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The interaction between rhizobacteria and the hyperaccumulator fern *Pteris vittata* in arsenic transformation

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Abstract

INTRODUCTION

Arsenic is an ubiquitous element which occurs naturally in the earth's crust. Arsenic is toxic to both plants and animals and inorganic arsenicals are proven carcinogens in humans (Ng, 2005). Arsenic toxicity to human health ranges from skin lesions to brain, liver, kidney, and stomach cancer (Smith *et al.*, 1992). Arsenic contamination of groundwater used for domestic supplies has been reported in over 70 countries, affecting health of an estimated 150 million people (Ravenscroft *et al.*, 2009). Because of its wide distribution and marked toxic effects, arsenic contamination is a problem of global concern. Inorganic arsenic is indeed regarded as the number one toxin in the USEPA list of priority pollutants (Ng *et al.*, 2003).

In this respect, an interesting case study is that discussed in this PhD thesis focusing on the Scarlino area, a Site of Regional Interest (SRI) which requires prominent attention from the environmental point of view. The contamination in this context is the result of dumping of ash from arsenopyrite roasting for more than 30 years as a consequence of sulfuric acid production by the Nuova Solmine Company operating with industrial facilities in this site. Disposal of such ash in addition to the natural arsenic background has thus provoked either a serious soil contamination or a diffuse pollution of aquifers within the whole industrial district, where As can be detected in an average concentration of 140 mg/kg.

An approach that might alleviate this problem is represented by the biotechnological option defined as microbe-enhanced phytoremediation. Phytoremediation is a low-cost and eco-friendly technology that uses vascular plants for *in situ* environmental restoration and reclamation of contaminated soils, sludges and sediments. Particularly, in the case of As contamination, phytoextraction – which is the removal of toxic metals/metalloids from soil or whatever other environmental matrix and their concentration into the harvestable plant portion – appears quite useful (Khan, 2005).

Microorganisms are known to play a very important role in this process. Actually, bacteria can enhance the mobility of arsenic in the soil matrix (eliciting the metalloid uptake by plants) while plant growth promoting rhizobacteria (PGPR) can improve plant biomass production (Abou-Shanab *et al.*, 2003a; Glick *et al.*, 1995; Glick, 2003).

AIMS OF THE STUDY

The research project here presented aimed at finding the best integrated plant-microorganisms system able to lower arsenic contamination in the Scarlino area below the concentration of 50 mg/kg established by law. In particular, the main objective of this study was to verify the possible influence of rhizosphere bacterial cenoses on both the dynamics of As uptake from the contaminated soil and the accumulation efficiency of the hyperaccumulator fern *Pteris vittata* considered as biological tool for phytoremediation. The investigation was carried out through three main phases:

1. Taxonomic characterization of the rhizospheric bacterial cenoses of autochthonous plant species, grown on aged heavily arsenic polluted soils with the adoption either of classic diagnostic methods or molecular techniques. In detail, both culture-independent methods (PCR-DGGE) and culture-dependent protocols (total viable count and isolation from enrichment cultures) were used.
2. Screening of the strains isolated relatively to:
    - As resistance (through MIC determination),
    - genotypic study of the mechanisms of arsenic transformation,
    - ability to reduce arsenate to more mobile chemical species.
    - presence of Plant Growth Promoting (PGP) traits (IAA and siderophores production and ACC deaminase activity),

These evaluations allowed to identify and select different functional groups such as PGP rhizobacteria (PGPR) as well as bacterial species resistant to high levels of arsenic and capable of mobilizing arsenic from the soil.
3. Development and testing of specific bacterial *inocula* with reference to their possible influence on phytoextraction efficiency of *Pteris vittata* in bioaugmentation experiments at lab scale. Plants grown in the presence of arsenic with or without bacterial *inocula* were evaluated through the analysis of their performance in terms of biomass produced and metal concentration in plant tissues.

MATERIALS AND METHODS

All the analyses described in the present study were performed on six soil samples from different collecting points: one from the matrix (M) consisting in a huge pile of about 1,600,000 metric tons of arsenopyrite ash while the other five samples from the rhizosphere of as many different autochthonous plants (P1, P2, P3, P4 and P5) grew close to the waste disposal site. These five plants were identified on the basis of morphological characteristic as *Lolium rigidum* (P1), *Daucus carota* (P2), *Trifolium angustifolium* L. (P3), *Populus alba* (P4) and *Sedum sediforme* (P5). The average concentrations of arsenic were 500 mg/kg and 140 mg/kg in the matrix and in the surrounding area respectively.

For each sample, the culture-dependent approach gave the number of the cultivable microorganisms. Meanwhile the isolation by means of enrichment cultures in presence of As(III) or As(V) allowed to identify arsenic resistant and metabolically interesting strains. After eight weeks of incubation in the dark, the strains selected were brought into pure culture and Operational Taxonomic Units (OTUs) were singled out. Samples from the ash dumping site (M) were also analyzed with a culture-independent approach, in order to evaluate the biodiversity of the bacterial community including the uncultivable fraction. Then total genomic DNA was extracted, V3 variable region of *16S rDNA* gene was amplified, and a Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis was performed.

Cultivation-based methods were also considered to characterize the strains isolated with respect

to their As resistance. Minimum Inhibitory Concentrations (MICs) for As(III) and As(V) were determined. Furthermore, isolates were screened for possible PGP characteristics such as the production of indoleacetic acid (IAA), siderophores, and 1-amino-cyclopropane-1-carboxylic acid (ACC)-deaminase activity of particular interest in the perspective of a phytoremediation approach. Some bacterial strains were even tested for the ability to reduce As(V) *in vitro*.

A molecular study was carried out to detect the presence of relevant genes for As resistance and transformation by using group-specific primer pairs to possibly identify arsenite transporters genes (Achour *et al*., 2007), As(V) reductase genes (Macur *et al*., 2004) and As(III) oxidase genes (Inskeep *et al.,* 2007 and Quéméneur *et al*., 2010).

As far as phytoextraction is concerned, two lab-scale trials were arranged with the fern *P. vittata* in association with two different bacterial *inocula*. Three strains - namely *Ochrobactrum cytisi* OTU E, *Pseudomonas putida* OTU N and *Achromobacter marplatensis* OTU P - were chosen for the phytoextraction tests and inoculated according to three different protocols: i) *inoculum* A: only strain P, ii) *inoculum* B: strains E + O, and iii) *inoculum* C: strains P + E + O. Fern without bacterial *inocula* were grown and used as negative control to evaluate the effect of PGPR on plants growth.

Analogously to the first trial, a second *inoculum* was selected and inoculated as follows: i) *inoculum* A: *P. putida* OTU N and *Delftia lacustris* OTU U, ii) *inoculum* B: *B. thuringiensis* OTU M, *Variovorax paradoxus* OTU AP*, Pseudoxanthomonas mexicana* OTU AS, and iii) *inoculum* C: strains N + U + M + AP + AS, iiii) not inoculated.

At the end of the experiments, plants were harvested and biomass production and total As content in plant tissues were measured.

RESULTS AND DISCUSSION

CHAPTER 1. Starting from samples of matrix (M) and autochthonous plant species grown and acclimated to the As polluted site, a total of 201 As-resistant bacteria were isolated, 126 from As(V)- and 75 from As(III)-enriched cultures respectively. These data are consistent with the fact that As(V) is less toxic than As(III), thus more species can adapt to this form of the metalloid (Styblo *et al*., 2000). Axenic cultures of morphologically different colonies were screened by ARDRA molecular technique, grouped into 55 different Operational Taxonomic Units (OTUs) and taxonomically identified through sequencing of *16S rDNA* gene. The results obtained indicated the speciation of an As tolerant bacterial community within the area, quite heterogenic in structure despite the harsh environmental conditions, with a rich biodiversity consisting of even 25 different *genera*. Actually, almost all the isolates (belonging to *Firmicutes*, *Actinobacteria,* Alfa-, Beta-Gamma-proteobacteria and *Flavobacteria*) showed high homologies with bacteria occurring in particular ecological niches such as contaminated soils and capable of degrading recalcitrant pollutants or tolerating high concentrations of heavy metals. In particular, sequencing of the isolated OTUs evidenced high homologies (99%-100%) to *genera* such as *Stenotrophomonas* and *Pseudomonas* of the Gamma-proteobacteria, *Microbacterium* of the *Actinobacteria*, *Delftia* of the Beta-proteobacteria, and *B. thuringiensis* of the *Firmicute*s, all well known for their catabolic

capabilities or high resistance to arsenic and heavy metals, thus suggesting the presence of a strong potential to cope with heavy metals and organic pollutants within the indigenous bacterial community (Abou-Shanab *et al*., 2007; Muller *et al*., 2007; Xiong *et al*., 2012). Enrichment conditions, however, do not normally reproduce the actual ones of an environmental sample and therefore select for microorganisms growing faster or adapting better to the culture conditions imposed.

Further information about the microbiological features of such an extreme environment as the arsenopyrite ash pile was gained by PCR-DGGE analysis. This investigation confirmed the selection of dominant members within the autochthonous bacterial community due to the long-lasting chronic contamination. The fact that the *phyla* of *Firmicutes* and *Actinobacteria* resulted highly represented is indeed a novel finding.

CHAPTER 2. The 2nd part of this PhD work was focused on the screening of the bacterial isolates to detect strains with suitable features to be exploited in possible bioremediation procedures. Starting from the Minimum Inhibitory Concentration (MIC) values for As(V) and As(III), 32 strains with high As resistance were singled out, showing in some cases MIC limits up to 40 mM for As(III) and higher than 100 mM for As(V). In particular, among others, *Stenotrophomonas rhizophila* OTU B*, Brevundimonas olei* OTU C*, Microbacterium liquefaciens* OTU AX*,* and *Arthrobacter nitroguajacolicus* OTU AY) are known in the literature for their arsenic resistance (Achour-Rokbani *et al*., 2010; Branco *et al*., 2008). At the best of the author's knowledge few strains have been described so far as more resistant to As(III) than those cited above showing MIC values ranking well above the average. Nevertheless, the results pointed out a selective speciation in the Scarlino site towards a community of gram-negative Proteobacteria and gram-positive *Actinobacteria* with members particularly resistant to high As concentrations .

A molecular investigation dealing with the identification of arsenic resistance and reducing/oxidating capabilities of (As(III) and As(V) oxides was performed to evaluate the resistance and degrading potential within the indigenous bacterial micro flora. Microbial metabolism of As is very complex and therefore the attention was focused in this study on only two different reaction mechanisms. In particular, either the As(III) efflux pump or the arsenate reductase (*ars* operon) and arsenite oxidase (*aox* operon) genes were taken into account. In general terms, among the 55 isolates, 21 showed successful amplifications of the arsenite transporter genes, 22 gave positive results for the arsenate reductase, and 13 for the arsenite oxidase. In some cases, high MIC values did not correspond to the presence of specific functional genes and - vice versa - strains showing low MIC values were carrying functional As genotypes. Evidently, besides the resistance mechanisms considered in this study others might be present giving an explanation of the observed discrepancies. For instance, As(III) can be detoxified by complexation with Cys-rich peptides, inorganic arsenic can be transformed into organic species through a methylation cascade and then volatilized, As(V) can be used as the ultimate electron acceptor during respiration but even adsorbed on the bacterial cell wall, and As(III) can be finally oxidated by membrane lipids (Abdrashitova *et al*., 1986; Frankenberger and Arshad, 2001;

Saltikov and Newman, 2003; Paéz-Espino *et al*., 2009). Moreover, horizontal gene transfer (HGT) seems a likely hypothesis to explain the presence of identical (or nearly identical) sequences in some distant bacterial lineages; this has been described for *arsC* genes (Saltikov and Olson, 2002; Jackson and Dugas, 2003), for arsenite efflux genes (Cai *et al*., 2009; Valverde *et al*., 2011), and for *aox* genes (Oremland *et al*., 2005) under arsenic-induced selective pressure.

As(V)-reducing bacteria have been found to mediate the transformation of As(V) into As(III) under highly aerobic conditions causing arsenic mobilization from limed mine tailings (Macur *et al*. 2001). The ability to reduce As(V) into As(III) represented – in this case – a net advantage from the bioremediation point of view since the more mobile and bioavailable As(III) could be easily absorbed by fern roots and therefore removed from contaminated soils and aquifers.

Ten different bacterial strains were tested for their ability to reduce As(V) into As(III). The best performances were exhibited by *Delftia lacustris* strain U that was capable of completely reducing 5 mM As(V) in 48 h of incubation and *Pseudomonas putida* strain N, able to totally reduce As(V) 5 mM in 72 hours of incubation under aerobic condition. Although direct roles of these bacteria in arsenic mobilization was not studied, the ability of the isolates to reduce As(V) suggests that these bacteria can potentially mediate arsenic transformation becoming relevant in a phytoextraction context. Additionally, As(III) removal efficiency could have even been increased by the synergism between microbial As(V) reduction in the rhizosphere and a faster As(III) root uptake.

The presence of PGP traits among the bacterial community members highly resistant to As was also investigated giving the following results:

- 24 bacterial isolates were able to produce IAA, a phyto-hormone with positive effects on plants growth (Glick, 1995);
- 24 bacterial isolates were able to produce siderophores, which are compounds that can bind ferric iron and enhance the bioavailability of this element in the rhizosphere (Garbisu and Alkorta, 2001);
- only one strain showed to produce ACC deaminase, an enzyme which cleaves the "stress hormone" ethylene produced in plants by using the reaction product (ACC) as the sole N source. As a consequence, the plants root system can have an improved development (Penrose and Glick, 2002).

The OTUs identified mostly possessed at least one PGP trait suggesting a synergistic potential role in a phytoremediation. Although PGPR were identified among members of the *taxa* resulted by enrichment cultures, it is interesting to notice how Gamma-proteobacteria represent the majority of rhizobacteria capable of producing IAA and siderophores. This is particularly true for the *genera Pseudomonas* and *Stenotrophomonas* which are dominant members of the autochthonous bacterial cenosis and already known in the literature for their PGP activity (Bloemberg and Lugtenberg, 2004; Pages *et al*., 2008).

CHAPTER 3. On the basis of the results achieved during the phases 1 and 2, the 3rd part of this

PhD project dealt with the identification of possible candidates to be used in a microbe-enhanced phytoextraction experiments. This investigation was planned as a bioaugmentation assay. Selected microorganisms either metal-resistant or able to produce plant growth-promoting compounds have previously been used as inoculants in phytoremediation processes (Rajkumar *et al*., 2012). Moreover, not only PGPR are known to be able to promote plant growth in heavy metal contaminated soils, but a variety of bacteria, including *Pseudomonas* sp. and *Delftia* sp., have demonstrated to enhance phytoextraction (Koo and Kyung-Suk, 2009; Rajkumar *et al*., 2012). These isolates were therefore chosen since they were all characterized for their high resistance to both the toxic species As(V) and As(III), by the presence either of the *ars* operon involved in As solubilization or of different PGP traits. Moreover the selected strains are well adapted to this particular soil and contamination, from which they have been isolated.

As already mentioned, three bacterial strains (*Ochrobactrum cytisi*, *Pseudomonas putida*, and *Achromobacter marplatensis*) were selected for the phytoextraction trial and inoculated according to different protocols. The first evident result was about biomass production and how all the plants grown on As-contaminated soil from Scarlino had higher values even without any kind of *inocula*. This finding is not surprising since other authors have previously observed that plant biomass increased after exposure to As and hypothesized that As might stimulate P uptake in *P. vittata* (Ghosh *et al*., 2011). Nevertheless, of particular interest was the promotion of plant growth elicited by the bacterial inoculants. In fact, biomass production resulted higher in all inoculated plants with a particular evidence in plants treated with the *inoculum* B (*Pseudomonas* + *Delftia*, IAA producers) and the *inoculum* C (*Pseudomonas* + *Delftia* + *Achromobacter*, IAA and ACC deaminase producers). On the other hand, uninoculated plants accumulated higher concentration of As in their epigeous portion in comparison with plants that had been treated with *inoculum* A and *inoculum* B. Essentially, it seems that the bacterial *inocula* caused a decrease in As uptake by plants. This was not in agreement with expectations since different studies have already demonstrated the increase of As (and other toxic metals) uptake in ferns inoculated with PGP bacteria (Yang *et al*., 2012; Kumar *et al*., 2008; Rajkumar and Freitas, 2008). Thus, it might be hypothesized that the bacterial *inocula* used to treat *P. vittata* rhizosphere in this study lowered As uptake due to a potential role in the phytostabilization of the metalloid in the contaminated soil. However, by considering both the biomass production and the metalloid uptake, the *inoculum* C evidenced an appreciable enhancement of the phytoextraction process. This result suggests for a synergistic activity of the bacterial inoculants bearing different PGP traits. Finally, As concentrations in soil at both starting and end of the trial in the different mesocosms prepared were measured. Remarkably, all the plants amended with bacterial *inocula* were able to extract a sufficient amount of As to remediate the polluted soil in exam and bring the value below 50 mg/kg (the limit by law for industrial area). Plants amended with PGPR abated As concentration in soil even more effectively than uninoculated plants (seven times for *inoculum* A, three times for *inoculum* B, and four times for *inoculum* C). Since only data concerning As concentration in shoots were available, it can only be hypothesized that a non-negligible amount of the metalloid

was accumulated also in fern roots or, although unusual, may have been methylated and volatilized by soil bacteria (Tsai *et al*., 2009).

Nevertheless these data pointed out the positive influence exerted particularly by soil autochthonous bacterial strains on both plant growth and phytoremediation efficiency. The positive influence of the autochthonous bacteria in *P. vittata* mesocosms was evidenced as far as both biomass production and As soil content at the end of the cultivation trial.

Bacterial *inocula* were applied to fern rhizosphere at a final concentration of 10^8 CFU/g of soil. During the experiment the presence and the persistence of inoculated strains were assessed in the different pots by means of PCR-DGGE analyses. All the inoculated bacterial species were retrieved from As contaminated pots. Conversely, some bands were missing in the controls. This might be explained by assuming that the inoculated strains autochthonous of the contaminated matrix (arsenopyrite ash), had probably difficulty in adapting to the control soil. Besides, since the bacterial communities in the samples were very complex, probably not all the bands were clearly visible on gels. This analysis however confirmed the presence of *Achromobacter marplatensis, Ochrobactrum cytisi*, and *Pseudomonas putida* in the rhizosphere of ferns grown on As contaminated soil, therefore confirming that the PGP effects observed on plants were associated with the bacterial *inocula*.

Analogously to the first trial, a second inoculum was selected to promote microbe-enhanced phytoextraction. The treatments were carried out as follows: i) *inoculum* A: *P. putida* OTU N and *Delftia lacustris* OTU U, ii) *inoculum* B: *B. thuringiensis* OTU M, *Variovorax paradoxus* OTU AP, *Pseudoxanthomonas mexicana* OTU AS, iii) *inoculum* C: strains N + U + M + AP + AS, and iiii) not inoculated. As the analyses are still in progress no data dealing with this experiment are reported in this presentation.

CONCLUDING REMARKS

On the basis of the results obtained and discussed in this PhD thesis it is therefore possible to draw the following main considerations:

- The high contamination due to arsenic and heavy metals present in the Nuova Solmine industrial area has determined a selection of the soil autochthonous bacterial cenoses towards a more tolerant and well adapted community, with wide biodiversity, resistance and As-transforming potential;
- Most of the strains identified in the area with the highest pollutant concentrations (M, the arsenopyrite ash dumping pile) belonged to Gram positive *Firmicutes* and *Actinobacteria*, including strains possessing interesting genotypic traits involved in As transformation;
- The isolates belonging to *Delftia lacustris,* and *Pseudomonas putida* specie reduced As(V) *in vitro* at high efficiency also showing the ability to solubilize arsenic in soils;
- Among the members of the above mentioned bacterial community isolated in pure culture, strains belonging mainly to Gamma-proteobacteria were carrying particular PGP traits useful in a phytoremediation perspective based on bioaugmentation;

- *Ochrobactrum cytisi*, *Pseudomonas putida* and *Achromobacter marplatensis* were selected and tested for a microbe-enhanced phytoextraction experiment in association with the hyperaccumulator fern *P. vittata*. They exerted positive effects on both plant biomass production and total phytoextraction efficiency when compared with the not inoculated plants. However, all the plants – with or without bacterial inoculants – were able to extract an appreciable amount of As; however, where the *inocula* were present, ferns lowered As concentration in soil more effectively.

The results of a second phytoextraction trial with another selection of As-resistant PGP strain (*P. putida, Delftia lacustris, B. thuringiensis, Variovorax paradoxus, Pseudoxanthomonas mexicana*) are coming. When completed, these preliminary evidences will allow either to evaluate the phytoextraction potential of the bacterial strains tested so far at lab scale or to schedule a future pilot trial at the Scarlino contaminated site.

In conclusion an integrated system of plants and bacteria would be perfected as a reliable remediation tool to be applied in the Scarlino industrial area.

Riassunto

INTRODUZIONE

L'arsenico è un elemento ubiquitario che ricorre con naturale abbondanza nella crosta terrestre. È tossico sia per le piante che per gli animali e ne è stato dimostrato il potere cancerogeno nell'uomo (Ng, 2005). La tossicità dell'As nei confronti della salute umana si esplica in un ampio range di patologie che vanno da lesioni cutanee a cancro ai reni, allo stomaco, al fegato (Smith *et al*., 1992). La contaminazione da As nelle acque ad uso domestico è stata riportata in più di 70 paesi diversi, e si stima che abbia compromesso la salute di più di 150 milioni di persone nel mondo (Ravenscroft *et al*., 2009). Data la sua ampia distribuzione ed elevata tossicità, la contaminazione da arsenico rappresenta oggigiorno un problema di importanza mondiale (Ng *et al*., 2003).

Un interessante caso di studio – in esame in questo lavoro di dottorato – è rappresentato dall'area di Scarlino, un Sito con priorità di risanamento di Interesse Regionale (SRI). In questo contesto la contaminazione di suoli e falde è stata provocata dall'accumulo di scorie di arsenopirite derivanti dall'incenerimento del minerale per la produzione di acido solforico presso l'impianto industriale Nuova Solmine operante nel sito. Aldilà del fattore di contaminazione antropico, tutta l'area di Scarlino è allo stesso tempo contaminata per cause naturali, i suoli di tutto il comune, al di fuori delle aree industriali, presentano infatti una concentrazione media di As pari a 140 mg/kg.

Dal punto di vista biotecnologico un approccio che potrebbe attenuare questa problematica è rappresentato dalla fitoestrazione assistita da batteri. La fitobonifica in generale è l'insieme delle tecnologie a basso costo e impatto ambientale che prevede l'uso di piante vascolari per il risanamento *in situ* di suoli, fanghi e sedimenti contaminati. In particolare, in caso di suoli contaminati da As o metalli, la fitoestrazione – che prevede la concentrazione dei contaminanti nei tessuti vegetali e la loro successiva raccolta – rappresenta la strategia di maggior successo (Khan, 2005).

I microrganismi hanno un ruolo fondamentale in tale processo. Di fatto i batteri possono incrementare la mobilità dell'arsenico dalla matrice suolo (stimolandone l'*uptake* da parte della pianta) mentre rizobatteri promotori delle crescita vegetale (PGPR) possono elicitare la produzione in biomassa vegetale (Abou-Shanab *et al*., 2003) migliorando in entrambi i casi l'efficienza del sistema.

OBIETTIVI

Tenendo presente l'obiettivo principale, mirato all'identificazione di un sistema integrato di piante e batteri in gradi di abbassare il livello della contaminazione al di sotto della concentrazione soglia prevista dalla legge di 50 mg/kg, si è voluto investigare sulla possibile influenza delle cenosi batteriche sia sulle dinamiche di *uptake* dell'arsenico che sull'efficienza di fitoestrazione da parte della felce iperaccumulatrice *Pteris vittata*. L'indagine si è articolata in tre principali fasi di analisi:

1. La prima parte si è focalizzata sullo studio e la caratterizzazione della comunità batterica

autoctona, selezionatasi e acclimatatasi alla contaminazione da arsenico inorganico presente nell'area da 50 anni, integrando sia un approccio *culture-dependent* che *culture-independent*.

2. La seconda fase ha previsto lo screening della collezione batterica relativamente a:

- La resistenza all'arsenico (attraverso la determinazione di MIC),
- Lo studio genotipico dei meccanismi coinvolti nella trasformazione degli ossianioni arsenito e arsenato,
- La capacità di ridurre l'As(V) a specie chimiche più mobili e biodisponibili,
- La presenza di tratti PGP (*Plant Growth Promotion*) quali la produzione di IAA e siderofori e l'attività dell'enzima ACC deaminasi.

