTAGGAGT	Amann et al. 1995	Bacteria	30	102
EUB338II	GCAGCCACCCGTAGGTGT	Daims et al. 1999	Planctomycetales	30	102
EUB338III	GCTGCCACCCGT AGGTGT	Daimset al. 1999	Verrucomicrobiales	30	102
NONEUB	ACTCCTACGGGAGGCAGC	Wallneret al. 1993	Negative control	30	102
ALF968	GGTAAGGTTCTGCGCGTT	Neef et al. 1999	α- Proteobacteria	20	215
BET42a	GCCTTCCCACTTCGT TT	Manz et al. 1992	β- Proteobacteria	35	70
GAM42a	GCCTTCCCACATCGT TT	Manz et al. 1992	γ- Proteobacteria	35	70
PSE227	AATCCGACCTAGGCTCATC	Watt et al. 2006	*Pseudomonas* spp.	30	102
CF319a	TGGTCCGTGTCTCAGTAC	Manz et al. 1996	Cytophaga-Flavobacterium cluster of CFB phylum	35	70
HGC69a	TATAGTTACCACCGCCGT	Roller et al. 1994	Gram-positive bacteria with high DNA G+C	25	149
LGCb	CGGAAGATTCCCTACTGC	Meier et al. 1999	Gram-positive bacteria with low DNA G+C	35	70

Competitors were used for: BET42a (5'-GCCTTCCCACATCGTTT-3'), GAM42a (5'-GCCTTCCCACTTCGTTT-3') and HGC69a (5'-TATAGTTACGGCCGCCGT-3') to improve *in situ* accessibility and specificity; FA, formamide in the hybridization buffer; NACl, concentration of NaCl in the washing solution

Table 3 Bacterial populations in the three fractions of the root-soil system from *C. arvense* grown in the site of Scarlino, and *D. caespitosa* grown in the site of Pestarena, by DAPI and FISH analysis. Results are expressed as Log of cell number (g dw^{-1}) ± SE (n=6). Means within a probe of the same fractions with different superscripts differ significantly (Upper case letters $p<0.01$; lower case letters $p<0.05$).

	Bulk		Rhizosphere		Rhizoplane	
	Scarlino	Pestarena	*C.arvense*	*D.caespitosa*	*C.arvense*	*D.caespitosa*
DAPI	9.14 ±0.17	8.99 ±0.07	9.93 ±0.13	9.90±0.02	7.30^{A}±0.15	7.90^{B}±0.07
EUB338	7.65^{a}±0.10 3.2*	8.02^{b}±0.12 10.1*	8.94 ±0.18 8.6*	8.65±0.09 9,1*	6.29^{A}±0.11 8.8*	7.27^{B}±0.05 20.1*
Bet42a	7.14^{a}±0.10 31.3**	6.80^{b}±0.07 5.7**	7.9 1±0.08 23.7**	7.76±0.11 14.3**	5.70^{A}±0.09 24.2**	6.46^{B}±0.09 16.9**
Gam42a	6.86^{a}±0.08 16.2**	7.28^{b}±0.16 21.4**	8.05 ±0.15 32.7**	7.92±0.08 18.4**	6.03^{A}±0.12 55.7**	6.59^{B}±0.07 21.9**
Pse227	6,96 ±0.08 20.8**	7.28 ±0.16 22.5**	8.13 ±0.17 39.7**	7.84±0.10 16.4**	5.95^{A}±0.13 49.9**	6.56^{B}±0.07 20.1**
Alf968	5.89^{A}±0.10 1.8**	6.67^{B}±0,09 4.4**	7.67 ±0.09 13.7**	7.37±0.10 5.6**	5.36^{A}±0.14 13.3**	6.25^{B}±0.11 10.9**
CF319a	4.62^{A}±0.22 0.1**	5.62^{B}±0.09 0.4**	5.96^{A}±0.20 0.3**	6.98^{B}±0.15 2.6**	3.99^{A}±0.17 0.6**	5.38^{B}±0.09 1.4**
HGC69a	5.25 ±0.30 0.4**	5.37 ±0.16 0.3**	5.68^{a}±0.40 0.1**	6.78^{b}±0.15 1.8**	4.12^{A}±0.20 0.8**	5.41^{B}±0.07 1.4**
LGCb	4.79^{A}±0.21 0.1**	5.53^{B}±0.10 0.3**	5.47^{A}±0.13 0.1**	6.53^{B}±0.14 0.8**	4.22^{A}±0.21 1.1**	5.40^{B}±0.09 0.8**

* Percentage of EUB-positive cells related to DAPI count. ** Percentage group-specific cells related to EUB-positive cell count. Probes used are listed in Table 2.