Queste valutazioni hanno quindi permesso di individuare e selezionare gruppi batterici funzionali come batteri in grado di promuovere la crescita vegetale o batteri in grado di solubilizzare l'As dal suolo da poter essere impiegati in una prova di fitoestrazione.

3. Nell'ultima fase si sono quindi testati specifici inoculi batterici in associazione con la felce iperaccumulatrice *Pteris vittata* in relazione all'efficienza di fitoestrazione di As dalla matrice contaminata di Scarlino. Le piante sono state fatte crescere sul suolo contaminato in presenza o assenza dell'inoculo batterico ed il sistema è stato valutato misurando la produzione di biomassa da parte della pianta e la concentrazione di As nei tessuti vegetali.

MATERIALI E METODI

Tutte la analisi descritte nel presente studio sono state condotte a partire da 6 diversi matrici: una proveniente dal cumulo di 1.600.000 tonnellate di ceneri di arsenopirite (M) e altre 5 provenienti dalla rizosfera di altrettante piante autoctone cresciute nelle vicinanze della massa di ceneri. Le piante sono state ricondotte in base a caratteristiche fenotipiche alle seguenti specie: *Lolium rigidum* (P1), *Daucus carota* (P2), *Trifolium angustifolium* L. (P3), *Populus alba* (P4) and *Sedum sediforme* (P5). I diversi punti di campionamento sono caratterizzati da diversi livelli di contaminazione da arsenico che si attestano rispettivamente attorno a 500 e 140 mg/kg nelle ceneri e nella rizosfera delle piante.

Per ogni campione analizzato, l'approccio *culture-dependent* ha previsto la determinazione della carica microbica e l'isolamento di specie batteriche a partire da colture di arricchimento in presenza di As(III) e As(V) che hanno quindi favorito la selezione di ceppi resistenti o metabolicamente interessanti. Dopo 8 settimane di incubazione al buio, i ceppi batterici sono stati isolati in coltura pura e raggruppati in *Operational Taxonomic Units* (OTUs). I campioni provenienti dal punto maggiormente contaminato (M) sono stati analizzati anche tramite un approccio *culture-independent*, allo scopo di valutare la potenziale biodiversità comprensiva anche dei ceppi incoltivabili in condizioni di laboratorio. Si è quindi estratto il DNA totale, si è amplificato il gene codificante per la regione V3 dell'*rDNA 16S* ed è stata effettuata un'analisi *Denaturing Gradient Gel Electrophoresis* (PCR-DGGE).

Metodiche di microbiologia classica sono state utilizzate anche per caratterizzare gli isolati in relazione alla loro resistenza all'As e quindi per determinare i valori di MIC (*Minimum Inhibitory Concentration*) per arsenito e arsenato. Gli isolati più resistenti sono stati valutati in relazione alla

presenza di importanti tratti PGP come la produzione di acido indolacetico (IAA) e siderofori o l'attività dell'enzima ACC deaminasi, di particolare interesse in prospettiva di interventi di fito-bonifica. Alcuni membri della collezione sono stati ulteriormente testati in relazione alla capacità di ridurre l'As(V) *in vitro* in condizioni aerobiche.

È stato condotto uno studio molecolare a livello genotipico, per identificare la presenza di determinanti genetici codificanti per geni coinvolti nella trasformazione degli ossianoni arsenito e arsenato, utilizzando *primers* gruppo-specifici con target l'arsenato reduttasi e la pompa di estrusione dell'arsenito (codificati dall'operone *ars*) e il gene della arsenito ossidasi (codificato dall'operone *aox*) (Achour *et al*., 2007; Macur *et al*., 2004; Inskeep *et al.,* 2007 and Quéméneur *et al*., 2010).

Considerando invece la terza parte della sperimentazione, sono stati allestiti due distinti esperimenti di fitoestrazione in *lab scale* con la felce *P. vittata* in associazione a due tipi di inoculo batterico. Per il primo esperimento sono stati utilizzati i ceppi *Ochrobactrum cytisi* OTU E, *Pseudomonas putida* OTU N and *Achromobacter marplatensis* OTU P inoculati secondo i seguenti protocolli: i) inoculo A: ceppo P, ii) inoculo B: ceppi E + N e iii) inoculo C: ceppi P + E + N. Sono state coltivate anche felci senza inoculo in modo da avere un controllo negativo per poter valutare l'influenza dei ceppi in studio nel processo di fitoestrazione.

Analogamente al primo esperimento, è stato selezionato anche un secondo inoculo batterico utilizzato in associazione con le piante seguendo questo assetto: i) inoculo A: *P. putida* OTU N e *Delftia lacustris* OTU U, ii) inoculo B: *B. thuringiensis* OTU M, *Variovorax paradoxus* OTU AP*, Pseudoxanthomonas mexicana* OTU AS, iii) inoculo C: ceppi N + U + M + AP + AS, iiii) piante non inoculate.

Alla fine delle prove le piante sono state raccolte ed è stata valutata la produzione in biomassa e il contenuto in arsenico all'interno dei tessuti vegetali.

RISULTATI

CAPITOLO 1. Relativamente alle colture di arricchimento allestite a partire dai campioni provenienti dalle ceneri e dai campioni rizosferici delle piante presenti nel sito è stato possibile isolare 201 ceppi batterici, rispettivamente 126 e 75 dalle colture in presenza di As(V) e As(III). Questi dati preliminari sono in accordo con il fatto che l'As(V) è meno tossico dell'As(III) e quindi più specie batteriche sono riuscite ad adattarvisi (Styblo *et al*., 2000). Tramite lo screening delle colonie ottenute in coltura axenica con la metodica ARDRA gli isolati sono stati raggruppati in 55 diverse OTUs (*Operational Taxonomic Units*) ed identificati tassonomicamente attraverso il sequenziamento del gene *16S rDNA*. I dati inerenti allo studio *culture-dependent* hanno attestato un buon adattamento e resistenza alla contaminazione da parte della microflora autoctona speciatasi e acclimatatasi alla contaminazione presente nell'area da diversi decenni, e caratterizzata da una struttura eterogenea e, per un suolo perturbato, da una ricca biodiversità. I ceppi ottenuti in coltura pura si riconducono, infatti, a 25 diversi generi (appartenenti ai *phyla Firmicutes, Actinobacteria*, Alfa-, Beta-, Gamma-proteobacteria e *Flavobacteria*), mostrando un'elevata omologia con specie associate a suoli contaminati, resistenti a metalli pesanti ovvero

caratterizzate da capacità degradative verso composti organici e recalcitranti. È inoltre interessante notare come dai risultati dei sequenziamenti questi isolati mostrino elevata omologia di sequenza con generi batterici ampiamente diffusi in varie nicchie ambientali, come *Stenotrophomonas* e *Pseudomonas* isolati da suoli contaminati da metalli pesanti, *Microbacterium*, un genere noto per resistenze multiple presenti all'interno del genoma, o generi come *Delftia* e *Bacillus,* noti per interessanti capacità metaboliche nei confronti di composti arsenicali (Abou-Shanab *et al*., 2007; Muller *et al*., 2007; Xiong *et al*., 2012). Bisogna tuttavia tenere in considerazione che le colture di arricchimento non riproducono esattamente l'ambiente d'origine ma tendono a selezionare microrganismi che crescono più velocemente e si adattano meglio alle condizione di coltura imposte.
Ulteriori informazioni sulla struttura tassonomica della matrice sono state ottenute tramite l'analisi PCR-DGGE. L'analisi molecolare condotta ha indicato la selezione di componenti dominanti all'interno della comunità batterica esercitata dalla contaminazione protratta nel tempo, svelando una inusuale preponderanza dei *phyla Firmicutes* e *Actinobacteria.*

CAPITOLO 2. La seconda parte di questo lavoro si è focalizzata sullo screening della collezione di isolati in relazione a importanti tratti da poter sfruttare in un protocollo di bonifica fito-assistita. Partendo dalla determinazione della MIC (*Minimum Inhibitory Concentrations*) per As(III) e As(V), sono stati individuati 32 ceppi altamente resistenti (tra cui *Stenotrophomonas rhizophila* OTU B, *Brevundimonas olei* OTU C, *Microbacterium liquefaciens* OTU AX, *Arthrobacter nitroguajacolicus* OTU AY), con valori in alcuni casi superiori a 20 mM per l'As(III) e superiori a 100 mM per l'As(V) che si attestano al di sopra della media. I ceppi isolati hanno evidenziato quindi una selezione all'interno del sito contaminato di Scarlino verso una popolazione arsenico-resistente, sia tra i membri dei Proteobatteri Gram negativi che tra gli attinomiceti Gram positivi.
È stata quindi condotta un'analisi molecolare in relazione ai determinati genetici per la resistenza all'As e alla capacità di trasformare As(III) e As(V) al fine di valutare il potenziale degradativo all'interno della comunità microbica. L'attenzione si è rivolta a due principali meccanismi di reazione coinvolgenti i seguenti determinanti genici: la pompa di estrusione dell'As(III) e l'arsenato reduttasi (appartenenti all'operone *ars*) e l'arsenito ossidasi dell'operone *aox*. In termini generali, tra i 55 isolati, 21 hanno dato un risultato positivo per i trasportatori dell'As(III), 22 per l'arsenato reduttasi e 13 per l'arsenito ossidasi. Confrontando questi dati con i risultati delle MIC è stato possibile osservare come in certi casi alti valori di MIC non corrispondessero alla presenza dei geni in analisi o, al contrario, ceppi con bassa resistenza all'As presentassero dei genotipi positivi. Si può quindi ipotizzare che altri meccanismi di resistenza siano presenti aldilà di quelli analizzati in questa sede e possano quindi spiegare quindi tali discrepanze. A tal riguardo occorre infatti menzionare che l'As(III) può essere anche complessato con peptidi ricchi in cisteine, sia As(III) che As(V) possono essere metilati e volatilizzati, l'As(V) inoltre può essere usato come accettore finale di elettroni durante la respirazione o assorbito dalla parete cellulare ed infine l'As(III) può essere ossidato da lipidi di membrana (Abdrashitova *et al*., 1986; Frankenberger and Arshad, 2001; Saltikov and Newman, 2003; Paéz-Espino *et al*., 2009). Per di più è stato riportato

che il trasferimento genico orizzontale potrebbe spiegare la presenza di sequenze identiche o comunque molto simili in specie filogeneticamente distanti, come è stato dimostrato per i geni *arsC* (Saltikov and Olson, 2002; Jackson and Dugas, 2003) *arsB* (Cai *et al*., 2009; Valverde *et al*., 2011) e *aox* (Oremland *et al*., 2005) sotto la pressione selettiva esercitata dalla presenza del metalloide.

È stato dimostrato che i batteri in grado di ridurre l'As(V) ad As(III) possono mediare la solubilizzazione dell'As all'interno di matrici contaminate (Macur *et al*., 2001) presentando quindi, in un contesto di bonifica, un notevole vantaggio. La forma trivalente As(III) è infatti molto più mobile e biodisponibile e quindi più facilmente assorbibile da parte della pianta per la successiva rimozione. Nel presente lavoro sono quindi stati testati 10 ceppi per la potenziale capacità di ridurre l'As(V) *in vitro*. La migliore performance è stata esibita dai ceppi *Delftia lacustris* OTU U, in grado di ridurre completamente una concentrazione iniziale di As(V) pari a 5 mM in 48 ore di incubazione, e da *Pseudomonas putida* OTU N in grado di completare la riduzione in 72 ore in condizioni aerobiche. Nonostante non sia stato chiarito il ruolo diretto di questi ceppi nella mobilizzazione dell'As, la loro capacità di riduzione suggerisce che potrebbero avere un ruolo chiave nel processo di fito-estrazione, facilitando l'*uptake* di As(III) da parte delle felci.

Considerando inoltre lo studio in relazione a tratti a tratti PGP (*Plant Growth Promotion*) condotto tra gli isolati risultati maggiormente resistenti ad As(V) e As(III), è stato possibile osservare che:

- 24 ceppi sono in grado di sintetizzare il fito-ormone IAA;
- 24 producono siderofori;
- un ceppo possiede l'enzima ACC deaminasi.

Dallo screening è quindi emerso che la quasi totalità degli isolati analizzati possiede almeno un tratto PGP, ad indice di una potenzialità sinergica dei componenti della comunità batterica in un una approccio di *phytoremediation*. Nonostante rizobatteri con tratti PGP siano stati rilevati in tutti i generi ottenuti dalle colture di arricchimento, la maggior parte dei PGPR appartiene alla classe dei Gamma-proteobatteri, sia per quanto riguarda la produzione di IAA che di siderofori. L'attenzione viene quindi portata ai generi *Pseudomonas* e *Stenotrophomonas*, membri dominanti della cenosi batterica, peraltro già noti in letteratura per la loro attività PGP (Bloemberg and Lugtenberg, 2004; Pages *et al*., 2008).

CAPITOLO 3. Sulla scorta dei risultati ottenuti nelle prime fasi di questo lavoro, si è potuto procedere all'identificazione di validi candidati da poter essere utilizzati in un protocollo di fitobonifica assistita da batteri. La prova si è svolta tramite un protocollo di *bioaugmentation* con i componenti selezionati della collezione batterica. È stato infatti dimostrato che selezionare microrganismi che al contempo siano resistenti al contaminante e PGPR aumenti l'efficienza di fitoestrazione da parte della piante (Koo and Kyung-Suk, 2009; Rajkumar *et al*., 2012).

In ragione di ciò tutti gli isolati scelti presentavano elevata resistenza all'arsenico, la presenza dell'operone *ars* all'interno del genoma coinvolto nella solubilizzazione dell'As e la presenza di diversi tratti PGP. Inoltre il fatto che siano stati isolati dalla stessa matrice garantisce una buona adattabilità al particolare tipo di suolo utilizzato durante l'esperimento.

Per il primo esperimento di fitoestrazione in mesocosmo sono stati utilizzati i ceppi *Ochrobactrum cytisi* OTU E, *Pseudomonas putida* OTU N and *Achromobacter marplatensis* OTU P che sono stati inoculati secondo i protocolli: i) inoculo A: ceppo P, ii) inoculo B: ceppi E + N, iii) inoculo C: tutti i ceppi E + N + P e infine un set di vasi che non è stato inoculato. Alla fine della prova le felci sono state raccolte per misurarne la biomassa e il contenuto in arsenico.

Per quanto concerne la produzione di biomassa è risultato innanzitutto evidente come le felci cresciute in presenza del contaminante (con o senza inoculo) presentassero valori più elevati delle piante cresciute su suolo controllo. Questa osservazione non è precisamente sorprendente, infatti anche altri autori hanno osservato che la biomassa di *P. vittata* aumenta in seguito all'esposizione con arsenico e si ipotizza che questo sia dovuto al fatto che l'As possa indurre nella pianta un incremento nell'*uptake* del fosforo (Ghosh *et al*., 2011). Tuttavia è ancor più degno di nota il fatto che la biomassa vegetale prodotta abbia subito un incremento in presenza di tutti gli inoculi batterici testati, in particolar modo con l' inoculo B (*Pseudomonas* e *Delftia*, produttori di IAA) e l'inoculo C (IAA-produttori e ceppo ACC deaminase positivo, *Pseudomonas, Delftia* and *Achromobacter*).

Per quanto invece concerne il contenuto in As le piante cresciute su suolo contaminato non inoculato hanno mostrato una concentrazione del metalloide nei loro tessuti aerei superiore a quella contenuta nelle piante associate agli inoculi A e B. Sostanzialmente tali inoculi potrebbero aver avuto un effetto detossificante per la pianta, abbassandone il contenuto di As nei tessuti, mentre per l'inoculo C si sono riscontrati dei valori leggermente più alti rispetto alle piante non trattate con batteri. Come dimostrato in vari studi riportati in letteratura si poteva sicuramente attendere un incremento nel contenuto di As in presenza degli inoculi utilizzati (Yang *et al*., 2012; Kumar *et al*., 2008; Rajkumar *et al.,* 2012). Si può quindi ipotizzare che tali ceppi inoculati nei mesocosmi delle felci producano un effetto di fitostabilizzazione dell'arsenico nella rizosfera, probabilmente ossidando l'As(III) ad As(V). Tuttavia, considerata sia la produzione di biomassa che l'accumulo di As, l'inoculo C (composto da tutti i ceppi selezionati) ha dimostrato un sostanziale miglioramento nell'efficacia di fitoestrazione, suggerendo quindi un'attività sinergica tra batteri associati a diversi tratti PGP.

In ultima analisi si è determinata la concentrazione iniziale di As nel suolo contaminato e la concentrazione residua alla fine dell'esperimento nella rizosfera delle felci. Né risultata una notevole diminuzione in tutti i suoli a seguito della crescita di *P. vittata*, sia inoculati che non. Tale effetto è stato particolarmente significativo in presenza degli inoculi nella rizosfera, dove la concentrazione residua del contaminante è scesa al sotto della soglia prevista dalla legge pari a 50 mg/kg per le aree industriali. In dettaglio le felci associate all'inoculo A, B e C hanno funzionato rispettivamente 7, 3 e 4 volte più efficacemente delle felci senza inoculo. Non avendo a disposizione i dati relativi al contenuto di arsenico nelle radici, può essere soli ipotizzato che una buona parte del metalloide sia stato accumulato nell'apparato radicale o, anche se insolito, sia stato metilato e volatilizzato dai batteri della rizosfera (Tsai *et al*., 2009).

Questi risultati comunque confermano l'influenza positiva esercitata specificatamente dalle specie batteriche autoctone in esame sul processo di fito-bonifica, sia per quanto riguarda gli effetti sullo stato di salute della pianta che per l'effettiva rimozione del contaminante dal suolo.

Gli inoculi batterici sono stati ammendati nei vasi con una concentrazione finale pari 10^8 CFU/g di suolo. Durante la sperimentazione la presenza e la persistenza dei ceppi è stata monitorata attraverso la delle analisi PCR-DGGE. Per quanto riguarda la matrice contaminata di Scarlino, tutti gli inoculi sono sempre stati rilevati nelle diverse tempistiche dei campionamenti tuttavia ciò non si è verificato anche nei terreni controlli. Questo potrebbe essere spiegato con il fatto che, essendo gli inoculi autoctoni della matrice, abbiano avuto dei problemi nell'adattarsi ai controlli popolati da una diversa comunità microbica; inoltre, essendo tali cenosi molto complesse, l'intrinseca alta biodiversità potrebbe non essere stata rilevata efficacemente dalla metodica PCR-DGGE.

Tuttavia le varie analisi confermano che in presenza dei ceppi *Achromobacter marplatensis, Ochrobactrum cytisi* and *Pseudomonas putida* sia chiaro il miglioramento della performance delle felci durante il processo di fitoestrazione.

Analogamente al primo esperimento, un secondo inoculo è stato selezionato per essere testato in una seconda prova di fitoestrazione in presenza *di P. vittata*. La pianta, cresciuta su suolo di controllo non contaminato e su suoli di Scarlino è stata così inoculata: i) inoculo A: *P. putida* OTU N e *Delftia lacustris* OTU U, ii) inoculo B: *B. thuringiensis* OTU M, *Variovorax paradoxus* OTU AP, *Pseudoxanthomonas mexicana* OTU AS, iii) inoculo C: ceppi N + U + M + AP + AS and iiii) non inoculata. Le relative analisi sono tuttavia ancora in corso e non è presentato in questa tesi alcun dato in merito all'esperimento.

CONCLUSIONI

Sulla base dei risultati ottenuti e discussi in questo lavoro di dottorato è possibile trarre le seguenti considerazioni finali:

- L'elevata contaminazione presente nell'area industriale ha esercitato una notevole pressione selettiva sulla cenosi batterica autoctona verso una comunità più tollerante, caratterizzata da un'elevata biodiversità, resistenza e potenziale abilità nella trasformazione degli ossianioni As(III) e As(V);
- All'interno dell'area, laddove la contaminazione è più severa si è evidenziata una prevalenza di batteri Gram positivi appartenenti a *phylum Firmicutes* e *Actinobacteria* e ceppi a cui si riconducono interessanti tratti coinvolti nel metabolismi dell'arsenico;
- Gli isolati riconducibili alla specie *Delftia lacustris, Pseudomonas putida,* in grado di ridurre l'As(V) *in vitro* con elevata efficienza, potrebbero potenzialmente solubilizzare l'As nel suolo;
- Tra i membri della comunità batterica isolati in coltura pura, per lo più tra i Gamma-proteobatteri, hanno evidenziato diversi tratti PGP risultando di particolare interesse in una prospettiva di fitobonifica secondo un protocollo di *bioaugmentation*;

- Gli isolati *Ochrobactrum cytisi* OTU E, *Pseudomonas putida* OTU N and *Achromobacter marplatensis* OTU P sono stati selezionati e testati in una prova di fitoestrazione assistita da batteri in presenza delle felce iperaccumulatrice di As *P. vittata*. Essi hanno esercitato effetti positivi sia per quanto riguarda la produzione in biomassa che per quanto riguarda l'efficienza di estrazione del contaminante dal suolo. Invero tutte le felci, con o senza inoculo, sono state in grado di estrarre delle discrete quantità di As anche se, in presenza dei ceppi PGP, si sono dimostrate più efficaci.

Si stanno momentaneamente attendendo i risultati di una seconda prova di fitoestrazione assistita da altri ceppi PGP selezionati (*P. putida, Delftia lacustris, B. thuringiensis, Variovorax paradoxus, Pseudoxanthomonas mexicana*). Queste stime preliminari consentiranno quindi di meglio valutare il potenziale del sistema di fitoestrazione in esami e di programmare quindi una prova in scala pilota nel sito industriale di Scarlino.

Concludendo, si ritiene che un sistema integrato di felci iperaccumulatrici e batteri PGP resistenti all'arsenico possa essere un valido strumento per attuare la bonifica nell'area.

1. Introduction

1.1 Arsenic: a problem of global concern

1.1.1 As general characteristics

The name Arsenic is derived from the Greek word *arsenikon*, meaning potent.

Arsenic (As, atomic number=33) is a ubiquitous element, which occurs naturally in the earth's crust. It ranks 20th in natural abundance, 14th in seawater, and 12th in the human body (Mandal and Suzuki, 2002). When taken together with its notoriety as a poison, its relative sparseness makes it an improbable element to focus upon when considering the pattern of microbial evolution on the early Earth, or in the context of microbial life's occurrence elsewhere in our Solar System. Yet arsenic is, paradoxically, an element that supports diverse microbial life, can accumulate to high concentrations in selected environments, and preliminary evidence suggests that it may have been of importance as an early means of energy generation on the primordial Earth (Olsen *et al.*, 1999; Hamaguchi *et al.*, 1969).

After the early accretionary phase of the Earth as it cooled and geologically differentiated, sulfur and chalcophilic elements like As were significantly enriched within its interior (core and mantle), relative to its crust because of the sinking of these denser metal(loid)-sulfides. Arsenic is often brought back to the Earth's surface from the interior by volcanism, a phenomenon that "distills" upwards the ore bodies of precious metals as well (Cabral *et al.* 2007; Witt-Eickschen *et al.* 2009). Hence, at the beginning of the Archean (~3.8 Ga), it is likely that considerably more arsenic occurred on the surface of our home orb than does today, thereby posing a biochemical challenge (and opportunity) for the early emergent life.

Arsenic exists in four oxidation states, +V (arsenate), +III (arsenite), 0 (arsenic), and –III (arsine). In addition to arsenite, arsenate, and their methylated derivatives, there are "fish arsenic" (arsenobetaine, AB and arsenocholine, AC) and arsenosugar compounds of environmental interest (Ng, 2005). Arsenic occurs mainly as inorganic species but also can bind to organic materials in soils. The As(V)/As(III) couple has a potential of + 130 mV making it a much stronger oxidant than sulfate (sulfate/sulfide = –220 mV) but considerably less than nitrate (NO^{-3}/NO^{-2} = +440 mV) or oxygen (O_2/H_2O= + 818 mV). Arsenite has greater hydrologic mobility and toxicity than As(V). Arsenate also tends to adsorb to more mineral surfaces than As(III), thereby making it less mobile than As(III) in fluvial environments like subsurface aquifers (Ferguson and Gavis, 1972).

The forms of arsenic present in soils, illustrated in Fig. 1.1, depend on the type and amounts of sorbing components of the soil, the pH and the redox potential (Ferguson and Gavis, 1972).

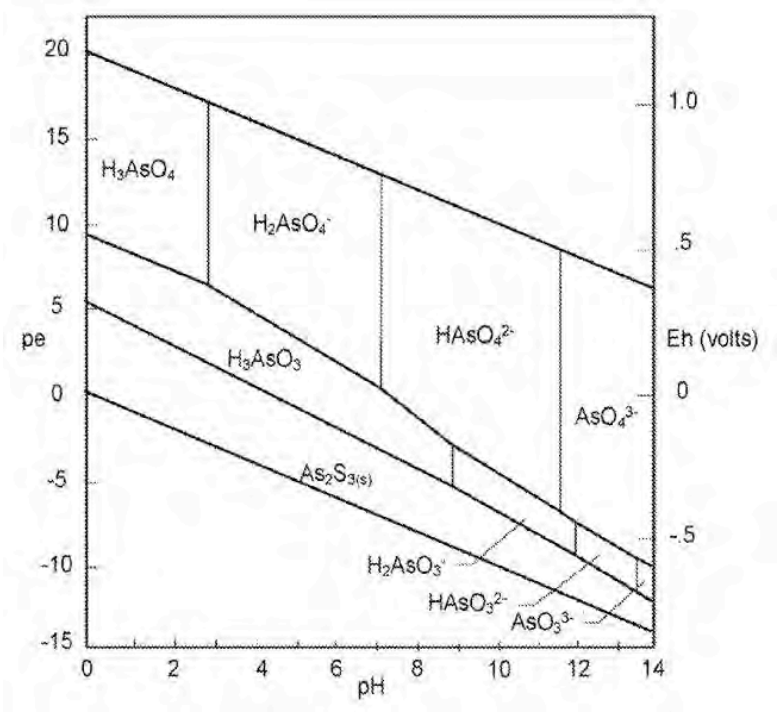


***Fig. 1.1** – The Eh–pH diagram for arsenic at 25 °C and 1 atmosphere (Ferguson and Gavis, 1972).*

Fig. 1.1 shows the Eh–pH diagram for inorganic As compounds in the natural environment. Under oxidizing conditions (high Eh values), inorganic arsenic occurs primarily as H_3AsO_4 (As(V)) at pH>2, and both $H_2AsO_4^-$ and $HAsO_4^{2-}$ species exist in the pH range from 2–11. At low Eh values, H_3AsO_3 is the predominant inorganic arsenic species (As(III)) under reducing conditions. If Eh values below –250 mV exist in the environment, arsenic compounds such as As_2S_3 in the presence of sulfur or hydrogen sulfide can be formed but these conditions are not environmentally relevant. The solubility of these compounds is very limited under neutral and acidic conditions (Ferguson and Gavis, 1972). Under very strong reducing conditions, arsine and elemental arsenic are formed but again, only rarely, if ever in the natural environment.

The inorganic species As(III) and As(V) can be adsorbed on the surface of metallic mineral in the soil and the strength of the bound is mostly on which As can be adsorbed correlated to iron and aluminum content (Goldberg, 2002). The most common complex in nature is represented by pyrite, a widespread mineral with high affinity for As (Bostick and Fendorf, 2005). In detail, in moderately reducing conditions As(III) is the most stable chemical specie and the most adsorbed, especially with high pH values; on the other hand, in oxidant conditions and acidic pH is As(V) to be the most represented and adsorbed onto mineral surface (Jain and Loeppert, 2000). The last one is the common situation in soils but there are many other factors that influence the speciation and the sequestration of As, i.e. the abundance of colloidal substances (fulvic and humic acids) that are responsible for a higher retention of the metalloid thanks to the bounds with iron ions (Macalady and Ranville, 1998). In contrast, when inorganic phosphate or sulfur are present in high concentrations they can compete for the same binding sites and thus allowing a desorption of As from minerals (Jain and Loeppert, 2000; Walsh and Keeney, 1975).

1.1.2 Arsenic toxicity and diffusion in the environment

Arsenic is a natural and ubiquitous element present in many environmental biotopes (sea and fresh waters, soils and rocks). It is released through various natural processes such as weathering, hydrothermal and volcanic emissions (Fig. 1.2). It naturally occurs in over 200 different mineral forms, of which approximately 60% are arsenates, 20% sulfides and sulfosalts and the remaining 20% includes arsenides, arsenites, oxides, silicates and elemental arsenic, but only few of these are commonly encountered in significant amounts in the environment (Onishi, 1969).

Anthropogenic sources exceed natural sources in the environment by 3:1. The major sources that contribute to arsenic release are metal smelting industry, coal combustion, semiconductor industry, mine tailings and pigment production for paints and dyes. The use of arsenic in the chemical weapons has resulted in the contamination of several former military bases in Eastern Europe. Use of arsenic in medicine as chemotherapeutic agent for protozoan diseases, in agriculture as pesticides and herbicide, animal feed additives particularly for poultry and in leather and wood treatments also contributes as additional sources of contamination (Welch *et al.*, 2000).

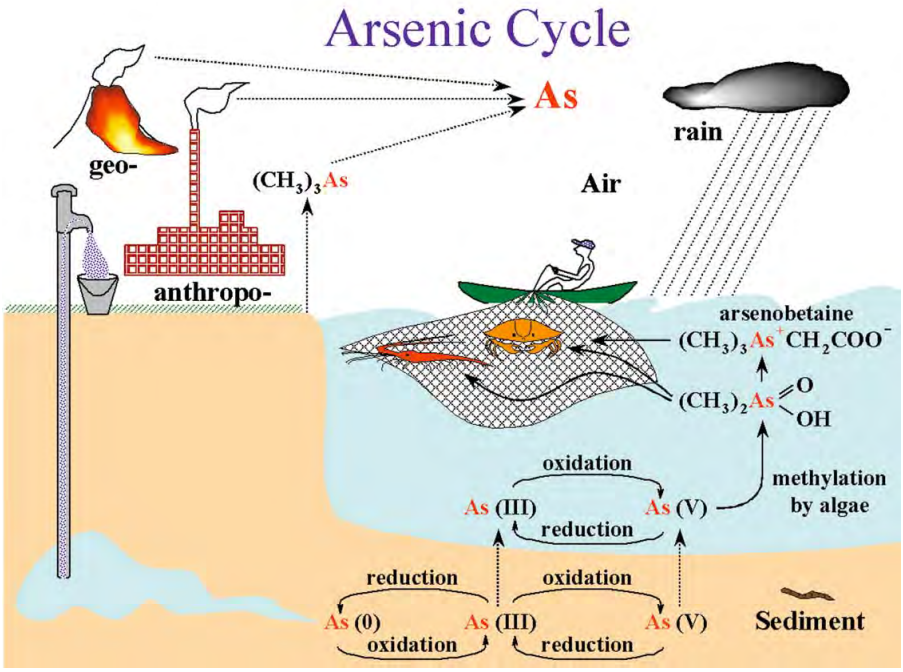


Fig. 1.2 *– The global As geocycle and food chain contamination (Mukhopadhyay et al., 2002).*

Arsenic is toxic to both plants and animals and inorganic arsenicals are proven carcinogens clastogens and teratogens in humans (Ng, 2005). The toxicity of arsenic to human health ranges from skin lesions to cancer of the brain, liver, kidney, and stomach and several heart, respiratory, gastrointestinal, liver, nervous and kidney diseases (Smith *et al.*, 1992). A wide range of arsenic toxicity has been determined that depends on arsenic speciation. Generally

inorganic arsenic species are more toxic than organic forms to living organisms, including humans and other animals and As(III) is usually more toxic than As(V) (Mass *et al.*, 2001).
Once in the cell, As(V) substitutes for phosphate leading to the production of unstable arsenical by-products. It interferes with the normal phosphorylation processes resulting in the uncoupling of oxidative phosphorylation, and thereby disruption of ATP synthesis (Goyer and Clarkson, 2001). Indeed, As(V) reacts enzymatically with the ADP to form an arsenate–phosphate bond. The arsenical ester formed is unstable and spontaneously hydrolyses to ADP and As(V) thus preventing ATP formation. In the same way, ATP-dependent transport, glycolysis, the pentose phosphate pathway and signal transduction pathways (two-component and phosphorelay systems, chemotaxis, etc.) could be impaired (Slyemi and Bonnefoy, 2012).
On the other hand, As(III) has a strong affinity for protein sulfhydryl groups. Indeed, the redox status of cysteine residues can affect both the structure and the activity of numerous enzymes, receptors and transcription factors. As(III) reacts also with dithiol groups present on active sites of many enzymes and on glutathione, glutaredoxin and thioredoxin. These last three metabolites have multiple functions, including intracellular redox homeostasis, deoxyribonucleotide synthesis and repair, protein folding, sulfur metabolism and xenobiotic detoxification. All these processes are therefore inhibited by As(III). Another cause of carcinogenicity of As(III) is likely linked to its capacity to oxidize reduced glutathione, which is the major cellular antioxidant. This oxidation leads to the increase of reactive oxygen species, known to damage macromolecules such as proteins, lipids and DNA (Liu *et al.*, 2001; Dopp *et al.*, 2010).

The World Health Organization recommends As guideline values of 10 µg/L in drinking water (WHO, 2001), but As concentrations in many parts of the world (i.e. Bangladesh, Cambodia, Vietnam, West Bengal, and the United States) often exceed this limit. Arsenic contamination of groundwater used for domestic water supplies has been reported from over 70 countries, affecting the health of an estimated 150 million people (Ravenscroft *et al.*, 2009). This situation is worse in Bangladesh and the West Bengal state of India, and the World Health Organization dubbed this as the “worst mass poisoning” event in human history. In these countries, the mortality rate due to arsenic was estimated recently to be higher than 20% (Argos *et al.*, 2010).
Because of its large distribution and high toxicity, arsenic contamination is a problem of global concern. Inorganic arsenic is indeed regarded as the number one toxin in the USEPA list of prioritised pollutants (Ng *et al.*, 2003).

1.2 Arsenic interaction with biological systems

1.2.1 The microbiology of arsenic

Prokaryotes have not only developed arsenic resistance mechanisms but some of them are even able to utilize arsenic as a source of energy (Silver and Phung, 2005a; Paez-Espino *et al.*, 2009; Tsai *et al.*, 2009). It has even been recently been found that arsenic can substitute for phosphorus in proteins and nucleic acids in *Halomodaceae* strain GFA-J (Wolfe-Simon *et al.*, 2011).

In nature, microorganisms cope with arsenic toxicity in a variety of different ways: the responses include precipitation, chelation, compartmentalization, extrusion or biochemical transformation (Paez-Espino *et al.*, 2009; Tsai *et al.*, 2009). Figure 1.3 shows various strategies that prokaryotes have developed to transform arsenic including arsenite methylation, arsenate reduction for detoxification or respiratory purposes, and arsenite oxidation (Mukhopadhyay *et al.,* 2002; Oremland and Stolz, 2003; Tsai *et al.*, 2009) (Fig. 2). The living organisms, especially microbes, play therefore an important role in the arsenic geocycle (Mukhopadhyay *et al.,* 2002).

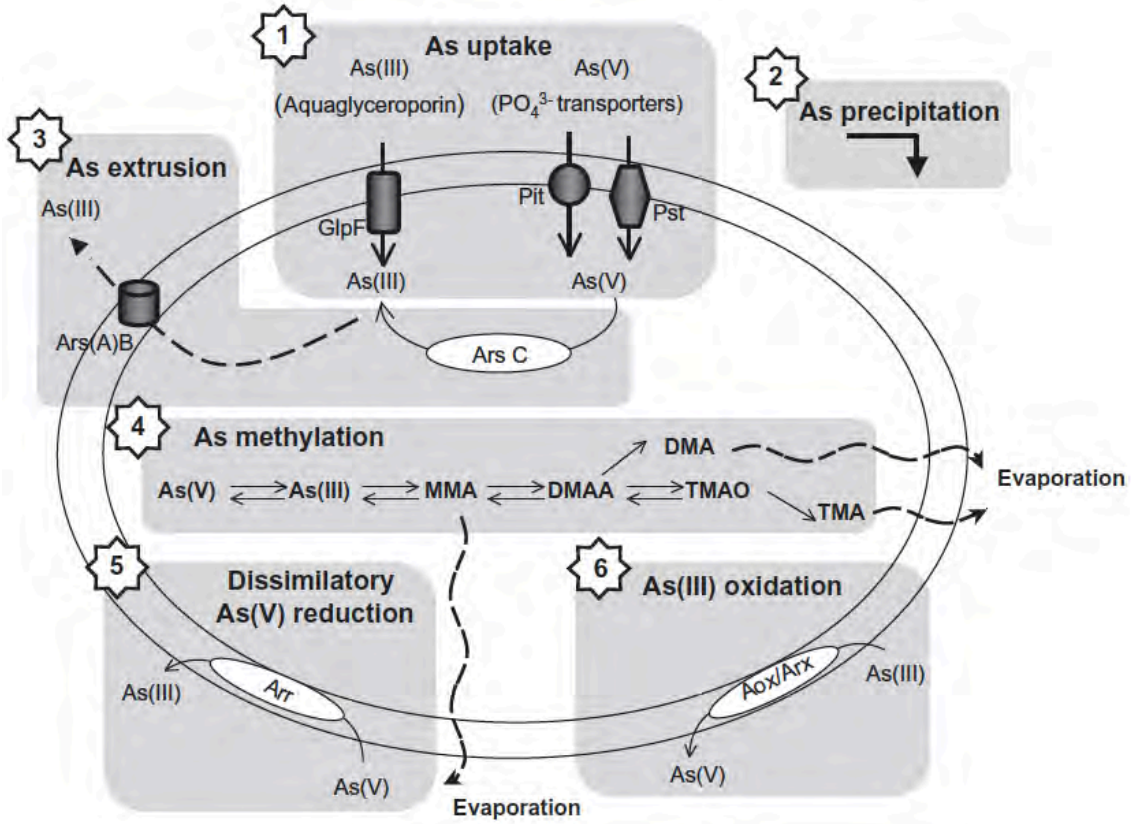


Fig. 1.3 *– Schematic representation of the different processes evolved by prokaryotes to cope with arsenic (Slyemi and Bonnefoy, 2012).*

1.2.1.1 Arsenic uptake

Because of the extreme toxicity of arsenic, specific uptake transporters dedicated to this metalloid are not likely to have evolved. Rather, it appears to enter the cell through the outer membrane by non-selective porins and through the cytoplasmic membrane adventitiously via transporters of chemically analogous molecules.

As(V), which is an analogue of phosphate, enters bacterial cells by phosphate transport systems (Rosen and Liu, 2009) (Fig. 1.3, point 1). Two phosphate transport systems are involved: the constitutive Pit (phosphate inorganic transport) and the phosphate inducible Pst (phosphate specific transport). It appears that As(V) uptake occurs mainly through the Pit system rather than the more specific Pst system.

At a pH lower than 9.3, As(III) exists in the uncharged form $As(OH)_3$ that is structurally similar to glycerol. Therefore, the major route of As(III) entry into bacteria is through aquaglyceroporins that are usually dedicated to the transport of water, glycerol and other small uncharged molecules (Bhattacharjee *et al*., 2008; Bienert *et al*., 2008b; Rosen and Liu, 2009; Rosen and Tamas, 2010) (Fig. 1.3, point 1).

1.2.1.2 Arsenic immobilization

Extracellular precipitation. One of the resistance mechanism is the immobilization of the toxic ion before it enters into the cytoplasm by precipitation outside of the cell, thus preventing its entry into cells and interaction with essential components (Fig. 1.3, point 2). As(V), which is the dominant species under oxidizing conditions, may be removed by sorption onto or precipitation with iron, manganese or aluminum (hydr)oxides, calcium and magnesium compounds as well as by organic matter (Sadiq, 1997; Smedley and Kinniburg, 2002; Bissen and Frimmel, 2003). In reducing and acid environments, the uncharged form $As(OH)_3$ becomes dominant and co-precipitates with oxides, sulfides (as orpiment, As_2O_3, or realgar, AsS) as well as iron sulfides (as arsenopyrite, FeAsS) (Cullen and Reimer, 1989; Carbonell-Barrachina *et al*., 2000). Thus, depending on the As oxidation state, sulfate reducers and iron oxidizers bacteria could maintain this metalloid outside of the cell by reaction with the product of their energy metabolism. In fact, the final product of sulfur and sulfate reduction is H_2S which precipitates with As(V) to form insoluble sulfide complexes (Rittle *et al*., 1995; Newman *et al*., 1997; Keimowitz *et al.,* 2007; Battaglia-Brunet *et al*., 2009). Bacteria mediating Fe(II) oxidation through nitrate reduction in anoxic conditions have been shown to play a key role in arsenic cycling by forming solid hydrous ferric oxide on which As(V) (Senn and Hemond, 2002; Hohmann *et al*., 2010) or As(III) (Gibney and Nusslein, 2007; Hohmann *et al*., 2010) sorbs.

As(III) can also be trapped with Fe(III) tightly bound to the extracellular polymeric substances (EPS) (Duquesne *et al*., 2003). Indeed, the EPS which surround bacteria could stably accumulate metallic ions (Harrison *et al*., 2007). Different bacteria have been shown to have this capacity to sequester metals outside of the cell, including arsenic like in the case of *Herminiimonas arsenicoxydans* (Muller *et al*., 2007; Marchal *et al.,* 2010). However, this

protection is limited, due to the saturation of the metal binding sites at relatively low level of arsenic.

Chelation. Once the metal(loid)s manage to reach the cytoplasm, one mechanism for limiting their accumulation and interaction with essential cellular components is chelation. Such chelation in eukaryote is mediated by peptides or proteins containing thiol ligands, such as glutathione, phytochelatins and metallothioneins (Tsai *et al*., 2009; Ngu and Stillman, 2009a,b). In *Cyanobacteria* and in *Pseudomonas* sp., metallothioneins have been detected and shown to coordinate different metals (Turner and Robinson, 1995; Robinson *et al*., 2001).

Intracellular sequestration. In the yeast *Saccharomyces cerevisae*, the vacuolar membrane-associated protein Ycf1p, a member of the ABC transporter superfamily, pumps a wide range of GSH-conjugated substrates, including $As(GS)_3$, into the vacuole allowing removal of arsenic from the cytosol (Tsai *et al*., 2009). Such intracellular sequestration of arsenic may not occur in prokaryotes since they are generally devoid of similar vacuoles but arsenite can be detoxified by complexation with Cys-rich peptides (Paez-Espino *et al.,* 2009).

1.2.1.3 Arsenic extrusion: the *ars* system

The most widely distributed and the best characterized system for As(III) detoxification is the *ars* system (Fig. 1.3, point 3), which may be either plasmid or chromosomal encoded (Rosen, 1999; Silver and Phung, 2005b; Stolz *et al*., 2006; Paez-Espino *et al*., 2009). *Ars* genes appear systematically co-transcribed by a large variety of genomic configurations arranged in a fashion that depends on the specific strain. The minimal gene set arranged in one operon *arsRBC* is found in the chromosome of *E. coli* (Carlin *et al.* 1995; Diorio *et al.* 1995) and *P. fluorescens* MSP3 (Prithivirajsingh *et al*. 2001a,b), as well as in the *Staphylococcus* plasmids pI258 and pSX267 (Silver, 1998). It consists of an arsenate reductase (ArsC), an arsenite efflux pump (ArsB) and a repressor (ArsR). An enlarged version of the core genes is found in *E. coli* plasmids R773 and R46 (Silver, 1998) and plasmid pKW301 of *Acidophulus multivurum* AIU301, (Suzuki *et al*. 1997; Suzuki *et al*. 1998). These enlarged operons are arranged as a gene cluster *arsRDABC*. ArsA is an ATPase that provides energy to ArsB for the extrusion of arsenite and antimonite. ArsD is identified as an arsenic chaperone for the ArsAB pump, transferring the trivalent metalloids As(III) and Sb(III) to the ArsA subunit of the pump (Lin *et al*. 2007) and increasing the affinity of ArsA for As(III). ArsD is a homodimer with three vicinal cysteine pairs in each monomer. Other strains harbor the core *ars* genes in different arrangements like in the case of *Acidithiobacillus ferroxidans*, in which the *ars* genes are arranged in two divergently transcribed operons, *arsRC* and *arsBH* (*arsH* function has not been clearly understood yet).

As(V) detoxification involving its enzymatic reduction to As(III), followed by As(III) export, is conserved from bacteria to plants (Ellis *et al*., 2006).

Besides ArsB, another family of As(III) extrusion pumps exists in prokaryotes: the Acr3 family. Acr3 is more widely distributed and is found in members of every kingdom while ArsB has not been found in eukaryotes (Mansour *et al*., 2007). Surprisingly, Acr3 appears to be selective for As(III) over Sb(III) although these two metalloids have similar chemical properties (Fu *et al*., 2009).

Synorhizobium meliloti presents a different mechanism of extrusion: it utilizes a unique arsenic detoxification pathway wherein As(III) flows out of the cell through an aquaglyceroporin, AqpS, that is encoded within the *ars* operon in place of *arsB* (Yang *et al*., 2005; Rosen and Tamas, 2010).

No As(V) extrusion pump has been identified to date. In fact, the less toxic As(V) is converted by the cytoplasmic arsenate reductase ArsC to the more toxic As(III), which is then pumped out of the cell (Rosen, 1999; Silver and Phung, 2005b; Stolz *et al*., 2006; Paez-Espino *et al*., 2009).

Concerning the other fundamental component of the *ars* operon, the arsenate reductase (ArsC), it has been observed that all these detoxifying enzymes are small, monomeric and cytoplasmic enzymes that reduce As(V) to As(III). However, two distinct, but related, families that have evolved convergently were identified in prokaryotes: the thioredoxin- and glutaredoxin-coupled arsenate reductases.

The thioredoxin-coupled arsenate reductase class is typified by the ArsC protein from pI258 plasmid of *Staphylococcus aureus*. These ArsC reductases use the cysteine thiol-coupling enzyme thioredoxin as the reductant and require the presence of thioredoxin reductase and NADPH to complete the catalytic cycle (Mukhopadhyay and Rosen, 2002; Messens and Silver, 2006). On the other hand, the glutaredoxin-coupled arsenate reductase family is represented by the product of the *arsC* gene of the *E. coli* R773 plasmid *ars* locus. It has only 12% identity with ArsC encoded by the *S. aureus* pI258 plasmid and their tertiary structures are not related at all. If the first type requires NADPH, these ArsC reductases require reduced glutathione (GSH) and the small thiol transfer protein glutaredoxin for their arsenate reductase activity (Mukhopadhyay and Rosen, 2002; Messens and Silver, 2006).

1.2.1.4 Arsenic methylation/demethylation

Methylation of arsenic is a widespread phenomenon in nature. From bacteria to humans, many organisms can methylate the element to different extents. The general picture (as shown in Fig. 1.3, point 4) is that methylation pathway(s) involve a series of steps in which the reduction of the pentavalent form of As is followed by the oxidative addition of a methyl group (Dombrowski *et al*. 2005) thereby generating a growingly methylated series of As chemical species: methyl arsenite (MMA), dimethyl arsenate (DMA-V), dimethyl arsenite (DMA-III) and trimethyl arsine oxide (TMAO).

Glutathione and other thiol-containing compounds are involved in the reduction steps. The methylation reactions require S-adenosylmethionine as the source of methyl groups while in anaerobic bacteria methylcobalamin may be utilized. An arsenite S-adenosylmethionine

methyltransferase (ArsM) catalysing transfer of methyl groups from S-adenosylmethionine to As(III) with glutathione as electron donor, has been characterized in *Rhodopseudomonas palustris* (Qin *et al*., 2006; Yuan *et al*., 2008). The *arsM* gene is widespread and has been detected in more than 120 prokaryotes (including Archaea) (Stolz *et al.,* 2006).

Microorganisms are thought to play a key role in regenerating As(V) by demethylation of methylated arsenic species to use them as carbon and energy sources. *Alcaligenes*, *Pseudomonas* and *Mycobacterium* have been proven able to demethylate mono- and dimethyl arsenic compounds (Bentley and Chasteen, 2002) and, at least, *Pseudomonas* sp., has been shown to use DMA as carbon source (Maki *et al*., 2004). However, the mechanism of demethylation is still completely unknown.

1.2.1.5 Dissimilatory As(V) reduction: the *arr* system

The other mechanism of As(V) reduction is carried out by dissimilatory arsenate reductases with the utilization of As(V) as a terminal electron acceptor in bioenergetic processes (Ahmann *et al*., 1994) (Fig. 1.3, point 5). As(V) reduction coupled with the oxidation of an electron donor with a lower redox potential provides sufficient energy to sustain bacterial growth and microbes respiring As(V) under anaerobiosis have been found throughout the prokaryotes, including Proteobacteria, Gram-positive Eubacteria, thermophilic Eubacteria and Crenarchaeota, from different environments (Silver and Phung, 2005a; Stolz *et al*., 2006; Paez-Espino *et al*., 2009; Oremland *et al*., 2009). These microorganisms can use a full range of electron donors for As(V) reduction including inorganics (hydrogen and sulfide), organics such as acetate, lactate, formate and pyruvate, and even more complex compounds such as benzoate, phenol and toluene (Stolz *et al*., 2006).