Table 4 Culturable counts in bulk- and rhizospheric soil fractions of *C. arvense* grown in the site of Scarlino, and *D. caespitosa* grown in the site of Pestarena. Results are expressed as Log of cell number (g dw^{-1}) ± SE (n=3).

	Bulk		Rhizosphere	
	Scarlino	Pestarena	*C. arvense*	*D. caespitosa*
Heterotrophic bacteria[a]	6.90 ± 0.07	6.90 ± 0.17	8.03 ± 0.12	8.45 ± 0.27
As(V)-Tolerant[b]	6.53 ± 0.06	6.66 ± 0.29	7.97 ± 0.13	8.08 ± 0.27
As(III)-Tolerant[c]	5.92 ± 0.09	5.68 ± 0.25	6.89 ± 0.27	7.06 ± 0.13

[a], bacteria grown on R2A medium; [b], bacteria grown on R2A supplemented with 15 mM arsenate; [c], bacteria grown on R2A supplemented with 3 Mm As(III).

25 **Table 5** Sequence homology of DGGE bands from bulk and rhizospheric soil fractions of *C*. *arvense* and *D*.

26 *caespitosa*.

Plant	Soil fraction	Band number	Identification	Homology (%)
C. *arvense*	Bulk	1	*Pseudoflavonifractor capillosus* ATCC 29799(T)	97.2
		4	*Flavobacterium hercynium* WB 4.2-33(T)	100
	Rhizospheric	2	*Pseudomonas mohnii* Ipa-2(T)	99.6
		3	*Pseudomonas taetrolens*	97.3
		5	*Pseudomonas fluorescens* AJ971392	100
		6	Uncultured *Pseudomonas* sp. AM232786	100
		7	*Pseudomonas rhodesiae* CIP 104664(T)	97.5
		8	Uncultured bacterium clone TF 88	98
D. *caespitosa*	Bulk	10	*Flexibacteraceae* bacterium JL007	98
		11	*Bacteroidetes* bacterium ONB11	97
		13	*Pedobacter borealis* G-1(T)	96
	Rhizospheric	9	*Sphingobacteriales* bacterium TP524	96
		12	*Cupriavidus necator* ATCC 43291(T)	100
		14	*Flavobacterium fluvii* H7(T)	97.9
		15	*Flavobacterium pectinovorum* DSM 6368(T)	97.7

Table 6 Characterisation of bacterial isolates from *C. arvense* (S isolates) and *D. caespitosa* (P isolates): As-resistance level and As-detoxification genes

Plant	Isolates	Closest 16S rRNA Genebank Match	Resistance level (mM)		As(V)[a] reduction (%)	As(III)[b] oxidation (%)	Presence of arsenic genes				
			As(V)	As(III)			*arsC*	*acr3(1)*	*acr3(2)*	*arsB*	*aioA*
C. arvense	SI-1	*Pseudomonas plecoglossicida* (99% KF285959)	310	16	93	0	-	+	-	+	-
	SI-2	*Bacillus firmus* (100% FN429099)	>360	16	90	0	-	-	-	+	-
	SI-3	*Pseudomonas plecoglossicida* (99% KF285959)	310	16	97	0	-	+	-	+	-
	SII-1	*Bacillus megaterium* (100% KJ534462)	150	12.5	90	0	-	-	-	-	-
	SII-2	*Pseudomonas plecoglossicida* (99% KF285959)	120	12.5	93	0	-	-	-	+	-
	SII-3	*Bacillus idriensis* (99% HF585035)	190	7.5	85	0	+	-	-	-	-
D. caespitosa	PI-1	*Bacillus simplex* (99% AJ628747)	310	15	85	0	-	-	-	+	-
	PI-2	*Bacillus simplex* (99% AJ628747)	310	15	80	0	-	-	-	+	-
	PI-3	*Buttiauxella aegrestis* (100% NR041968)	240	7	70	0	-	-	-	+	-
	PI-4	*Buttiauxella aegrestis* (100% NR041968)	240	7	70	0	-	-	-	+	-
	PI-5	*Buttiauxella aegrestis* (100% NR041968)	240	7	90	0	-	-	-	+	-

[a], As(V) 3 mM after 7 d incubation; [b], As(III) 1 mM after 7 d incubation