Dissimilatory arsenate reduction is carried out by a terminal reductase, fundamentally different from the detoxifying arsenate reductases. Arsenate reductase ArrAB, characterized in *Chrysiogenes arsenatis* (Krafft and Macy, 1998), *Bacillus selenitireducens* (Afkar *et al*., 2003), and *Shewanella trabarsenatis* strain ANA-3 (Malasarn *et al*., 2008), is a heterodimer composed of a large and a small subunits of about 100 kDa (ArrA) and 30 kDa (ArrB) respectively (Fig. 1.4). ArrA, which belongs to the DMSO reductase family, contains the As(V) binding site and the catalytic site with a bismolybdopterin guanine dinucleotide cofactor and one [4Fe-4S] cluster while ArrB is an iron–sulfur protein with four [4Fe-4S] clusters.

It has to be pointed out that no archaeal Arr representatives have been detected so far (Duval *et al*., 2008).

1.2.1.6 As(III) oxidation

Because As(V) exhibits stronger reactivity with specific surface minerals (ferrihydrite, ferric oxide, calcite, aluminum, etc.) and humic substances than As(III), arsenite oxidizers play an important role in the immobilization of arsenic. A wide range of phylogenetically diverse prokaryotes isolated from various habitats (cattle-dipping baths, raw sewage, fresh and marine

waters, geothermal environments, mine tailings, arsenic deposit) was reported (Ehrlich, 2002; Oremland and Stolz, 2003; Stolz *et al.*, 2006; Oremland *et al.*, 2009).

As(III) oxidation (Fig. 1.3, point 6) is mediated by two distinct enzymes: the AoxAB (also named AroBA or AsoBA), which has been extensively studied, and ArxAB, which has been found only recently and which has closer phylogenetic relatedness to the dissimilatory arsenate reductase ArrAB than to AoxAB arsenite oxidase. The ArxAB enzyme has been detected so far only in *Ectothiorhodospiraceae* family (Kulp *et al.*, 2008; Richey *et* al., 2009; Oremland *et al.*, 2009) while the first is widespread.

The AoxAB-type arsenite oxidase consists of two heterologous subunits: a large subunit (AoxB) belonging to the DMSO reductase family with a molybdenum cofactor together with a [3Fe-4S] center and a small subunit (AoxA) containing a 'Rieske' [2Fe-2S] center (Ellis *et al.,* 2001; Duval *et al.*, 2010). Interestingly, both the phylogenetic tree of the molybdopterin subunits of the DMSO reductase family and that of the Rieske proteins indicate that the arsenite oxidase has emerged before the *Archaea*/*Bacteria* split, that is more than 3 billion years ago (Lebrun *et al.*, 2003). Arsenite oxidation might be one of the 'ancient' bioenergetics pathways in hydrothermal environments known to be rich in arsenic and considered as vestiges of the primordial earth when life has appeared (Lebrun *et al.*, 2003).

Furthermore, the phylogenetic relatedness of this type of arsenite oxidase is more closely related to ArrAB respiratory arsenate reductases than to ArxAB arsenite oxidases (Fig. 1.4) (Zargar *et al.*, 2009) and it's been proposed to be an ancestral form of the ArrAB respiratory arsenate reductases and ArxAB arsenite oxidases (Oremland *et al.*, 2009).

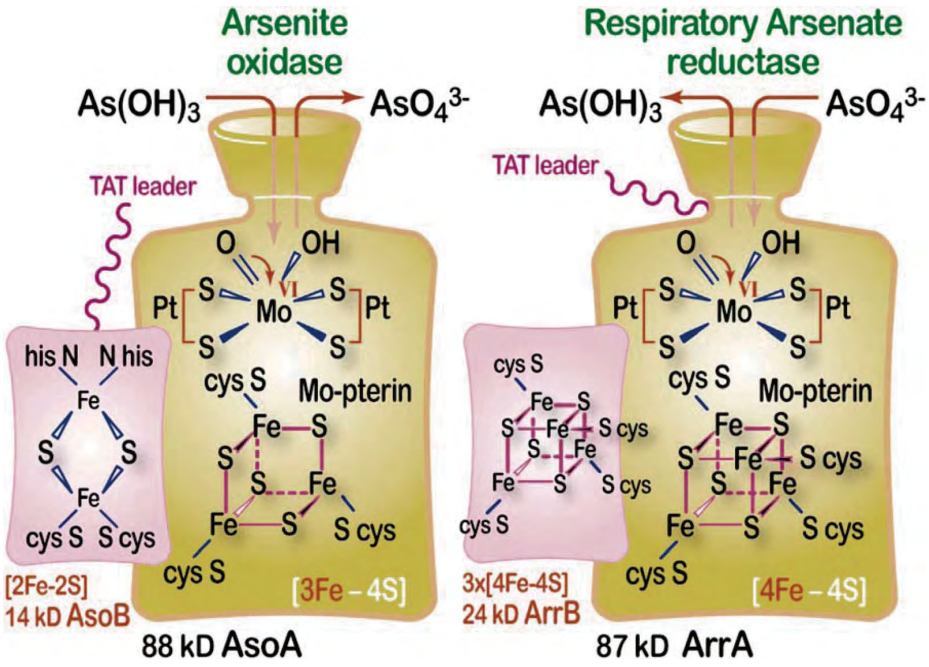


Fig. 1.4 *– The arsenite oxidase and arsenate reductase of prokaryotes (Oremland et al., 2009).*

No homologues for the *aoxAB* genes have been detected in the arsenite oxidizers *Alkalimnicola ehrlichii* (Hoeft *et al*., 2007; Rhine *et al*., 2007), *Azoarcus* sp. strain DAO1 (Rhine *et al*., 2007) and *Ectothiorhodospira* sp. strains PHS-1 and MLP2 (Kulp *et al*., 2008), all of which oxidize As(III) in anoxic conditions, suggesting that an alternative mechanism is utilized in these conditions. Indeed, the *arxA* and *arxB* genes coding for a novel arsenite oxidase were identified in these microrganisms. ArxB has been predicted to be a small subunit with four [4Fe-4S] iron–sulfur clusters and ArxA to be a large subunit containing a molybdenum cofactor and a [4Fe-4S] cluster (Richey *et al*., 2009). This *arx* operon is only expressed under anaerobic conditions in the presence of As(III) (Zargar *et al*., 2010).

1.2.2 Biochemistry of arsenic in higher plants

1.2.2.1 Arsenate and arsenite uptake

Plants cope with As(V) and As(III), the two biologically important species, using mechanisms that are partly similar to those discovered in microbes (Tripathi *et al*., 2007). The main route of As(V) uptake in plants is through the phosphate transporters as a phosphate analogue (Asher and Reay, 1979) whereas As(III) is transported in the neutral $As(OH)_3$ form through aquaglyceroporins (Meharg and Jardine, 2003) (Fig. 1.5). There are over 100 phosphate transporters in the Phosphate transporter 1 (Pht1) family, most of which are strongly expressed in roots and are likely to be involved in phosphate uptake from the external medium (Bucher, 2007). Reduced uptake of arsenate is a well-known mechanism of arsenate resistance employed by some plant species (*Holcus lanatus* for instance), which is achieved through a suppression of the high-affinity phosphate/arsenate uptake system in the resistant plants (Meharg and Hartley-Whitaker, 2002).

Little was known about the mechanisms of arsenite uptake in plants until recently. Research on arsenite uptake mechanisms in plants has benefited greatly from the knowledge gained from microbial studies. In *Escherichia coli*, yeast and humans, some aquaglyceroporins, a subfamily of the aquaporin superfamily with larger pores to allow passage of neutral molecules such as glycerol, can transport arsenite (Bhattacharjee and Rosen, 2007). Recently, evidence that some plant aquaporin channels can mediate arsenite influx, has been reported in *A. thaliana, Lotus japonicas* and *Oriza sativa* (Bienert *et al.,* 2008b). Short-term (20 min) uptake experiments with excised rice roots showed that the maximum (V_{max}) of arsenite influx was comparable to that of arsenate in the absence of phosphate and, at high concentrations (> 100 µM), arsenite influx was substantially faster than arsenate (Abedin *et al*., 2002b; Meharg and Jardine, 2003).

Low-level uptake of organic As species, such as monomethylarsonic acid and dimethylarsinic acid, has also been observed in rice but the underlying transport pathways are unknown (Meharg, 2004).

1.2.2.2 Arsenic efflux

Following uptake of arsenate by roots, it can be lost from the cells via efflux to the external medium (Mimura, 1999) or be rapidly converted to arsenite by the roots cells (Xu *et al*., 2007). It appears that arsenite efflux by roots is very rapid immediately following arsenate uptake, and diminishes once the arsenate supply is withheld, possibly because cellular arsenite is complexed with thiols and sequestered in the vacuoles (Par. 1.2.2.3). Some characteristics of arsenite efflux by tomato roots remind microbial As(III) extrusion pump, suggesting a possibility of ArsB- or Acr3-like carriers for the efflux (Xu *et al*., 2007) however, direct evidence has still to be obtained.

Another possible mechanism responsible for arsenite efflux from plant roots involves aquaporin channels, some of which allow bidirectional passage of solutes (Mitani *et al*., 2008). Similarly in the legume symbiont *Sinorhizobium meliloti*, the arsenic resistance (*ars*) operon includes an aquaglyceroporin (*aqpS*) in place of *arsB*, which confers arsenate resistance possibly through arsenite efflux (Yang *et al*., 2005).

Furthermore in aerobic soils, arsenite is oxidized rapidly to arsenate either chemically by reactions with manganese oxide (Oscarson *et al*., 1981) or by arsenite-oxidizing microbes (Macur *et al*., 2004). Thus, soil, plant roots and microbes are likely to be engaged constantly in the reduction–oxidation cycle of arsenate – arsenite.

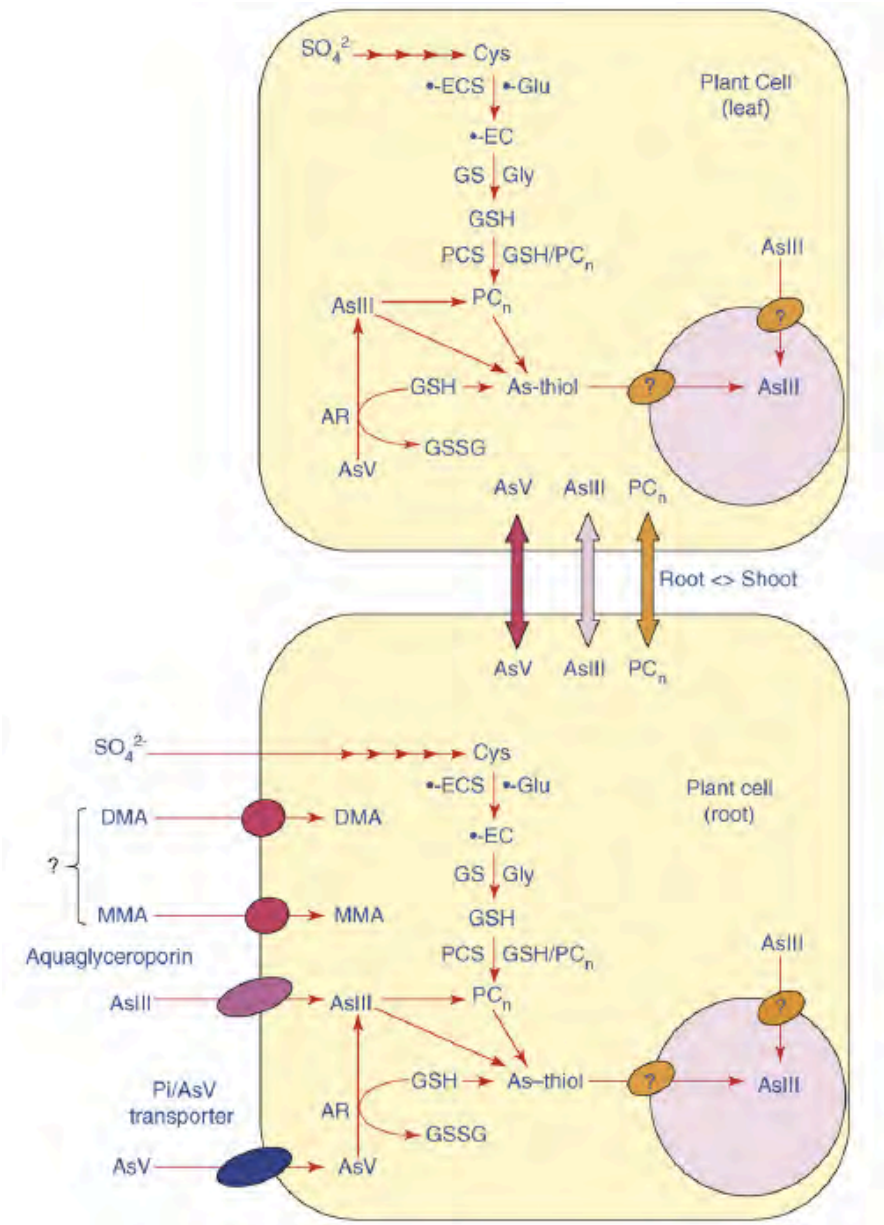


Fig. 1.5 *– Schematic mechanisms of arsenic uptake, translocation and detoxification in plants (Tripathi et al., 2007).*

1.2.2.3 Arsenic metabolism in plants

The main mechanisms involved in As resistance and transformation in plants are illustrated in (Fig. 1.5) and described in the following paragraph.

Arsenate reduction. Analysis of As speciation in plant tissues generally shows that As is predominantly present in the As(III) oxidation state, even though plants had been exposed to arsenate (Pickering *et al*., 2000; Dhankher *et al*., 2002). This means that, following uptake, arsenate is reduced efficiently to arsenite in plant cells, and that most plants have a high capacity for arsenate reduction. Based on the sequence homology to the yeast As(V) reductase Acr2p, plant homologues (ACR2) have been cloned and characterized from *A. thaliana* (Dhankher *et al*., 2006), *H. lanatus* (Bleeker *et al*., 2006), rice (Duan *et al.,* 2007) and the As hyperaccumulator *P. vittata* (Ellis *et al*., 2006).
It is possible that other enzymes or pathways of arsenate reduction exist in plants. Recently, Rathinasabapathi *et al*. (2006) reported that a cytosolic triosephosphate isomerase (TPI) isolated from *P. vittata* may be involved in arsenate reduction directly or indirectly.

Complexation and sequestration of arsenic. Arsenite has high affinity to the sulphydryl groups of peptides such as GSH and phytochelatins (PCs). These complexes are stable in the pH range from 1.5 to 7.0–7.5, but dissociates at higher pH (Delnomdedieu *et al*., 1994).
There is strong evidence that complexation of arsenite by PCs is an important mechanism of As detoxification, and hence tolerance, in As non-hyperaccumulating plants. Exposure to arsenate or arsenite induces a large response in the synthesis and accumulation of PCs in plants (Srivastava *et al*., 2007; Schulz *et al*., 2008) and, by contrast, inhibition of PC synthesis by treatment with L-buthionine-sulphoxime (BSO), a potent inhibitor of γ-glutamylcysteine synthetase, leads indeed to hypersensitivity to As (Schmöger *et al*., 2000; Hartley-Whitaker *et al*., 2002; Schat *et al*., 2002).

Methylation. In field-collected samples, methylated As species may originate from the soil. However, in hydroponic cultures where no methylated As species were present in the medium, DMA and/or MMA was found in plant tissues or xylem sap at low concentrations, usually < 1%, of the total As concentration (Quaghebeur and Rengel, 2003; Raab *et al*., 2007a; Xu *et al*., 2007), suggesting that *de novo* methylation of As occurs in plants.

1.2.2.4 Arsenic translocation: from roots to shoots

Unlike P, As has, generally, a low mobility with respect to translocation from roots to shoots in plants except the hyperaccumulators (Quaghebeur and Rengel, 2004). The explanation is probably that arsenate is reduced to arsenite rapidly in roots, followed by complexation with thiols and possibly sequestration in the root vacuoles (Dhankher *et al.,* 2006).

In all of the plant species studied, inorganic arsenite, not complexed with –PC or –GS, is the predominant form of As in the xylem sap (Ma *et al*., 2008; Su *et al*., 2008). The fact that arsenite is the dominant form of As in xylem sap is not surprising, considering that arsenate is reduced rapidly in roots. The ratio of As concentration in the xylem sap to that in the external nutrient solution vary widely among plant species. This ratio is well below 1 in non-hyperaccumulating plants, among which rice stands out as the most efficient in transporting As to the xylem, and above 1 for hyperaccumulating plants.

1.2.2.5 Arsenic hyperaccumulation: the fern *Pteris vittata*

In 2001 Ma *et al.* discovered a brake fern, *Pteris vittata*, extremely efficient in extracting arsenic from soils and translocating it into its above-ground biomass (Fig. 1.6). This fern was growing on a site in Central Florida contaminated with chromated copper arsenate in concentration up to 1600 ppm and it was found to contain 1,442–7,526 ppm arsenic in its fronds.

Fig. 1.6 *– Brake fern growing on an abandoned wood preservation site contaminated with chromated copper arsenate.*

In this work (Ma *et al*., 2001) was reported that *P. vittata*, as well as being tolerant of soils containing as much as 1,500 ppm arsenic, it can take up large amounts of arsenic into its fronds in a short time. Indeed arsenic concentration in fern fronds growing in soil spiked with 1,500 ppm arsenic increased from 29.4 to 15,861 ppm in only two weeks while As concentrations in brake fern roots were less than 303 ppm. Furthermore, in the same period, ferns growing in soil containing just 6 ppm arsenic accumulated 755 ppm of arsenic in their fronds, a 126-fold enrichment.

As well as removing arsenic from soils containing different concentrations of arsenic, brake fern also removed arsenic from soils containing different arsenic species ($FeAsO_2$, $AlAsO_2$, Na_2HAsO_4, K_2HAsO_4, NaMMA, $NaAsO_2$, CaMMA and $Ca_3(AsO_4)_2$) and again, up to 93% of the arsenic was concentrated in the fronds.

Compared with the non-hyperaccumulator *Pteris ensiformis, P. vittata* possesses a higher antioxidant capacity and also maintains a lower concentration of reactive oxygen species (Srivastava *et al*., 2005; Singh *et al*., 2006). By contrast to non-hyperaccumulators, which rely on PC complexation for As detoxification and tolerance, very little of the As accumulated in the roots and fronds of *P. vittata* and *Pteris cretica* (~1–3% of the total As) is complexed with PCs (Raab *et al*., 2004; Zhang *et al*., 2004). The majority of As (60–90% of the total As) in the fronds of *Pteris* species is indeed inorganic arsenite (Francesconi *et al*., 2002; Webb *et al*., 2003; Su *et al*., 2008), which appears to be stored in the vacuoles (Lombi *et al*., 2002; Pickering *et al*., 2006). Vacuolar sequestration of arsenite is therefore the key mechanism of As detoxification in the hyperaccumulator ferns too.

But the most striking difference between non- and hyperaccumulators arsenate lies in the efficiency of root to shoot translocation, exemplified by the large ratios of shoot to root As concentrations in the As hyperaccumulators (typically 5–25) (Tu and Ma, 2002; Zhao *et al*., 2002). The ratio of the As concentration in the xylem sap of *P. vittata* to that in the nutrient solution is usually about 2 orders of magnitude higher than that in the non-hyperaccumulators. Su *et al.* (2008) showed that the majority (93–98%) of the As in the xylem sap of *P. vittata* was in the form of arsenite, regardless of whether the plant was treated with arsenate or arsenite. Roots or rhizoids of *P. vittata* are likely to be the main location of arsenate reduction, with arsenite being preferentially loaded into the xylem (Duan *et al*., 2005).

Also, hyperaccumulators differ from non-hyperaccumulators in that there is minimal efflux of arsenite from the roots of *P. vittata* to the external medium (Su *et al.,* 2008). This, together with little PC complexation of arsenite in *P. vittata* roots (Zhao *et al*., 2003), may explain the highly efficient xylem transfer in hyperaccumulator ferns.

Moreover, brake fern is mesophytic and is widely cultivated and naturalized in many areas with a mild climate. In the United States, it grows in the southeast and in southern California (Jones, 1987). The fern is versatile and hardy, and prefers sunny and alkaline environments (where arsenic is more available). It has considerable biomass, and is fast growing, easy to propagate, and perennial. These characteristics suggest a high potential of *P. vittata* in remediating arsenic-contaminated soils cheaply.

1.3 Possible exploitation of biological processes for the treatment of As polluted soils

Conventional remediation technologies like physical, chemical and thermal processes have been used to clean up metal-contaminated sites because they are relatively insensitive to the heterogeneity in contaminated matrix and can function over a wide range of oxygen, pH, pressure, temperature, and osmotic potentials (Cunningham and Berti 1993; Mohan and Pittman 2007). However, they are expensive and time-consuming, often hazardous to workers, and produce secondary wastes that may not be environmental friendly. Thus the disadvantages of conventional remediation methods represent the forces behind the search for alternative remediation technologies, such as phytoremediation (Bhattacharya *et al*. 2002; Naidu *et al.* 2006).

The United States Environmental Protection Agency (USEPA) defines bioremediation as a treatability technology which uses biological activity to reduce the concentration and/or toxicity of a pollutant. Bioremediation in fact uses biological systems to remediate contaminated soil and water, by destroying or rendering harmless various contaminants using natural biological activity (Shukla *et al.,* 2010). Bioremediation technologies offer many advantages over traditional remediation technologies as they can be applied *in situ* without the need for removal and transport of contaminated soil, are less expensive and require low-technology input. Moreover, these technologies are environmentally friendly, in fact they are non-intrusive, they do not alter the soil matrix and cause no collateral destruction of the site material or its indigenous flora and fauna (Salt *et al*. 1998). Besides they help preventing landscape destruction and enhances activity and diversity of soil microorganisms, maintaining healthy ecosystems. Moreover they generally have a high level of public acceptance and are aesthetically pleasing (Zhuang *et al*., 2007; McGuinness and Dowling, 2009).

1.3.1 Phytoremediation

Phytoremediation is defined as the use of green plants to remove pollutants from the environment or to render them harmless (Kramer, 2005; Salt *et al*., 1998). This technique involves a number of processes and, in case of As polluted soil, phytostabilization and phytoextraction represent the best solutions. The former is used to reduce the bioavailability of pollutants in soils and the latter involves the cultivation of tolerant plants that concentrate soil contaminants in their above-ground tissue. At the end of the growth period, plant biomass is harvested, dried or incinerated, and the contaminant-enriched material is deposited in a special dump or added into smelter. After harvest, a lower level of the pollutant will remain in the soil, so the growth/harvest cycle must usually be repeated through several crops to achieve a significant cleanup. After the process, the cleaned soil can support other vegetation (Shukla *et al.,* 2010; Karami and Shamsuddin, 2010).

Although these technologies are still in developmental stages, in particular there only few reports of the application in case of As polluted soil, they appear to have great potential for the clean-up of contaminated soils (Sharma and Dubey, 2005; Chaudhry *et al.*, 2005).

The efficiency of a phytoremediation approach depends on both metal concentration reached in plant tissues and on biomass production. Therefore the success of phytoextraction depends upon the identification of a suitable plant species, able to tolerate and hyperaccumulate the heavy metals, and to produce large amounts of biomass using established agricultural techniques (Manousaki and Nicolas, 2009; Karami and Shamsuddin, 2010).

In the cases of As contaminated soils, the ideal candidate is represented by the fern *Pteris vittata* for all the reasons described previously (par 1.2.2.5).

1.3.2 How to improve As phytoremediation: plant-microbe interaction

Since 1904, when the term 'rhizosphere' was first coined by Hiltner (1904), rhizosphere processes of plants have been widely investigated; however, little attention has been paid to the microbial community of rhizosphere of plants growing on metal contaminated sites. Soil microorganisms, including plant root associated free-living as well as symbiotic rhizobacteria and mycorrhizal fungi, are integral part of the rhizosphere biota. The overall result of plant–rhizosphere microbe interactions is a higher microbial density and their metabolic activity in the rhizosphere, even in metal contaminated soils (van der Lelie, 1998). In exchange plant root exudates provide nutrition to rhizosphere microbes, thus increasing microbiological activity in the rhizosphere, which in turn stimulate plant growth.

1.3.2.1 Plant Growth Promoting Rhizobacteria (PGPR)

PGPR may promote plant growth directly – usually by either facilitating resource acquisition or modulating plant hormone levels – or indirectly – by decreasing the inhibitory effects of various pathogenic agents on plant growth and development, that is, by acting as biocontrol bacteria (Glick, 1995). In the present work, attention has been paid mainly to the direct plant growth promoting traits, of which a schematic illustration in metal-contaminated soils is presented in Fig. 1.7 and, to the ways of PGPR to enhance metal uptake in phytoextraction protocols.

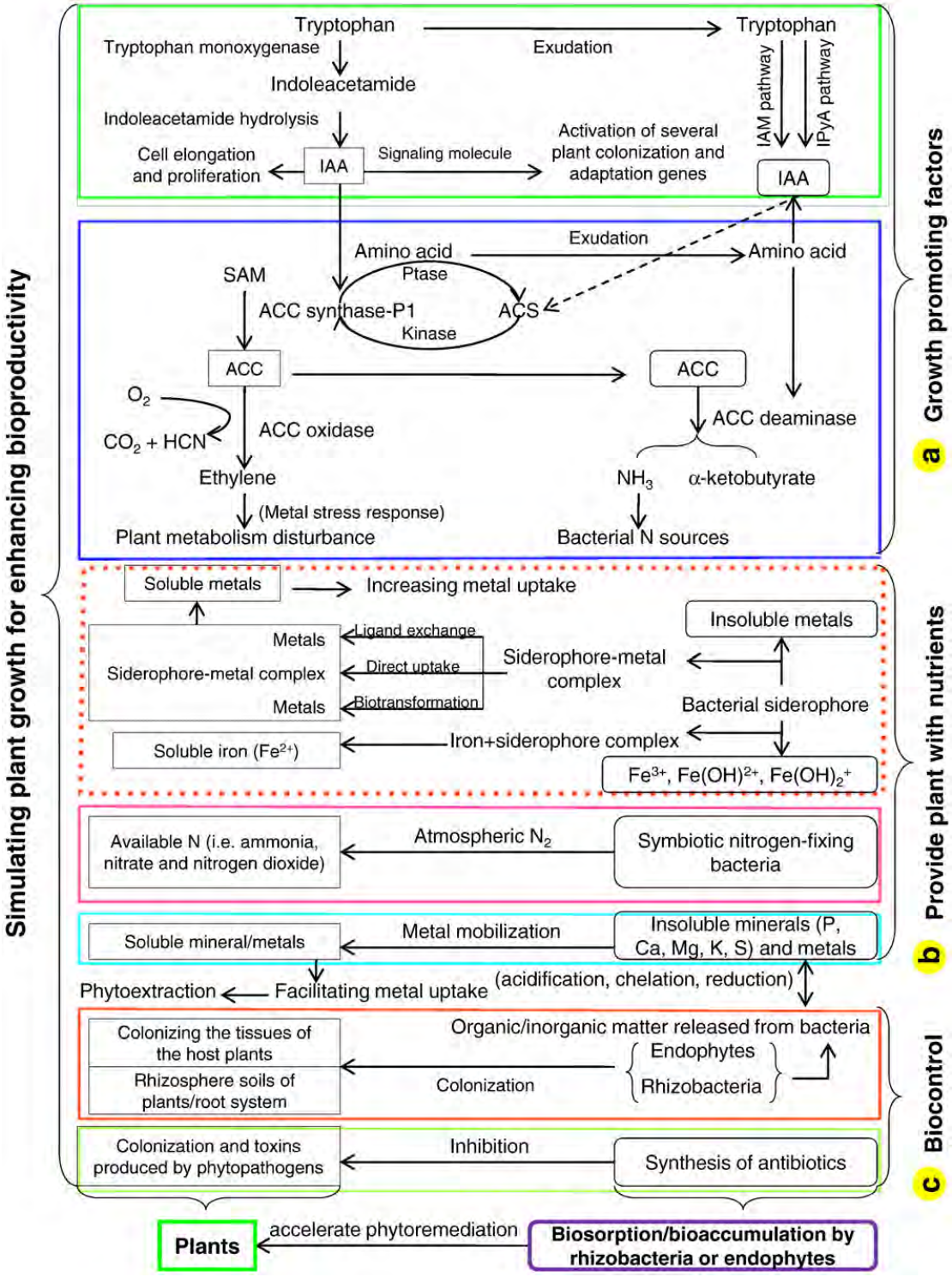


Fig. 1.7 *– Plant growth promoting rhizobacteria and endophytes accelerates phytoremediation of metalliferous soils though modulation of (a) plant growth promoting parameters, (b) by providing plants with nutrients, and (c) controlling disease through the production of antifungal metabolites (Ma et al., 2011a).*

1.3.2.1.1 Facilitating Resource Acquisition.

The best-studied mechanisms of bacterial plant growth promotion include providing plants with resources/nutrients that they lack such as iron, and phosphorus.

Phosphorus solubilisation. Despite the fact that the amount of phosphorus in the soil is generally quite high (between 400 and 1,200 mg/kg of soil) most of this phosphorus is insoluble and therefore not available to support plant growth (Feng *et al*., 2004). Thus, solubilization and mineralization of phosphorus by phosphate-solubilizing bacteria is an important trait in PGPR as well as in plant growth-promoting fungi such as *mychorrizae* (Richardson, 2001; Rodríguez and Fraga, 1999). Typically, the solubilization of inorganic phosphorus occurs as a consequence of the action of low molecular weight organic acids such as gluconic and citric acid, both of which are synthesized by various soil bacteria (*Bacillus* sp., *Rhodococcus* sp., *Arthrobacter* sp., *Serratia* sp., *Delftia* sp.) (Bnayahu, *et al.,* 1991; Rodriguez *et al*., 2004; Chen *et al*., 2006). On the other hand, the mineralization of organic phosphorus occurs through the synthesis of a variety of different phosphatases, catalyzing the hydrolysis of phosphoric esters (Rodriguez and Fraga, 1999). Importantly, phosphate solubilization and mineralization can coexist in the same bacterial strain (Tao *et al*., 2008).

Sequestering iron. Despite the fact that iron is the fourth most abundant element on earth, in aerobic soils, iron is not readily assimilated by either bacteria or plants because ferric ion or Fe(III), which is the predominant form in nature, is only sparingly soluble so that the amount of iron available for assimilation by living organisms is extremely low (Ma, 2005). To survive with such a limited supply of iron, bacteria synthesize low-molecular mass siderophores (~400–1500 Da), molecules with an exceptionally high affinity for Fe(III) (K_a ranging from 10^{23} to 10^{52}) as well as membrane receptors able to bind the Fe-siderophore complex, thereby facilitating iron uptake by both microorganisms and plants (Neilands,1981; Hider and Kong, 2010).

The provision of iron to plants by soil bacteria is even more important when the plants are exposed to an environmental stress such as heavy metal pollution. In this case, bacterial siderophores help to alleviate the stresses imposed on plants by high soil levels of heavy metals (Belimov *et al*., 2005; Braud *et al*., 2006; Diels *et al*., 2002). The ability to synthetize this molecules is quite diffused among soil bacteria; at the present time, there are over 500 known siderophores. It has been detected in several species growing on heavy metal polluted environment i.e. *Variovorax paradoxus*, *Flavobacterium* sp., *Rhodococcus* sp., *Ralstonia* sp., *Arthrobacter* sp., *Stenotrophomonas* sp., *Pseudomonas* sp., *Microbacterium* sp., *Sphingomonas* sp., *Bacillus* sp., *Paenibacillus* sp. and many other (Delorme *et al*., 2001; Barzanti *et al*., 2007; Dell'Amico *et al*., 2005).

1.3.2.1.2 Modulating phytohormone levels

Indoleacetic acid production. Although several naturally occurring auxins have been described in literature, indole-3-acetic acid (indoleacetic acid, IAA) is by far the most common as well as the most studied auxin (Patten and Glick, 1996; Spaepen *et al*., 2007). IAA affects plant cell division, extension, and differentiation; stimulates seed and tuber germination; increases the rate of xylem and root development; controls processes of vegetative growth; initiates lateral and adventitious root formation; mediates responses to light, gravity and florescence; affects photosynthesis, pigment formation, biosynthesis of various metabolites, and resistance to stressful conditions (Spaepen and Vanderleyden, 2011; Tsavkelova *et al*., 2006).

Overall, bacterial IAA increases root surface area and length, and thereby provides the plant has greater access to soil nutrients. In addition, bacterial IAA loosens plant cell walls and as a result facilitates an increasing amount of root exudation that provides additional nutrients to support the growth of rhizosphere bacteria (Patten and Glick, 2002; Xie *et al*., 1996). Recent examples of IAA production by plant-associated microbes and the consequent successful improvement of the phytoremediation process were proved in *Bacillus cereus*, *Bacillus* sp., *Pseudomonas* sp., *Agrobacterium radiobacter*, *Enterobacter* sp., *Bacillus megaterium*, *Achromobacter xylosoxidans*, and *Burkholderia* sp. just to cite some of them (Wu *et al*., 2006; Jiang *et al*., 2008; Kumar *et al*., 2008; Ma *et al*., 2009; Azcón *et al*., 2010; Luo *et al*., 2011; Wang *et al*., 2011; Ma *et al*., 2011a)

Modulating ethylene levels: The 1-aminocyclopropane-1-carboxylate (ACC) deaminase (E.C.4.1.99.4) is a cytoplasmically localized enzyme produced by some soil bacteria to catalyze the degradation of ACC, a precursor of ethylene, as their source of nitrogen (Jacobson *et al.* 1994; Glick, 1995). ACC degradation will ultimately reduce ethylene biosynthesis in the plant. Various studies have shown that biosynthesis of ethylene at early stage of plant growth inhibits root development (Glick 1995; Mayak *et al*. 1997; Shah *et al*. 1997) and nodulation of various legumes (Ma *et al*. 2003), and in most cases, weakens plant defense against plant pathogens (Wang *et al*. 2000; Dey *et al*. 2004). The ethylene that is synthesized as a response to various stresses is called “stress ethylene” and it describes the increase in ethylene synthesis that is typically associated with various environmental stresses including the presence of toxic metals and organic pollutants (Abeles *et al.,* 2002). The importance of ACC deaminase-producing bacteria for plant growth is to control ethylene biosynthesis in the plants (Husen *et al*., 2009). As a direct consequence of ACC deaminase’s activity, the amount of ethylene produced by the plant is reduced. Therefore, root or seed colonization by PGPR that synthesize this enzyme prevents plant ethylene levels from becoming growth inhibitory (Glick *et al*., 1998). In the short term, the main visible effect of seed or root inoculation with ACC deaminase-producing bacteria is the enhancement of plant root elongation; promotion of shoot growth is generally seen in longer term experiments. These effects have been observed to be produced by isolates of *Pseudomonas putida*, *Mesorhizobium* sp., *Burkholderia* sp., *Enterocabter cloacae*,

Achromobacter xylosoxidans, *Flavobacterium* sp., *Rahnella aquatis* (Nie *et al*., 2002; Contesto *et al*., 2008; Hall *et al*., 1996; Nascimento *et al*., 2012; Onofre-Lemus *et al*., 2009; Shaharoona *et al*., 2009; Ma *et al*., 2009; Kumar *et al*., 2009; He *et al*., 2010).

1.3.2.1.3 Enhancing metal (As) uptake

Although several conditions, for example, the plant growth, metal tolerance/accumulation, bacterial colonization, and plant growth promoting potentials must be met for microbe assisted phytoremediation to become effective, the concentration of bioavailable metals in the rhizosphere greatly influences the quantity of metal accumulation in plants, because a large proportion of heavy metals are generally bound to various organic and inorganic constituents in polluted soil and their phytoavailability is closely related to their chemical speciation (McBride, 1994). Plant-associated bacteria can potentially improve phytoextraction by altering the solubility, availability, and transport of heavy metal and nutrients by reducing soil pH, release of chelators, P solubilization, or redox changes. Among the various metabolites produced by PGPR, the siderophores play a significant role in metal mobilization and accumulation (Dimkpa, *et al*., 2009; Rajkumar *et al*., 2010), as these compounds produced by PGPR solubilize unavailable forms of heavy metal-bearing Fe but also form complexes with bivalent heavy metal ions that can be assimilated by root mediated processes (Carrillo-Castañeda *et al*., 2003). Recently, Braud *et al*. (2009) investigated the release of Cr and Pb in soil solution after inoculation of various PGPR and found that the siderophores producing PGP *Pseudomonas aeruginosa* was able to solubilize large amounts of Cr and Pb in soils solution. Similarly, the role of siderophores produced by *Streptomyces tendae* F4 in Cd uptake by bacteria and sunflower plant was investigated (Dimkpa *et al*., 2009). Bacterial culture filtrates containing hydroxamate siderophores secreted by *S. tendae* F4 significantly enhanced uptake of Cd by the plant, compared to the control. This study showed that siderophores can help to reduce metal toxicity in bacteria while simultaneously facilitating the uptake of such metals by plants.

In addition, certain PGPR have been shown to increase heavy metal mobilization by the secretion of low-molecular-mass organic acids comprising gluconate, 2-ketogluconate, oxalate, citrate, acetate, malate, and succinate, etc. An example is the release of 5-ketogluconic acid by endophytic diazotroph *Gluconacetobacter diazotrophicus*, which is able to dissolve various Zn sources such as ZnO, $ZnCO_3$, or $Zn_3(PO_4)_2$, thus making Zn available for plant uptake (Saravanan *et al*., 2007).

Besides the fact that a large proportion of As species are unavailable for the root uptake by plants, there is another factor to take into account. Plant roots can release oxygen to their rhizosphere through aerenchyma, resulting in the oxidation of ferrous iron (Fe(II)) and the formation of iron plaques, consisting mainly of ferrihydrite, on the root surfaces. The Fe oxides formed have a strong adsorptive capacity for arsenate and concentrations of As in the iron plaques can be about 5-fold higher than those in root tissues (Liu *et al*., 2006). In these

conditions in which the presence of iron plaques decrease arsenate uptake, probably as a result of the adsorption effect, arsenite uptake is enhanced (Chen *et al.*, 2005).
For all these reasons and because of the higher mobility and bioavailability of As(III) compared to those of As(V), bacteria able to reduce As(V) to As(III) may enhance As uptake by plants too.

The direct benefits of bacterial PGP traits have been demonstrated in several different types of experiments (Tab. 1.1) and a clear example is illustrated in Fig. 1.8.

Bacterial strain	**Plant**	**Metal**	**Effects/Mechanism**	**Reference**
Enterobacter cloacae	Canola	As	Increased biomass; ACC deaminase	Nie *et al.*, 2002
Pseudomonas fluorescens	Sunflower	As	Increased growth; mechanism unknown	Shilev *et al.*, 2006
Agrobacterium radiobacter	Poplar	As	Increased metal uptake and shoots translocation; IAA, siderophores	Wang *et al.*, 2011
P. diminuta, Brevundimonas diminuta, Nitrobacteria irancium, Ochrobacterum anthropi, B. cereus	Water hyacinth	Cr	Increased metal uptake; mechanism unknown	Abou-Shanab *et al.*, 2007
Variovorax paradoxus, Rhodoccus sp., Flavobacterium sp.	Indian mustard	Cd	Increased root length; IAA, siderophores, ACC deaminase	Belimov *et al.*, 2005
Achromobacter xylosoxidans	Indian mustard	Cu	Increased root and shoot length and biomass; ACC deaminase, phosphate solubilization, IAA	Ma *et al.*, 2009
Burkholderia sp.	Indian mustard, corn, tomato	Pb, Cd	Increased biomass and metal uptake; IAA, siderophores, ACC deaminase	Jiang *et al.*, 2008

Tab. 1.1 – Plant-bacteria combinations in various metal phytoremediation studies.

Authors (Dary *et al.*, 2009) studied the effect of inoculation with a consortium of PGPR resistant to heavy metals, on the phytoremediation capacity of *Lupinus luteus*. Inoculation with only *Bradyrhizobium* increased biomass yield by 29% and inoculation with the consortium of metal resistant PGPRs increased biomass production by 109%, and the yield and the aspect of inoculated plants was much better than those without the PGPR inoculation treatment in adjacent plots (Fig. 1.8). Furthermore the combined use of this legume with *Bradyrhizobium* and metal resistant rhizobacteria, improved the Zn phytoremediation capacity of the plant of 10 times (Dary *et al.*, 2009).

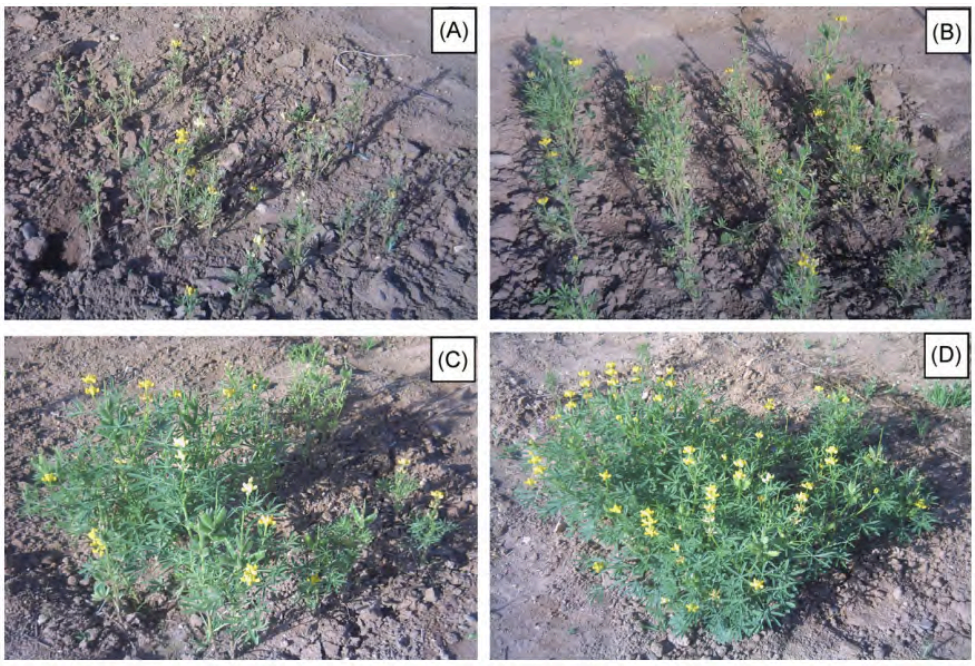


Fig. 1.8 *– Effect of inoculation with Bradyhizobium sp. 750 and a consortium of PGPR resistant to As and heavy metals on the growth of Lupinus luteus on a multi-metal contaminated soil. A: non inoculated, B: inoculated with Bradyrhizobium sp. 750, C: inoculated with Bradyrhizobium sp. 750 + Ochrobactrum cytisi Azn6.2, D: inoculated with Bradyrhizobium sp. 750 + Ochrobactrum cytisi Azn6.2 + Pseudomonas sp. Az13.*

In conclusion, the role of soil micro biota, specifically rhizospheric microorganisms, is fundamental in the development of phytoremediation techniques in order to speed up the process and to optimize the rate of mobilization/absorption/accumulation of pollutants. To efficiently phytoremediate As-contaminated soils, the bioavailability of metals to plant roots is considered to be a critical requirement for plant metal bioconcentration to occur. In this regard, it may be possible to employ beneficial bacteria to alter the bioavailability of metals for improving phytoremediation of As contaminants on large scale in the environment. Based on the foregoing account, microbe assisted phytoremediation is a reliable and dependable process.

1.4 Scarlino's study case

Scarlino is a medieval town located in a partially filled wetland in the south west of Tuscany. In 1962, in this area was located a big plant for the production of sulfuric acid from pyrite combustion, and for 40 years since that time, the waste of the process have been stored in the ground without any form of control or protection. Only after a municipal ordinance, in 2003 a plastic baffle was placed in order to isolated the toxic cinders from the natural background.

The pyrite ores were produced in a minefield of the inner lands (Colline Metallifere) that are located about 20 km east of the site. In the last decades it has been clear that a large area around the industrial site was strongly contaminated by arsenic compounds (AQUATER, 1985; Nuova Solmine, 1997). This finding was not surprising taking into account that about two millions cubic meters of hematite cinders – coming from pyrite combustion – were disposed on the ground prone to environmental dispersion (Fig. 1.9. (A)). Moreover, this mass was subsiding of at least 6 m under the previous ground level and before the grounding of the baffle the groundwater soaked it completely. From analysis performed by the current owner of the plant, Nuova Solmine Company, results that average As concentration in this material is about 500 mg/kg of arsenic (Tab. 1.2), and the total mass of this metalloid potentially available for diffusion in the environment, is of about 900 cubic meters. Besides, As isn't the only metal present in toxic concentration in the ashes cumulus: cadmium, lead, zinc and iron contaminate the site as well (Tab. 1.2). The lack of heavy metals (Zn and Pb) in the soil below the interface between ashes and the natural background can be explained with the fact that other metals are more adsorbed onto soil particulates than As in anaerobic conditions. This hypothesis is corroborated by the analysis performed on aquifers (Nuova Solmine, 1999) in which it's observed how As moves into solutions more easily than other metals and where a concentration of 1700 ppm has been found.

Sampling points	**As mg/kg**	**Cd mg/kg**	**Fe mg/kg**	**Pb mg/kg**	**Zn mg/kg**
Top	**588**	**10.30**	**310,700**	**1,606**	**1,843**
- 0.70 m	**476**	**6.13**	**289,500**	**1,070**	**1,794**
- 1.50 m	**540**	**7.79**	**382,800**	**1,299**	**1,935**
- 2.50 m	**413**	**6.55**	**270,700**	**1,400**	**1,188**
- 3.40 m	**635**	**5.58**	**361,500**	**1,072**	**1,363**
- 4.00 m	**561**	**6.06**	**339,300**	**1,279**	**1,324**
- 4.80 m	**308**	**5.39**	**308,200**	**759**	**1,540**
Interface ashes/natural soil					
- 5.40 m	**293**	0.39	22,600	65	221
- 6.50 m	**390**	0.36	52,100	73	238
- 7.50 m	**509**	0.44	53,800	56	233
- 8.50 m	**315**	0.19	59,100	< 30	135
- 10.00 m	**293**	0.23	63,400	< 30	183
Natural soils	**1-20**	0.1-0-5	5,000-100,000	5-40	10-300

***Tab. 1.2** – Metal concentrations in the main waste disposal at Scarlino site.*

Fine particle of pyrite, contained in the milling dust and in ores-enrichment sludge, constitutes the other two stocks of residues wastes (Fig. 1.9 (B) and (C)). These wastes were also disposed on the ground without any protection for the groundwater and no plastic baffle was afterward applied under these two stocks of contaminated material.

Fig. 1.9 *– Topographic map of the Scarlino plan. Letters shows the main contaminated deposits of the area: pyrite cinders waste stock (A); (B) and (C) fine pyrite particle and pyrite enrichment sludge stock.*

Nowadays the main waste disposal (A) has been in part dispatched but a huge amount of 800,000 m^3 (corresponding to 1,600,000 tons) of toxic ashes are still located in the original position.

But the unusual aspect of the arsenic contamination of this area concerns both its horizontal and vertical distribution in the soil. In fact, despite the arsenic concentration decreases with the increasing distance from the plant, some remote sample shows high concentrations, and the vertical profile of As level is very different from point to point.

These features induced some geologists and chemists to formulate the hypothesis of the natural occurring As contamination besides the anthropic causes (Nuova Solmine, 1999; ARPAT, 2001). This hypothesis was based in the idea that, in the past, the river coming from the mining district, upstream the industrial site, transported contaminated sediments which contributed to form the present alluvial Scarlino plan.

Natural arsenic background along with ash disposal has thus provoked a serious soil contamination and pollution of aquifers within the whole district where an average concentration of 140 mg/kg As can be found (Fig. 1.10).

Fig. 1.10 *– Contamination spread in Scarlino soils and aquifers in the plan and outside the industrial area.*

1.4.1 Remediation necessity

Nuova Solmine plant is located in the industrialized pole in Scarlino (Grosseto, Tuscany) area – named GR66 – which is characterized by high levels of metalloid and heavy metal pollution besides arsenic. Given the huge level of contamination and its spread in the area Regione Toscana introduced GR66 site in the Regional Plan of Reclamation with decree G.R.T. n° 1117 on 6th October 1997. In this area, As indeed represents a high health risk and for which remediation is a priority. As far as arsenic is concerned, the limits laid down by Italian regulations (Legislative Decree 152/2006 part IV) for green use of soil is 20 mg/kg d.w and for commercial or industrial use is 50 mg/kg d.w. In this particular study case, a combined approach of conventional and biological remediation is required. Whereas phytoextraction can be applied to clean up the soil in condition of moderate contamination level (outside the chemical plant area or near the main stocks of As contaminated cinders), phytoremediation is not applicable at the highest level of contamination as plant survival is affected by the toxicity and general condition of the soil (stocks illustrated in Fig. 1.9). Therefore interest has been drown by the concept of a combined approach of conventional treatments (deposit in special dump or added to smelter or concrete) at the highest contamination level and the application of a protocol of phytoextraction (with the use of *Pteris vittata* assisted by rhizobacteria) at more moderate conditions.

1.5 Aims of the study

Inorganic arsenic is nowadays regarded as the number one toxin in the USEPA (US Environmental Protection Agency) list of prioritised pollutants (Ng *et al*., 2003) and the World Health Organization recommends As guideline values of 10 µg/L in drinking water (WHO, 2001). Anyway arsenic contamination of groundwater used for domestic water supplies has been reported from over 70 countries (i.e. Bangladesh, Cambodia, Vietnam, West Bengal, and the United States), affecting the health of an estimated 150 million people (Ravenscroft *et al*., 2009). Inorganic arsenicals are proven carcinogens, clastogens and teratogens in humans (Ng, 2005). Their toxicity ranges from skin lesions to cancer of the brain, liver, kidney, and stomach and several heart, respiratory, gastrointestinal, liver, nervous and kidney diseases (Smith *et al*., 1992).

Although physical-chemical remediation technologies can be effective and applicable at high contamination levels, they are expensive and invasive, disrupting both soil structure, biological activity and fertility (Kirpichtchikova *et al*., 2006). On the other side bioremediation – which is the use of microorganisms and/or plant able to degrade, remove or detoxify the contaminant - is an interesting alternative or complement to conventional technologies. In particular the phytoextraction approach enhanced by microorganisms - based on the use of plant in synergy with microorganisms – offers a low cost *in-situ* applicable method to remediate and restore perturbed areas (Shukla *et al*., 2010; Manousaki and Nicolas, 2009; McGuinness and Dowling, 2009).

In this context the present PhD study deals with a real case of a soil contaminated by inorganic arsenic in the industrial area Nuova Solmine Company in Scarlino (Grosseto, Tuscany). In 1962, in this area was located a big plant for the production of sulfuric acid from pyrite combustion, and since that time, the waste of the process have been stored in the ground without any form of control or protection. This resulted in the accumulation of 2 million metric tons of As contaminated hematite cinders (nowadays reduced to 800 000 m^3) and reporting an esteem of approximately 800 tones of total arsenic.
Natural arsenic background along with ash disposal has thus provoked a serious soil contamination and pollution of aquifers within the whole district where an average concentration of 140 mg/kg As can be found.
Due to the contamination of soil and aquifers, Regione Toscana introduced GR66 site in the Regional Plan of Reclamation with decree G.R.T. n° 1117 on 6th October 1997 – i.e. implying high environmental risk and priority remediation.

The present work examined the soil autochthonous bacterial community selected by and

acclimated to the contamination of inorganic As present in this industrial area for fifty years. At the same time this study also focused on the interaction of the indigenous micro flora and the hyperaccumulating fern *Pteris vittata* in relation to a phytoremediation approach. This plant is known to be able to accumulate very high concentration of inorganic As in its fronds and to be characterized by a rapid biomass growth, thus representing an optimal candidate in a phytoextraction context (Ma *et al*., 2001).

Microorganisms are known to play a very important role in the phytoremediation process. Actually, bacteria can enhance the mobility of arsenic in the soil matrix (eliciting the metalloid uptake by the plants) while plant growth promoting rhizobacteria (PGPR) can improve the plant biomass production (Abou-Shanab *et al*., 2003; Glick *et al*., 1995; Glick, 2003). Therefore the main aim of this work is finding the best integrated plant-microorganisms system able to lower arsenic contamination in the Scarlino area below the concentration of 50 mg/kg established by law.

This study is a part of a bigger project named R.E.P.E.T (Rhizosphere Enhanced Phytoextraction Technology) realized in collaboration with University of Pisa and University of Florence and funded by Regione Toscana. All the operative units took care of different aspects of the project: basically, University of Florence characterized the level and extension of the contamination in the site object of study; University of Pisa attended to all the aspects concerning the vegetal species and University of Verona performed the analysis on the bacterial community and isolates.

In detail, with the main purpose of set up a phytoextraction protocols for the remediation of Scarlino area, the aims of the present work were:

- to study the biodiversity and composition of the soil autochthonous bacterial community at different sampling points within the area, to evaluate the impact of a long-term exposure to inorganic As, and the resistance and bioremediation potential of the selected micro flora;
- to isolate and characterize members of the soil microbial community relatively to arsenic resistance, PGPR traits (IAA and siderophores production and ACC deaminase activity), genotypic study of the mechanisms of arsenic transformation, ability to reduce arsenate to more mobile chemical species, in the perspective of a phytoremediation application in a bioaugmentation protocol;
- finally, to evaluate the interaction of some selected bacterial isolates and their possible synergistic role in a plant-rhizobacteria system in relation to As phytoextraction with the vegetal species *Pteris vittata*.

2. Materials and Methods

2.1 Chemicals and culture media

Chemicals purchased from Sigma-Aldrich (Milan, Italy) were all analytical grade. Nutrient Broth, Yeast Extract and Bacteriological Agar were supplied by Oxoid Italia S.p.A (Garbagnate Milanese, Italy).

2.1.1 Growth media

All cultural media, except when specified otherwise, were arranged by weighting different powdered components and mixing them in appropriated volume of bi-distilled water. pH of solutions was adjusted as specified by producers with solutions 2M of NaOH or HCl and sterilized by autoclaving at 121°C for 15 minutes under a pressure of 105 kPa. Solid media were obtained by adding Technical Agar n° 3 (Oxoid) at 1.2 % before the sterilization step.

Nutrient Broth was supplied by Oxoid Italia S.p.a.

Minimal defined Medium (DM): 3 g/l NH_4NO_3, 2.2 g/l Na_2HPO_4, 0.8 g/l KH_2PO_4 and the appropriate source of carbon in concentration 0,1%. After sterilization in autoclave, filtered Wolfe's mineral solution (10 ml/l) and Vitamin solution (1 ml/l) were added.

Minimal defined Medium Low Phosphate (DMLP): was prepared as previously described in Minimal defined Medium (DM) and supplemented with 0.1% of yeast extract. KH_2PO_4 was totally depleted and Na_2HPO_4 added in low concentration (0,23 g/l).

Reasonar's 2 Agar (R2A): yeast extract 0.5 g/l, Peptone pancreatic digest of casein 0.5 g/l, casein hydrolysate (acid) 0.5 g/l, D-glucose anhydrous 0.5 g/l, starch 0.5 g/l, sodium pyruvate 0.3 g/l, K_2HPO_4 0.3 g/l, $MgSO_4$ (anhydrous) 0.05 g/l.

Malt Medium: malt broth 20.0 g/l, yeast extract 5.0 g/l. After sterilization, the solution was supplemented with 15 mg/l of rifampicin (broad spectrum antibiotic).

Waksman Medium (pH 7): glucose 10.0 g/l, sodium chloride 5.0 g/l, bacteriological peptone 5.0 g/l, Lab Lemco Powder 3.0 g/l. After autoclave Waksman Medium was supplemented with a mix of antibiotics, namely 50 mg/l of nistatin (with fungistatic and fungicidal action against a wide variety of yeasts and yeast-like fungi), 5 mg/l of polymixin and 4 mg/l of anfotericin B (antifungal agents).

Luria Bertani (LB): 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl.

SOC broth: 10 g/l tryptone, 5 g/l yeast extract, 0.5 g/l NaCl, 10 ml/l KCl 250 mM, 5 ml/l $MgCl_2$ 2M, 5 ml/l glucose 1M.

Dworkin-Foster (DF) salts minimal medium was prepared as previously described in Penrose and Glick (2003).

Tris Minimal Medium (TMM) was prepared as described in Sokolovská *et al*. (2002).

2.1.2 Solutions

Salkowski's reagent: 150 ml concentrated H_2SO_4, 250 ml deionised H_2O, 7.5 ml 0.5 M $FeCl_3 \cdot 6H_2O$.

Tris-Acetate EDTA 50X (TAE): 242 g tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA (pH 8). The volume of the solution was adjusted to 1 liter with deionised H_2O.

Tris-Borate EDTA 10X (TBE): 54 g tris base, 27.5 g boric acid, 20 ml 0.5 M EDTA (pH 8). The volume of the solution was adjusted to 1 liter with deionised H_2O.

Wolfe mineral solution (100X): 1.6 g/l nitrilotriacetic acid, 3.5 g/l $MgSO_4 \cdot H_2O$, 1 g/l NaCl, 0.076 g/l $Fe_2(SO_4)_3$, 0.13 g/l $CoSO_4 \cdot 7H_2O$, 0.13 g/l $CaCl_2 \cdot 2H_2O$, 0.11 g/l $ZnSO_4 \cdot 2H_2O$. Ingredients were mixed together and then filter-sterilized with 0.2 µm nitrocellulose filter (Millipore).

Vitamin solution (1000X): 2 mg/l biotine, 2 mg/l folic acid, 5 mg/l chlorohydrate thiamine 5 mg/l riboflavin, 5 mg/l chlorohydrate pyridoxine, 10 mg/l cyanocobalamin, 0,1 mg/l nicotininc acid, 5 mg/l calcium pantothenate, 5 mg/l lipoic acid. Ingredients were mixed together and then filter-sterilized with 0.2 µm nitrocellulose filters.

Inorganic As(III) was supplied by Sigma-Aldrich as solution 50 mM; **inorganic As(V)** was prepared as a 500 mM stock solution in deionized water and sterilized by filtration.

2.2 Sampling within Scarlino industrial area

All the analyses described in the present study were performed on soil from 6 different sampling points: one from the matrix (M) consisting in a huge pile of about 1,600,000 arsenopyrite ashes tons and 5 samples from the rhizosphere of 5 different autochthonous plants (P1, P2, P3, P4 and P5) grew adjacent to the waste disposal site (Fig. 2.1). The 5 plants were identified on the basis of morphological characteristic as *Lolium rigidum* belonging to *Graminaceae* family (P1), *Daucus carota*, an *Umbelliferae* (P2), *Trifolium angustifolium* L. (P3), *Populus alba* (P4) and *Sedum sediforme* belonging to *Crassulaceae* family (P5). Arsenic average concentrations were of 500 mg/kg and 140 mg/kg in the matrix and in the surrounding area respectively.

Fig. 2.1 *– Sampling within the contaminated industrial area: the carrots (A) from the arsenopyrite ashes dump illustrated in (B) and from the vegetal samples found nearby (C).*

2.3 Culture-dependent analysis

2.3.1 Enumeration of cultivable micro flora

In 250-ml Erlenmeyer flasks 10 g of sieved soil - from each of the sampling point - were suspended in 90 ml of physiologic solution (0.9% wt/vol NaCl) and magnetically stirred for 1 hour, to separate microbial cells from the soil. After decanting for 15 minutes, 10-fold serial dilutions were spread on solid agar plates. Nutrient rich-medium and low-nutrient medium R2A agar allowed enumeration of heterotrophic aerobic eubacteria, Waksman medium was used for *Actinobacteria* enumeration and Malt medium for Fungi. For all media, agarized plates were inoculated in duplicate at each dilution and the visible colonies were enumerated as colony forming units (CFUs), previous incubation at 27 °C for 5 days. An extended incubation of 7 days was required for *Actinobacteria* growth on Waksman medium.

2.3.2 Isolation of soil bacterial strains from enrichment cultures

Enrichment cultures for bacterial isolation were arranged inoculating the soil samples from each sampling point (P1, P2, P3, P4, P5 and MP) in 100 ml R2A medium amended with 2 mM As(III) or 2 mM As(V) respectively.

Both enrichments – (As(III) and As(V) – were carried out in 250-ml Erlenmeyer flasks and incubated at 27° C on an orbital shaker (250 rev/min) in the dark for 8 weeks. Every 2-weeks of incubation, a 10 ml aliquot from each flask was transferred in a new one with 90 ml of the same fresh culture substrate amended with As(III) or As(V).

After 8 weeks the isolation of the selected resistant microorganisms was made plating 100 µl of serial dilutions on selective medium agar plates; the medium used was the same of the respective enrichment culture. These plates were incubated at 27 °C for 7 days and, single colonies were then isolated and axenic cultures of morphologically different bacteria were obtained.

2.3.3 Minimum inhibitory concentration (MIC) determination for inorganic As(III) and As(V)

Each bacterial isolate has been streaked from liquid cultures onto R2A agar medium with the addition of increasing concentrations of arsenate (from 10 mM to 100 mM) and arsenite (from 1 mM to 40 mM). The plates have been incubated at 27 °C for 5 days. After the incubation, each plate has been checked to see the eventual growth of the colonies. The lowest concentration of As(III) and As(V) that prevented growth corresponded the MIC.

2.3.4 Plant growth promoting (PGP) traits analysis

2.3.4.1 Assay for 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase activity

The enzymatic activity of ACC deaminase enables the bacteria to use ACC as the sole N source (Penrose and Glick, 2003). Therefore the bacteria were first grown for 48 h in 4 ml of minimal medium DF supplemented with N as Ammonium Sulfate $(NH_4)_2SO_4$ (2 g/l). Afterwards the cells were collected by centrifugation (5000 rpm for 5 min at 4 °C), washed twice with physiologic solution (0.9% wt/vol NaCl) and supplied to 30 ml of DF with no N source, to obtained an initial OD=0.1. After 2 days 1 ml aliquot was removed from the culture and transferred to a second flask containing 30 ml DF, this step was repeated until, by measuring OD at 600 nm, no further growth was detected in absence of any nitrogen source. Next, bacterial cells were harvested by centrifugation (5000 rpm for 5 min at 4°C) and divided among 3 flasks containing 30 ml solution of DF salts minimal medium, DF amended with ammonium sulfate and DF amended with 3.0 mM ACC. The ACC, which is heat-labile and labile in solution, was prepared as a 0.5 M stock, sterilized using 0.2 µm membrane filter (Millipore), aliquoted and frozen at -20 °C. Just prior to inoculation, the ACC solution was thawed and an aliquot was added to a final ACC concentration of 3.0 mM. The cultures were incubated on an orbital shaker (250 rev/min) in the dark and the eventual growth was checked after 7 days. The use of DF supplied with ammonium sulfate as a positive control allowed to check for cells viability and the absence of growth in the negative control - DF with no N source - allowed to verify the ability of a strain to utilize ACC as a source on nitrogen and of not being a diazotrophic strain.

2.3.4.2 Assay for indoleacetic acid (IAA) production

Bacterial strains were cultured for 5 days in R2A medium with 0.5 mg/ml tryptophan, a precursor of IAA. After 2 and 5 days of incubation respectively, 1ml of the cell suspension was transferred into a tube and then mixed vigorously with 2 ml Salkowski's reagent and kept at room temperature for 20 min, afterwards the cell suspensions color was checked for the development of pink color indicates IAA production (Cavalca *et al.,* 2010).

2.3.4.3 Assay for siderophores production

Siderophores secretion by strains was detected by the ''universal'' method of Schwyn and Neilands (1987) streaking the isolates on blue agar plates containing the dye Chrome azurol S (CAS) (Sigma-Aldrich). Plates were incubated at 27 °C and after 5 days they were checked for orange halos around the colonies, indicative of siderophores excretion.

2.3.5 Arsenate reduction test *in vitro*

To test the ability of the isolates to reduce As(V) the strains were inoculated into vials each containing 30 ml of Tris Minimal Medium with As(V) 5 mM (Sokolovská *et al*., 2002). Control flasks without inoculum were incubated to check abiotic transformation of arsenic. At each sampling

time, 1 ml of suspension was removed to measure cell growth by OD600 nm and to determine As(V) and As(III) concentration by spectrophotometric analysis according to the method described by Cummings *et al*., (1999).

2.4 Culture-independent analysis

The molecular technique ARDRA (Amplified Ribosomal DNA Restriction Analysis) and the partial *16S rDNA* gene sequencing were used to determine the genetic diversity of the cultivable soil bacteria, isolated from enrichment cultures.

2.4.1 ARDRA (Amplified Ribosomal DNA Restriction Analysis)

Each isolate was analyzed through ARDRA technique, based on the PCR amplification of the *16S rRNA* gene and subsequent restriction digestion of the amplicon (De Baere *et al*., 2002). The analysis includes the following steps:

2.4.1.1 Genomic DNA extraction with the bead-beater method

Microbial genomic DNA of the analyzed strains was extracted following the protocol of bead-beater method. One axenic colony was inoculated in a tube with 4 ml of rich growth broth at 27 °C in agitation over-night. 2 ml of the liquid culture was centrifuged at 8000 rpm for 10 min and the supernatant was discharged. 0.2 ml of extraction buffer (1% SDS, 0.02 g/ml Triton X-100, 0.1 M NaCl, 5 mM EDTA), 0.3 g of glass beads (diameter 0.4-0.5 mm sterilized in autoclave) and 0.2 ml of Phenol:Chloroform:Isoamyl Alcohol 24:25:1 (saturate with 10 mM Tris, 1 mM EDTA, pH 8.0) were added and the microtubes were vortexed for 2 min. Afterwards the samples were centrifuged at 10.000 rpm for 5 min. The watery phase was transferred in a clean microtube, isopropanol 1:1 was added and the samples were incubated for 5 min at room temperature. Then the samples were centrifuged at 10.000 rpm for 5 min and the supernatant was discharged. The pellet was washed with ethanol 70%, centrifuged at 10.000 rpm for 5 min and the supernatant removed. Finally, the pellet was air-dried and resuspended in 50 µl of deionised water. To determine the quality and quantity of DNA extracted, 5 µl of genomic DNA were loaded 1% (wt/vol) agarose gel containing 0.5 µg/ml of ethidium bromide, and run in TAE 1X buffer at 100 V for 20 min.

2.4.1.2 PCR amplification of *16S* ribosomal RNA gene

The gene encoding for *16S rRNA* (1500 bp) was amplified using primers R11/F8 (Tab. 2.1) (Weisburg *et al*., 1991). PCR reaction were performed in a final volume of 25 µl containing 5-20 ng of target DNA, 0.4 µM of each primers, 1 mM of dNTPs mix, 1 U of GoTaq^{TM} DNA polymerase and 5 µl of 5X PCR-buffer (Promega).

Primer	sequence 5'-3'
R11	ACGGCTACCTTGTTACGACT
F8	GAGTTTGATCCTGGCTCAG

Tab. 2.1 *– Primers used for amplification of 16S rDNA.*

The following cycling conditions were used: 94 °C for 2 min; by 30 cycles of 94 °C for 45 s, 45 °C for 30 s, and 72 °C for 2 min; with a final extension at 72 °C for 5 min.

2.4.1.3 Restriction digestion

The digestion of the PCR amplification products was performed separately with *AluI* and *HhaI* restriction enzymes (Promega) at 37 °C for 4 hours in a volume of 50 µl. Afterwards, the digested products were loaded in 1.5% (wt/vol) agarose gel with 0.5 µg/ml of ethidium bromide and run in TBE 1X buffer at 100 V for 60 minutes. The gel was visualized with the UV transilluminator UVITEC Cambridge (Eppendorf).

2.4.1.4 OTUs determination

All restriction profiles obtained for the analyzed strains by the two restriction enzymes *AluI* and *HhaI* were compared in order to group isolates according to the similarity of their banding patterns (ribotypes) obtained in the electrophoresis agarose gels. Next, the morphological comparison of isolates belonging to the same ribotypes, allowed the identification of different Operational Taxonomic Units (OTUs). For each OTU one representative strain was chosen for the sequencing.

2.4.1.5 Sequence and statistical analysis

For each OTU total DNA of one representative strain was selected, the gene encoding for *16S rRNA* (1500-pb) was amplified and PCR products were purified by gel elution using QIAEX II gel extraction Kit (Qiagen), following the manufacturer's instructions. The purified PCR products were sequenced by PRIMM Srl at PRIMM center, S.Raffaele Hospital in Milan (Italy), using a DNA analyzer (ABI 3730, Applied Biosystems, USA) capillary sequencer.

The sequences of about 500 bp were compared to the NCBI database using both the Basic local alignment search tool (BlastN) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1997) and the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012).

PAST (PAleontological STatistics v 1.19) software from the website http://folk.uio.no/ohammer/past/ was used for the calculation of different diversity indices (Rarefaction analysis, Coverage, Shannon-Wiener Diversity Index and Simpson) for the isolates of the different enrichment cultures. To perform rarefaction analysis, the total number of isolates compared with the number of OTU representing each unique phylotype was used to produce the rarefaction curves. Rarefaction curves and coverage values were calculated to determine how

efficiently the enrichments described the complexity of a theoretical community like an original bacterial community. The coverage (Good 1953) value is given as C = 1 - (n1/N) where n1 is the number of unique strains that occurred only once among the isolates.

2.4.2 PCR-DGGE (Denaturing Gradient Gel Electrophoresis) analysis

In order to evaluate the biodiversity of the soil bacterial community comprehensive of the uncultivable fraction, a PCR-DGGE approach was adopted on the matrix samples (M).

DGGE exploits the fact that 2 otherwise identical DNA molecules, which differ by only one nucleotide within a low melting domain, will have different melting temperatures. In fact the two strands of a DNA molecule separate, or melt, when heat or a chemical denaturant is applied and the melting temperature (Tm) is determined by the nucleotide sequence.

During electrophoresis in a gradient of increasing chemical denaturant (usually formamide and urea), the mobility of a DNA molecule is retarded at the concentration at which the DNA strands of low melting domain dissociate. The branched structure of the single stranded of the molecule becomes entangled in the gel matrix and no further movement occurs. Complete strand separation is prevented by the presence of a high melting domain, which is usually artificially created at one end of the molecule by incorporation of a GC clamp. This is accomplished during PCR amplification using a PCR primer with a 5' tail consisting of a sequence of 40 GC (Muyzer *et al.*, 1993).

The result of the separation of PCR products by DGGE is a pattern of bands, for which the number of bands correspond to the number of predominant members in the analyzed microbial communities. Moreover the sequencing of DNA eluted from excised DGGE bands allows identifying the respective community member.

2.4.2.1 Total DNA extraction

Total DNA from ashes samples (M) was extracted with Fast DNA® SPIN for Soil Kit (MP Biomedicals) as described in the manufacturer's instructions and purified using QIAEX II gel extraction Kit (Qiagen). Finally it was checked by electrophoresis on 1% (w/v) agarose gel in TAE buffer containing 0.5 µg/ml of ethidium bromide.

2.4.2.2 Amplification of bacterial V3 hypervariable region

Total extracted DNA was used as template for PCR amplification of the gene encoding for the *16S rRNA* (see par. 2.4.1.2). A suitable dilution of these reactions were used as template for a nested-PCR reaction. The universal bacterial primers targeting *16S rDNA* V3 hypervariable region, P3 and P2, were used to amplify fragments sized 200 bp (Tab. 2.2) (Muyzer *et al.*, 1993). PCR reaction were performed in a final volume of 25 µl containing 5-20 ng of target DNA, 0.4 µM of each primers, 1 mM of dNTPs mix, 1 U of GoTaq^{TM} DNA polymerase and 5 µl of 5X PCR-buffer (Promega). The following cycle conditions were used: 95°C for 5 min; followed by 30 cycles of

95°C for 30 s, 57°C for 30 s, and 72°C for 35 s; and a final extension at 72°C for 5 min.

Primer	sequence 5'-3'
P2	(40bp-GCclamp) CCTACGGGAGGCAGCAG
P3	ATTACCGCGGCTGCTGG

Tab. 2.2 *– Primers used for amplification of V3 region*

2.4.2.3 Electrophoretic run in denaturing gel

The PCR products, from both bacterial and archaeal community, were separated on polyacrylamide gels (8%(wt/vol), 37.5:1 acrylamide-bis-acrylamide) with a 30% to 60% linear gradient of denaturant (100% denaturant: 40% (vol/vol) formamide plus 42% (wt/vol) urea). The DGGE was performed using DcodeTM Universal Mutation Detection System (Bio-Rad) and gels were run for 16 h at 50 V in TAE 1X buffer at 65 °C. Afterwards the gels were stained for 30 min in ethidium bromide (1 mg/l) in TAE 1X buffer and visualized by UV illumination. The image was detected by the software UVIband - 1D gel analysis software with the UV transilluminator UVITEC Cambridge (Eppendorf).

2.4.2.4 Bands' cloning, sequencing and phylogenetic tree construction

Major bands in the DGGE profiles were excised and incubated for 4 hours in 50 µl sterile water, and successively re-amplified using the same set of primers (P2/P3). PCR products were ligated and cloned in pGEM®-T Easy vector system, following the manufacturer's instructions (Promega). Bacterial V3 regions sequences were sequenced at PRIMM Biotech Custom service and compared with GenBank by BLASTn program (Altschul *et al*., 1997).

Before proceeding with the identification of the bacterial strains, all the obtained sequences were analyzed with the web tool 'DECIPHER's Find Chimeras' to uncover the eventual chimeras sequences (http://decipher.cee.wisc.edu/index.html).

Neighborjoining phylogenetic trees were constructed with the Molecular Evolutionary Genetics Analysis package (MEGA version 4.1). Sequences were aligned with ClustalW, distances were calculated with the Jukes-Cantor algorithm and the robustness of the phylogeny was tested by bootstrap analysis with 1000 iterations.

2.5 PCR analysis targeting arsenic transformation genes

Arsenic microbial metabolism is very complex; it indeed influences the biogeochemical cycle of As, affecting both its speciation and toxicity. In this study attention has been paid on to different and – from a chemical point of view – opposite mechanisms of reaction. The presence of the *ars* operon

and the *aox* operon were indeed investigated. The first one, in its basic components consists of a cytoplasmic arsenate reductase (AsV ⇒ AsIII) and an arsenite efflux pump; conversely the second operon contains a membrane arsenite oxidase (AsIII ⇒AsV) (Cai *et al*., 1998; Silver and Pung, 2005a).

2.5.1 PCR reactions

This study was performed by primer-specific PCR amplifications starting from genomic DNA obtained from all OTUs (extracted as reported in par 2.4.1.1.). All the PCR reaction were carried out in a final volume of 25 µl containing 0.4 µM of each primers, 0.4mM of dNTPs, 1U of GoTaq DNA polymerase and 5 µl of 5x PCR-buffer (Promega). Approximately 5-20 ng of target DNA was added to each reaction. All PCR amplifications were performed with Mastercycler® personal (Eppendorf) and PCR product were checked on agarose 2% (wt/vol) gel with 0.5 µg/ml of ethidium bromide in Joule Box Mini Gel Electrophoresis System (Stratagene), and visualized with ultraviolet illumination. All reagents were from Promega.

For the analysis of the genotypes several primers sets were used (listed in Tab. 2.3):

• darsB1F and darsB1R for the amplification of *arsB* gene in *Firmicutes* e Gamma-proteobacteria *phyla* (Achour *et al*., 2007);

• dacr1F and dacr1R for the amplification of *ACR3(1)* gene, a variant of *arsB* described in *Actinobacteria* (Achour *et al*., 2007);

• dacr5F and dacr5R for *ACR3(2)* variant of *arsB* described in Alpha-proteobacteria (Achour *et al*., 2007);

• P810f and P1019r for the amplification of *arsB* gene in *Bacillus* genus (Cavalca *et al.,* 2010);

• #1F and #1R and #2F and #2R for the amplification of *aoxA* (Inskeep *et al*., 2007);

• aoxBM1 and aoxBM2 for the amplification of *aoxB* (Quéméneur *et al*., 2010);

• arsCb-1F and arsCb-1R for the amplification of arsenate reductase *arsC* gene in *Firmicutes phylum* (Macur *et al*., 2004);

• arsCp-6F and arsCp-6R for the amplification of arsenate reductase *arsC* gene in *Pseudomonadales* family (Macur *et al*., 2004);

• arsCe-F and arsCe-R for the amplification of arsenate reductase *arsC* gene in *Enterobacteriaceae* family (Macur *et al*., 2004);

• arsCa-F and arsCa-R for the amplification of arsenate reductase *arsC* gene in *Agrobacterium tumefaciens* (Macur *et al*., 2004).

Primer	Sequence 5'-3'
darsB1F	GGTGTGGAACATCGTCTGGAAYGCNAC
darsB1R	CAGGCCGTACACCACCAGRTACATNCC
dacr1F	GCCATCGGCCTGATCGTNATGATGTAYCC
dacr1R	CGGCGATGGCCAGCTCYAAYTTYTT

dacr5F	TGATCTGGGTCATGATCTTCCCVATGMTGVT
dacr5R	CGGCCACGGCCAGYTCRAARAARTT
P810f	CACTSGCAARGTRMTCC
P1019r	GTMGGCATGTTGTTCATG
#1F	GTSGGBTGYGGMTAYCABGYCTA
#1R	TTGTASGCBGGNCGRTTRTGRAT
#2F	GTCGGYTGYGGMTAYCAYGYYTA
#2R	YTCDGARTTGTAGGCYGGBCG
aoxBM1	CCACTTCTGCATCGTGGGNTGYGGNTA
aoxBM2	GGAGTTGTAGGCGGGCCKRTTRTGDAT
arsC-1F	GTAATACGCTGGAGATGATCCG
arsC-1R	TTTTCCTGCTTCATCAACGAC
arsC-6F	ATGAACAATATGCCTACCGT
arsC-6R	CCCCTTCGTCTTTATTTCAG
arsCe-F	ATGAGCAACATYACCAT
arsCe-R	TTATTTCAGYCGTTTACC
arsCa-F	ATGTCCGATTTTTCACAATCCG
arsCa-R	TTTCCTTCATTGTCGAGGACCTGC

Tab. 2.3 *– Primers used for the partial amplification of the As resistance determinants under study.*

2.5.2 PCR cycling conditions

Specific PCR-amplification were performed as listed below:

• ***arsB***: 94 °C for 5 min; 30 cycles of 94 °C for 45 s, 53 °C for 45 s, and 72 °C for 1 min; and final extension at 72 °C for 5 min.

• ***ACR(2)*** and ***ACR(3)***: 94 °C for 5 min; 30 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s; and final extension at 72 °C for 5 min.

• ***arsC***: 94 °C for 5 min; 30 cycles of 94 °C for 45 s, annealing at 37 °C for 45 s for primers arsC-1F/R and arsC-6F/R, at 42 °C for primers arsCe-F/R ,40 °C for primers arsCa-F/R and 72 °C for 30 s; and final extension at 72 °C for 5 min.

• ***aox/aso***: 94 °C for 5 min; 30 cycles of 94 °C for 45 s, 52 °C for 45 s, and 72 °C for 30 s; and final extension at 72 °C for 5 min.

PCR product were checked on agarose 2% (wt/vol) gel for the appropriate size, as indicated in Tab. 2.4.

Target gene	*arsB*	*ACR(2)*	*ACR(3)*	*arsC*	*aox/aso*
Amplicons length	700 bp	700 bp	700 bp	400 bp	500 bp

Tab. 2.4 *– Amplicons length.*

2.6 Microbe-enhanced phytoextraction experiments

Two different lab-scale trials have been set up with the hyperaccumulator fern *Pteris vittata* using a bioaugmentation protocol (in collaboration with University of Pisa). Bacterial strains used in these experiments were selected on the base of their PGP traits, on the ability to reduce As(V) to the mobile form As(III) or the presence of important genes involved in As metabolism.

2.6.1 *Pteris vittata* propagation

The plants/spores were propagated as illustrated in Fig. 2.2.

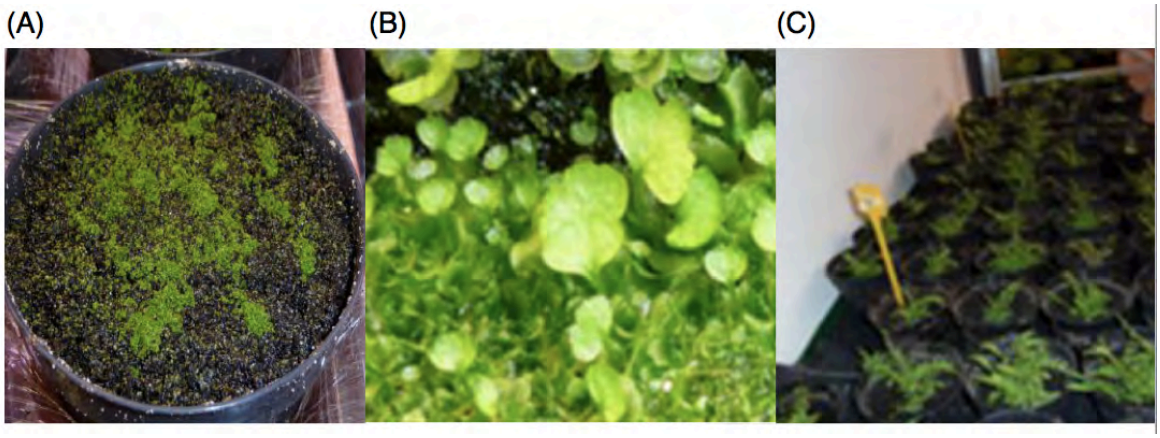


Fig. 2.2 *– Propagation of Pteris vittata plants (C) starting from spores and proceeding through gametophytes (A), sporophytes (B).*

The spores were collected and seeded in a mix of sterilized sand and loam in 1:2 proportion. 15-20 days and one month after the seeding the gametophytes and the sporophytes respectively were obtained (Fig. 2.2 (A) and (B)).

During the following months the sporophytes were thinned and replanted until, six months later, little *Pteris vittata* ferns were obtained (Fig. 2.2 (C)).

2.6.2 1st phytoextraction experiment

These first cultivation experiments were carried out in 6 kg pots in a temperature-controlled glasshouse (24/28 °C) for six months. Plants were grown on unpolluted soil (control) and on contaminated soil collected at the Scarlino area spiked with compost (30%). For each soil, four different trials were set up in triplicate (Tab. 2.5):

n.i. - non inoculated plants,

A - plants inoculated with *Achromobacter* sp. (ACC deaminase producer),

B - plants inoculated with *Ochrobactrum cytisi* + *Pseudomonas putida* strain (IAA-producers),

C - plants with inoculum A + B.

The experiment lasted six months and the different *inocula* were applied every 2 months at a final

concentration of 10^8 CFU/g of soil.

Soil	n.i.	*Inoculum A*	*Inoculum B*	*Inoculum C*
Control Soil	Not inoculated	*Achromobacter marplatensis*	*Ochrobactrum cytisi* + *Pseudomonas putida*	All 3 strains
Polluted Matrix	Not inoculated	*Achromobacter marplatensis*	*Ochrobactrum cytisi* + *Pseudomonas putida*	All 3 strains

***Tab. 2.5** – Experimental set up for the 1st phytoextraction trial in lab scale.*

2.6.3 2nd phytoextraction experiment

Similarly in the previous trial, cultivation experiments have been carried out in 1 kg pots in a temperature-controlled glasshouse (24/28 ˚C). Ferns were planted on unpolluted soil (control) and on contaminated soil collected at the Scarlino area spiked with compost (30%). For each soil, four different trials were set up in quadruplicate (Tab. 2.6):

n.i. - not inoculated plants,

A – plants inoculated with *Pseudomonas putida* and *Delftia lacustris* (IAA producers and able to reduce As(V) to As(III) in vitro),

B – plants inoculated with *Bacillus thuringiensis*, *Variovorax paradoxus* and *Pseudoxanthomonas mexicana* (siderophore producers),

C – plants with inoculum A + B.

Soil	n.i.	*Inoculum A*	*Inoculum B*	*Inoculum C*
Control Soil	Not inoculated	*P. putida* and *D. lacustris*	*B. thuringiensis*, *V. paradoxus* and *P. mexicana*	All 5 strains
Polluted Matrix	Not inoculated	*P. putida* and *D. lacustris*	*B. thuringiensis*, *V. paradoxus* and *P. mexicana*	All 5 strains

***Tab. 2.6** – Experimental set up for the 2nd phytoextraction trial in lab scale.*

The experiment lasted four months and the different *inocula* were applied every six weeks at a final concentration of 10^8 CFU/g of soil.

For both the experiments important parameters for the phytoextraction process were determined. The following analysis were indeed performed:

2.6.4 Determination of biomass production and total proteins content

Plants collected at the end of the experiments were washed with deionised water, separated into root and shoot portions, and oven dried at 50˚C until constant weight was reached. Dry weight (d.w.) values were recorded to determine biomass production.

Total proteins content was measured as described in Bradford, 1976.

2.6.5 Determination of arsenic content in plants tissues

At the end of each trail dry vegetal samples were harvested and kept at 120 °C for 24 hours in a solution of HNO_3 65%. The solutions were analyzed for total As content by ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry) Optima 2000 DV- Perkin-Elmerwith the method described in USEPA 1986.

2.6.6 Determination of arsenic content in soil samples

At the beginning of each trail 5 g of each soil sample were soaked in 30 ml $CaCl_2$ 0,05M for two hours on a rotary shaker. The suspension was centrifuged at 13,000 rpm and the pellet was discharged. The supernatant was evaluated for its As content by ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry) Optima 2000 DV- Perkin-Elmerwith the method described in USEPA 1986.

2.6.7 Monitoring microbial inocula

During both the phytoextraction PCR-DGGE analyses was performed on *Pteris vittata* rhizosphere soil samples collected at different sampling times in order to detect the presence and the persistence of the inoculated strains during the phytoremediation experiments.

DNA extraction from soil and PCR-DGGE analyses were carried out as described in paragraphs 2.4.2.

3. Results and discussion

Subdivision of the thesis study

This PhD work can ideally be divided into 3 main parts:

Chapter 1. The first part was focused on the taxonomic characterization of bacterial cenoses selected by and acclimated in aged arsenic polluted soils. Either classic diagnostic methods and molecular tools have been adopted. In detail culture-dependent protocols (total viable count and isolation from enrichment cultures) and culture-independent methods (PCR-DGGE) have been be used to assess bacterial composition and biodiversity richness.

Chapter 2. The second part basically consisted in the screening of the bacterial isolates in relation to:

- As resistance (through MIC determination),
- The genotypic study of the mechanisms of arsenic transformation,
- The ability to reduce arsenate to more mobile chemical species.
- The presence of Plant Growth Promoting traits (IAA and siderophores production and ACC deaminase activity),

These evaluations allowed to identify and select different functional groups such as PGPR as well as bacterial species resistant to high levels of arsenic and able to mobilize arsenic from the soil.

Chapter 3. Afterwards, in the third part, specific bacterial *inocula* have been developed and tested for their possible influence on the phytoextraction efficiency of the hyperaccumulator fern *Pteris vittata* in a bioaugmentation experiment in lab scale. Plants have grown in presence of arsenic with or without bacterial *inocula* and the system has been evaluated through the analysis of plant performance, i.e. by measuring plant biomass and metal concentration in plant tissues.

These preliminary estimations allowed to evaluate the influence of the bacterial strains under study on the phytoextraction efficiency of *P. vittata* in the perspective to program a future field scale trial in Scarlino contaminate area.

CHAPTER I:
Characterization of the soil autochthonous micro flora

All the analysis described in this PhD study were performed on soil from 6 different sampling points: one from the arsenopyrite ashes disposal (M) that consists in a cumulus of arsenopyrite-contaminate ashes of 1.600.000 tons and 5 samples from the rhizosphere of 5 different autochthonous plants (P1, P2, P3, P4 and P5) grew adjacent to the waste disposal. The 5 plants were identified on the basis of visual characteristic as *Lolium rigidum* belonging to *Graminaceae* family (P1), *Daucus carota*, an *Umbelliferae* (P2), *Trifolium angustifolium* L. (P3), *Populus alba* (P4) and *Sedum sediforme* belonging to *Crassulaceae* family (P5).

3.1 Enumeration of the cultivable bacterial community

Even though the results obtained by plate enumeration underestimate the actual soil microbial population, the evaluation of the cultivable bacteria fraction in a contaminated soil can provide useful information about the impact of the contamination on the autochthonous micro flora and on the size of the acclimated community.

Enumeration of total heterotrophic aerobic microorganisms at the six sampling points was determined by plating their dilutions onto 3 different culture media: Malt (to detect the presence of fungi), Waksman (specific for the growth of *Actinobacteria*) and Nutrient (for all heterotrophic bacteria).

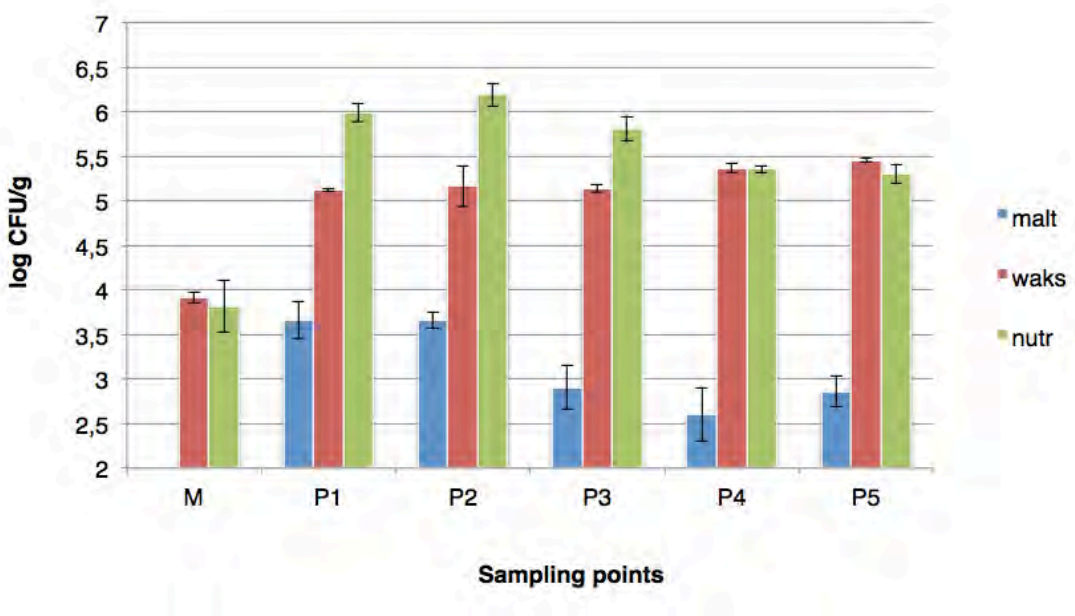


Fig. 3.1 *– Cultivable counts at the 6 examined sampling points (M, P1, P2, P3, P4, P5).*

The abundance of culturable heterotroph bacteria, *Actinobacteria* and fungi of the different samples is indicated in Fig. 3.1 respectively by the count on Nutrient, Waksman and Malt

medium. As represented in the chart microbial load is very low, ranging from 10^4 to 10^6 for bacteria cells and from 0 to $10^{3.6}$ for fungi. These results were quite expected given the high level of contamination in this site of study. Notably no fungi were detected in M sample and this is probably due to the lack of organic matter in the ashes cumulus. In general it was also observed that bacteria were of the order of 10^4 CFU (g dry wt)$^{-1}$ in the ashes fraction and from 10^5 up to 10^6 CFU (g dry wt)$^{-1}$ in the rhizosphere fractions. In both cases these values are very low although a higher count for the plants' rhizosphere was expected; it's indeed well documented how the presence of the plants can promote the proliferation of bacterial species (Abou-Shanab *et al*., 2005). Plant root exudates can indeed provide nutrition to rhizosphere microbes, thus increasing microbiological activity in the rhizosphere up to 1000 times, even in metal contaminated soils (van der Heijden, 2008; van der Lelie, 1998).

Having that one gram of soil can contain approximately 10^7 up to 10^{10} microorganisms of possibly thousands of different species (Daniel, 2004), this first datum doesn't seem to suggest a good adaptation to the contamination by the established heterotrophic bacterial population but a selection exerted by the presence of the contaminants.

3.2 Isolation of soil bacteria from As(III) and As(V) enrichment cultures and taxonomic characterization

Enrichment cultures were arranged in R2A medium by further adding to soil samples of each sampling point (P1, P2, P3, P4, P5 and M) 2 mM As(III) or As(V). The enrichment was aimed at pushing the selection further on the strains within the soil community which are most resistant to inorganic As or potentially able to transform the two toxic forms.

After 8 weeks of incubation, the isolation of the microbial species selected within each enrichment culture (As(III) and As(V)) was made by plating serial dilutions on agar plates of respectively the same selective medium.

A total of 201 As-resistant bacteria were isolated, 126 from As(V)- and 75 from As(III)-enriched cultures respectively. Data are consistent with the fact that As(V) is less toxic than As(III), thus more species can adapt to this form of arsenic (Styblo *et al*., 2000). Besides, being As(V) the dominant chemical specie in the analyzed soils, more bacterial taxa have been isolated in As(V) enrichment culture than in As(III) one. Axenic cultures of morphologically different colonies were screened by the molecular technique ARDRA – some exemplificative profiles obtained are reported in Fig. 3.2 – grouped into different Operational Taxonomic Units (OTUs) and taxonomically identified through sequencing of *16S rRNA* gene.

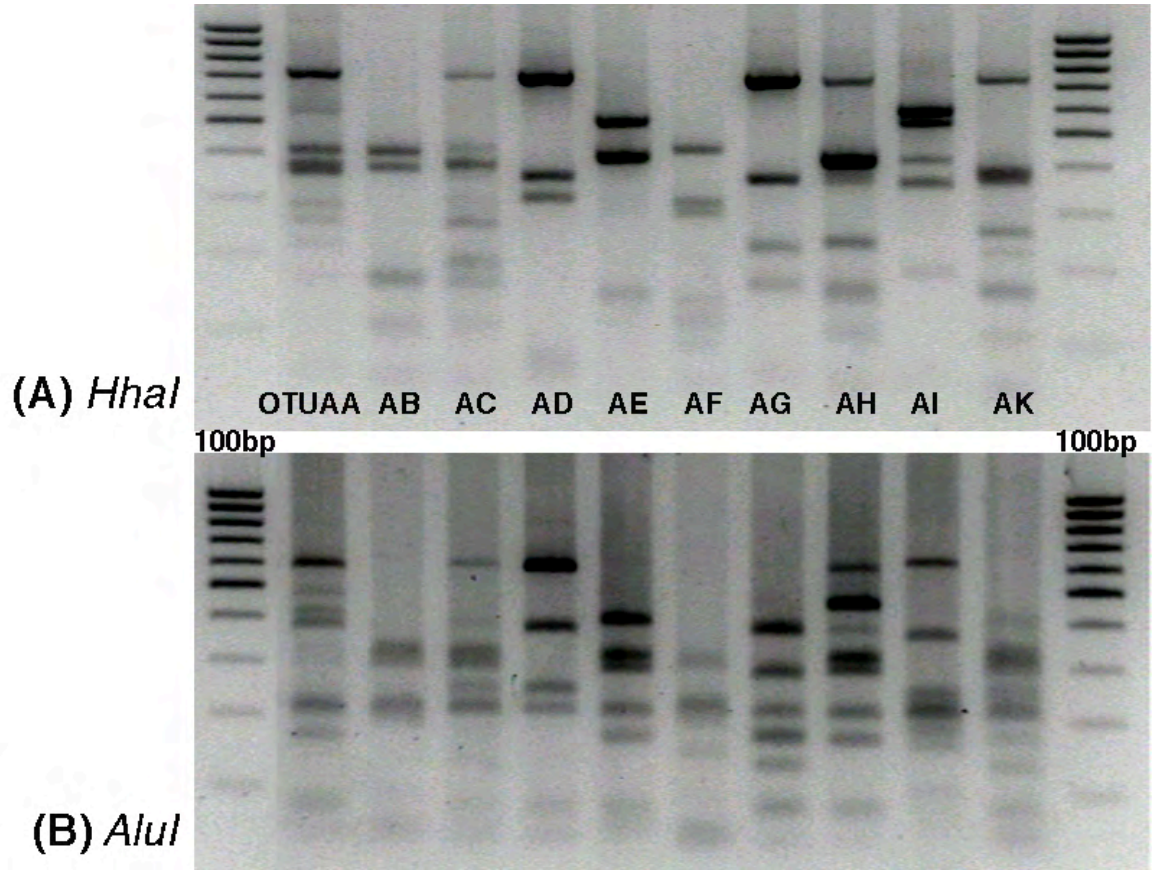


Fig. 3.2 *– Main restriction profiles obtained with HhaI (A) and AluI (B) enzymes.*

Restriction analysis enabled to cluster the 201 isolates into 55 different operational taxonomic units distributed among the different samples (12 in the matrix sample and 43 in the plants rhizosphere). Sequencing results of obtained OTUs are reported in Tab. 3.1 with percentage homology to EzTaxon-e Database and GenBank relatives.

SAMPLING POINT	OTU	Taxonomic Reference ID	Phylogenetic group	Homology %
MATRIX	A	*B. weihenstephanensis* FN796835	*Firmicutes*	100%
	B	*Stenotrophomonas rhizophila* AJ293463	Gamma-proteobacteria	99.35%
	C	*Brevundimonas olei* GQ250440	Alfa-proteobacteria	100%
	D	*Lysinibacillus fusiformis* AB271743	*Firmicutes*	100%
	E	*Ochrobactrum cytisi* AY776289	Alfa-proteobacteria	99.01%
	G	*Microbacterium oxidans* Y17227	*Actinobacteria*	98.37%
	H	*Brevibacterium simplex* AB363738	*Firmicutes*	100%

	I	*Brevibacterium frigoritolerans* KC355256	*Firmicutes*	100%
	J	*Rhizobium mesosinicum* DQ100063	Alfa-proteobacteria	99.68%
	K	*Bacillus flexus* AB021185	*Firmicutes*	100%
	L	*Ochrobactrum cytisi* AY776289	Alfa-proteobacteria	100%
	M	*Bacillus thuringiensis* ACNF01000156	*Firmicutes*	100%
PLANT 1 *(Lolium rigidum)*	N	*Pseudomonas putida* Z76667	Gamma-proteobacteria	100%
	O	*Pseudomonas fuscovaginae* AB021381	Gamma-proteobacteria	100%
	P	*Achromobacter marplatensis* EU150134	Beta-proteobacteria	99.42%
	Q	*Arthrobacter ureafaciens* X80744	*Actinobacteria*	99.35%
	R	*Arthrobacter nitroguajacolicus* AJ512504	*Actinobacteria*	100%
	S	*Pseudoxanthomonas japonensis* AB008507	Gamma-proteobacteria	99.59%
PLANT 2 *(Daucus carota)*	T	*Stenotrophomonas chelatiphaga* EU573216	Gamma-proteobacteria	100%
	U	*Delftia lacustris* EU888308	Beta-proteobacteria	100%
	V	*Brevundimonas bullata* D12785	Alfa-proteobacteria	100%
	W	*Microbacterium yannici* FN547412	*Actinobacteria*	99.05%
	X	*Bacillus megaterium* HQ285923	*Firmicutes*	100%
	Y	*Microbacterium arabinogalactanolyticum* AB004715	*Actinobacteria*	100%
	Z	*Flavobacterium chungbukense* HM627539	*Flavobacteria*	100%
	AA	*Bacillus niacini* AB021194	*Firmicutes*	99.18%
	AB	*Microbacterium arabinogalactanolyticum* AB004715	*Actinobacteria*	99.59%
PLANT 3 *(Trifolium angustifoliu m* L.)	AC	*Rhodococcus ruber* X80625	*Actinobacteria*	100%
	AD	*Devosia insulae* EF114313	Alfa-proteobacteria	100%
	AE	*Massilia oculi*	Beta-proteobacteria	97.47%

		FR773700		
	AF	*Xanthomonas vasicola* Y10755	Gamma-proteobacteria	100%
	AG	*Bacillus aryabhattai* HQ009875	*Firmicutes*	100%
	AH	*Brevundimonas alba* AJ227785	Alfa-proteobacteria	99.67%
	AI	*Microbacterium niemengense* JN408293	*Actinobacteria*	99.68%
	AJ	*Agromyces aurantiacus* AF3894342	*Actinobacteria*	100%
	AK	*Arthrobacter humicola* AB279890	*Actinobacteria*	100%
	AL	*Brevundimonas olei* GQ250440	Alfa-proteobacteria	99.06%
	AM	*Bacillus bataviensis* AJ542508	*Firmicutes*	99.67%
PLANT 4 *(Populus alba)*	AN	*Comamonas testosteroni* AHIL01000001	Beta-proteobacteria	99.71%
	AO	*Delftia acidovorans* NR074691	Beta-proteobacteria	100%
	AP	*Variovorax paradoxus* HQ231964	Beta-proteobacteria	100%
	AQ	*Microbacterum invictum* AM949677	*Actinobacteria*	99.31%
	AR	*Bacillus drententis* AJ542506	*Firmicutes*	98.64%
	AS	*Pseudoxanthomonas mexicana* GU908488	Gamma-proteobacteria	100%
	AT	*Pseudomonas plecoglossicida* AB009457	Gamma-proteobacteria	100%
	AU	*Flavobacterium cauense* EU5211691	*Flavobacteria*	100%
PLANT 5 *(Sedum sediforme)*	AV	*Bacillus nealsonii* EU656111	*Firmicutes*	98.02%
	AW	*Stenotrophomonas maltophilia* AB008509	Gamma-proteobacteria	100%
	AX	*Microbacterium liquefaciens* HM104368	*Actinobacteria*	100%
	AY	*Arthrobacter nitroguajacolicus* AJ512504	*Actinobacteria*	100%
	AZ	*Pseudoxanthomonas mexicana* AF273082	Gamma-proteobacteria	100%
	BA	*Brevibacillus brevis* AP008955	*Firmicutes*	99.43%
	BB	*Lysobacter yangpyeongensis* DQ191179	Gamma-proteobacteria	100%
	BC	*Microbacterium suwonense*	*Actinobacteria*	99.37%

		GQ246683		
	BD	*Sphingopyxis alaskensis* CP000356	Alfa-proteobacteria	99.68%

Tab. 3.1 *– Taxonomic collocation of OTUs isolated from As(III) and As(V) enrichment cultures.*

Despite of the high level of contamination this bacterial community showed a high degree of biodiversity at *genus* level, being the isolates allocated to 25 different *genera*.

In the sequencing results for the isolated OTUs it is noteworthy a high homology (99%-100%) to bacterial *genera* widely diffused within different environmental niches including contaminated soils such as *Stenotrophomonas* and *Pseudomonas* of Gamma-proteobacteria, *genera* reported for high resistances to arsenic and heavy metals such as *Microbacterium* of *Actinobacteria* and reporting interesting metabolic capabilities towards inorganic arsenic compounds such as *Delftia* of Beta-proteobacteria or *B. thuringiensis* of *Firmicutes* lineage, thus suggesting a high resistance and degrading potential within the indigenous bacterial community (Abou-Shanab *et al*., 2007; Muller *et al*., 2007; Xiong *et al*., 2012). The enrichment conditions, however, do not reproduce real conditions of an environmental sample and select the microorganisms growing faster and adapting better to the growth conditions imposed.

In the preliminary taxonomic analysis emerged that Gram positive bacteria accounted for more than one half of the totality of isolated strains with 36% of *Firmicutes* and 16% of *Actinobacteria*. On the other hand, Proteobacteria belonging to Alfa-, Beta- and Gamma- classes represented 10%, 12% and 24% respectively. Lastly, *Flavobacteria* were the less represented class with only 2% of the As resistant strains (Fig. 3.3).

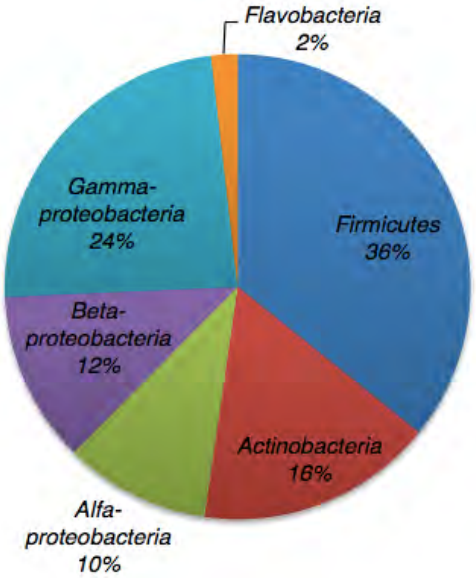


Fig. 3.3 *– Phyla distribution of the totality of isolated As resistant strains.*

Firmicutes

Considering the sequencing data of all the isolated strains, *Firmicutes taxon* appeared the most abundant in the site object of study. Although in other studies (Halter *et al*., 2011; Sheik *et al*., 2012; Xiong *et al*., 2012) *Firmicutes* represent the minority among the various *phyla*, in this case most of the isolated OTUs belonged to *Bacillus genus* as *B. megaterium, B. cereus, Brevibacillus, B. nealsonii and B. niacini* (Fig. 3.4). PGP activities, abundance in heavy metals polluted soil and resistance to conditions of stress including heavy metal are reported in literature for these species and their recovery is not surprising although it was higher than expected (Kusum and Kumar, 1998; Ellis *et al*., 2003; Belimov *et al*., 2001). It could be hypothesized that the disadvantages conditions of Scarlino, i.e. extreme temperature or the lack of water in some period of the year or poor organic matter content in some spot of the area, had promoted the survival of sporulating microorganisms. Thus, the high abundance of *Bacillus* species confirmed the strong resistance of this *genus* to conditions of stress (Ellis *et al*., 2003; Valverde *et al*., 2011).

Moreover some *Bacillus* isolates exhibited a high resistance to inorganic As and also showed the ability to reduce As(V) to As(III) (Cavalca *et al*., 2010; Bachate *et al.,* 2008; Achour *et al*., 2007). In addition, determinant sequences, such as the *ars* operon, for arsenic resistance are well known for *Bacillus* sp. (Sato and Kobayashi, 1998).

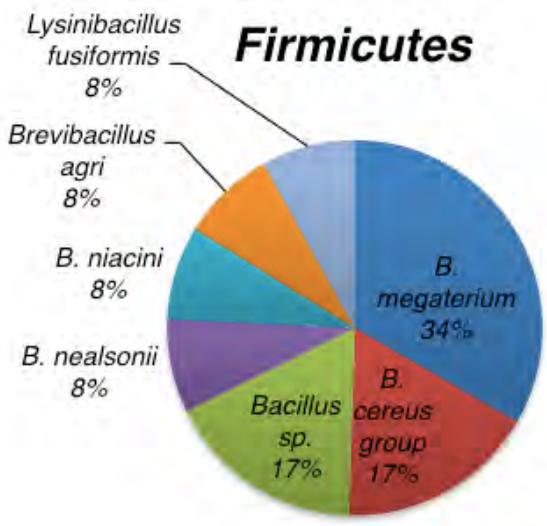


Fig. 3.4 *– Taxa distribution of OTUs within Firmicutes phylum.*

Actinobacteria

Even if less represented than *Firmicutes phylum*, an interesting potential can relays in the gram-positive *Actinobacteria* community members, with OTUs belonging to *genera* such as *Microbacterium* and *Arthrobacter* (Fig. 3.5), for which tolerance and resistance to various heavy metals such as As, Pb, Ni, Cr, U is reported (Abou-Shanab *et al*., 2007).

In the present study *Microbacterium genus* is in general the most represented among all the isolated strains; isolates showing high homology with *M. oxidans, M. yannicii, M. invictum, M. arabinogalactanolyticum, M. neimengenses, M. suwonense* and *M. liquefaciens* species have

indeed been isolated from the examined samples. The *genus Arthrobacter* was also very represented, with OTUs belonging to *A. ureafaciens, A. nitroguaiacolicus* and *A. humicola* species. Actually, towards arsenic, the species belonging to *Microbacterium genus* are among the most resistant known in literature (Achour-Rokbani *et al.,* 2010) and new examples of arsenic-resistant isolates of *Microbacterium* and *Arthrobacter* are continuously being reported (Cai *et al*., 2009; Chen and Shao, 2009; Drewniak *et al*., 2008).

Other isolated strains of this *phylum* most closely related to *Rhodococcus*, *Brevibacterium* and *Agromyces genera*, to those species different multiple heavy metal resistance characteristics and Plant growth promoting (PGP) activities have been related (Ma *et al*., 2011a; Sarkar *et al*., 2013; Rajkumar *et al*., 2012). Consequently these isolates may be particularly interesting in a phytoremediation approach.

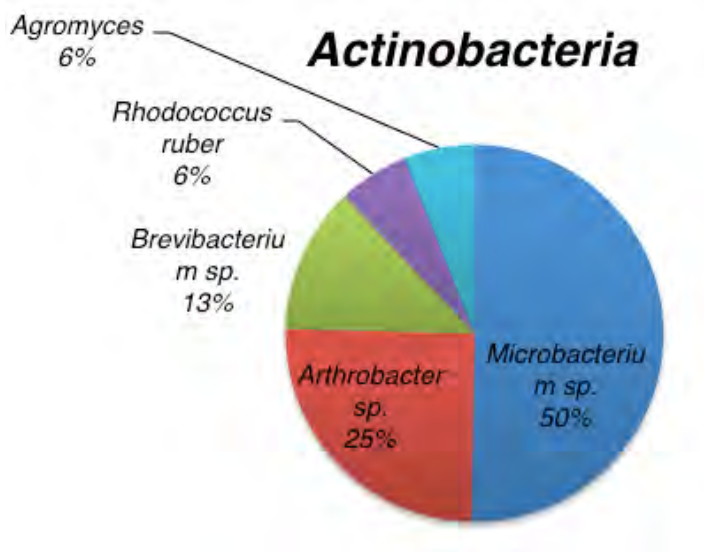


Fig. 3.5 *– Taxa distribution of OTUs within Actinobacteria phylum.*

Proteobacteria

As far as Proteobacteria are concerned, the Gamma class is most represented. This is not surprising since the easy cultivability of Proteobacteria and of the Gamma class in particular. As shown in Fig 3.6, most of the isolated strains affiliated to the Gamma-proteobacteria class are associated with members of the *genera Pseudomonas* and *Stenotrophomonas* whose members are able to grow well under laboratory culture conditions (Kim *et al*., 2008).

Their distribution has been reported to be affected by metal toxicity both negatively (Ellis *et al*., 2003; Gillan *et al*., 2005) and positively (Feris *et al*., 2003; Zhang *et al*., 2007), and the dominance of this class - mainly *Stenotrophomonas* and *Pseudomonas genera* - was detected by clone library screening in sub-superficial soil levels and connected to the heterotrophic feature of these *genera* (Zhang *et al*., 2007). Analogously species like *S. maltophilia, Pseudomonas putida* and *Pseudoxanthomonas mexicana* were isolated from this As contaminated site. These are well known for their As intrinsic resistance and for PGP activities and their recovery was expected (Sarkar *et al*. 2013; Srinivas *et al*., 2011; Xiong *et al*., 2012).

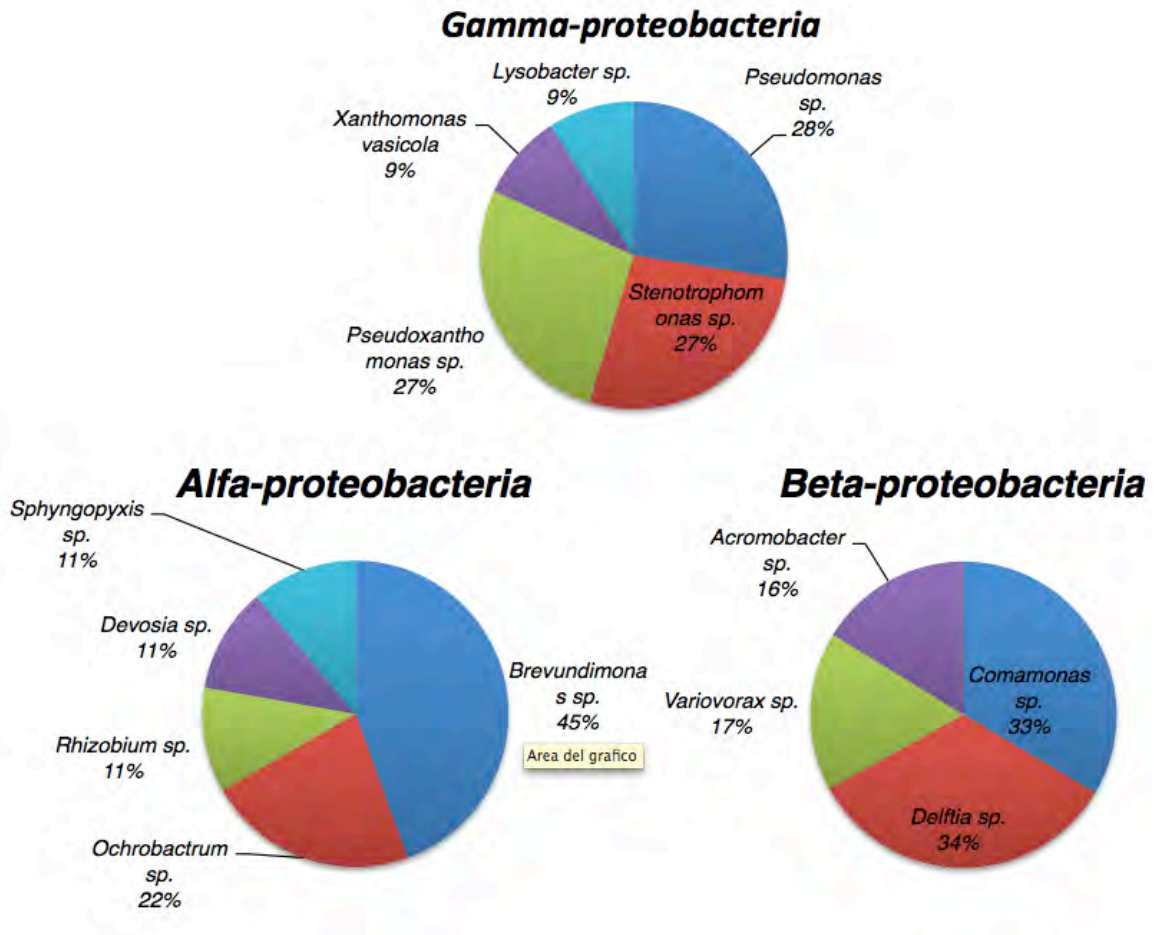


Fig. 3.6 *– Taxa distribution of OTUs within Proteobacteria phylum.*

Analogous considerations concerning As resistance of environmental isolates can be taken for Alfa- and Beta- class of Proteobacteria (Fig. 3.6). *Genera* isolated in this study include *Brevundimonas* and *Ochrobactrum* (Alfa-Proteobacteria) and *Delftia*, *Comamonas* and *Variovorax* (Beta-Proteobacteria) that are known for high resistance to arsenic and for their ability to transform the toxic compounds thanks to the presence of *ars* and *aox* operon in their genome (Branco *et al*., 2008; Jackson *et al*., 2005; Chen *et al*., 2005; Muller *et al.,* 2007; Rajkumar *et al*., 2012). Studies have moreover reported the predominance of both Gamma and Beta classes of Proteobacteria in oil-contaminated areas and in lead contaminate environments (Ben Said *et al*., 2008; Ferrari, 2012).

This suggests, as expected under a constant long-term As stress in soil, the selection towards more resistant population.

Flavobacteria

Considering the *Flavobacteria phylum*, its really low representation can be partly connected with its difficulty of cultivation. Only two OTUs of this class, belonging to *F. chungbukense* and *F. cauense* species, have been identified. Species of this *genus* have been detected in the

rhizosphere of plants colonizing on mine tailings, in deep sea sediments, heavy metals contaminated soils (Cavalca *et al*., 2010; Chen and Shao, 2009; Zhang *et al*., 2007).

It has to be considered that along with arsenic contamination other physicochemical factors may also influence the microorganisms distribution. Soil in fact can be regarded as very heterogeneous with respect to conditions for microbial growth and for the distribution of microorganisms and matrix substances, resulting in a wide variety of microbial niches and a high diversity of soil microorganisms (Daniel, 2004). Nutrients and the quality of organic matter may in fact also influence microbial communities, as well as the presence of other heavy metals (Pb, Fe, Al, Zn) in the site object of study.
However these factors are implied in all study of field samples and on long-term exposure to particular contaminants, where many environmental variables are thus implicated and it is very difficult to take all of them into adequate account.

Differences between the two enrichments

As(III)-resistant isolates

From partial *16S rDNA* genes sequencing from the isolates of the two different enrichments and homology searches emerged that the majority of the As(III)-resistant isolates showed a typical distribution of bacterial *phyla* associated with contaminated soils, with a preponderance of Proteobacteria. As shown in Fig. 3.7 mostly Gamma- (33%) and Beta- (28%) classes like *S. maltophilia* specie and *Pseudomonas* and *Delftia genera* have been isolated. This is not surprising, as Gamma-proteobacteria have previously been found in various arsenic-contaminated environments to represent the largest clade of As resistant isolates (Chen and Shao, 2009; Cavalca *et al*., 2010; Liao *et al*., 2011).

As(V)-resistant isolates

On the other hand most of the isolates from As(V) enrichment cultures belonged to the Gram-positive *phyla* of *Firmicutes* (43%) and *Actinobacteria* (24%) with *Bacillus* sp. (in particular belonging to *B. cereus* group and *B. megaterium*) and *Microbacterium* sp. being the most abundant *genera* (Fig. 3.7). Gram negative bacteria of other phylogenetic groups like Alfa-Proteobacteria, Beta-Proteobacteria, and Gamma-proteobacteria were expected to be the most represented but they actually appeared to constitute the minor part (13%, 0% and 17% respectively). As with any cultivation method, the microorganisms selected under specific enrichment conditions, in this case on R2A media, supplemented with As(III) or As(V) 2 mM, do not necessarily represent all the populations of the soil samples. A preponderance of *Firmicutes* and *Actinobacteria* lineages in soil samples was detected from direct isolation by Achour *et al*. (2007) and Valverde *et al*. (2011) noticed that the proportion of *Firmicutes* increases as soil arsenic levels increase but, to the best of authors' knowledge, similar relative abundance of Gram positive bacteria in enrichment culture in presence of As(V) in comparison to As(III) enrichments has never been detected. In general the preponderance of Gram positive species could be explained with the fact that As(V) concentration in the soil samples is higher than As(III) one (70%

> 30%). Having that As(V) is easily adsorbed on the bacterial cell wall, mostly Gram positive bacteria have been selected in this condition (Slyemi and Bonnefoy 2012).

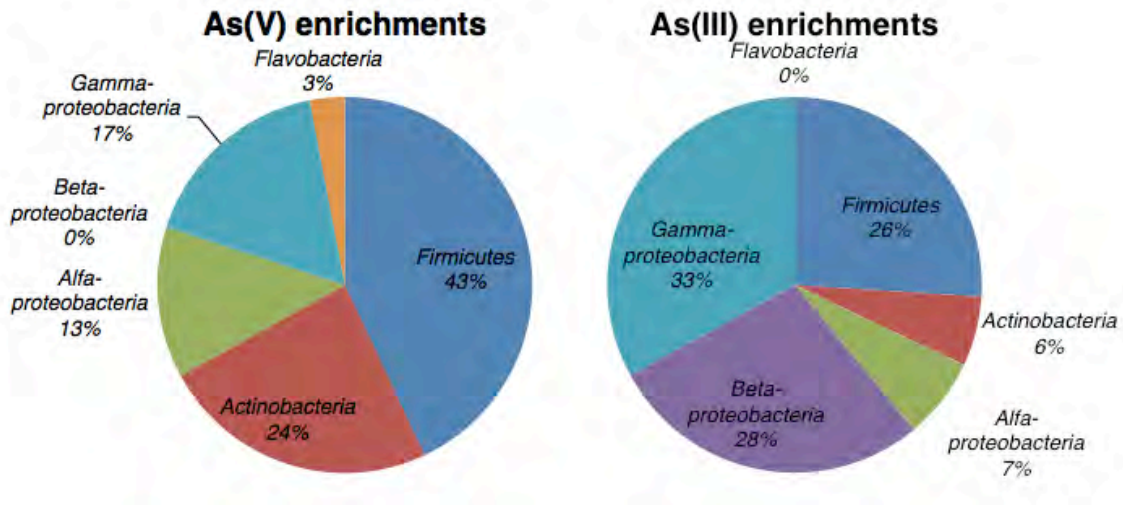


Fig. 3.7 *– Phyla distribution of the different strains isolated in presence of As(III) and As(V) respectively.*

Differences between the different samples

Phyla distribution in the rhizosphere of the analyzed plants in comparison to the highly contaminated matrix was also evaluated (Fig. 3.8). Results indicate that in the polluted matrix the most part of the isolates belonged to *Bacillus genus* (54%). Otherwise in the rhizosphere samples (P1, P2, P3, P4 and P5) the most abundant *phyla* were those belonging to Proteobacteria, in particular to Gamma and Beta class again with the most abundant *genera Stenotrophomonas*, *Pseudomonas* and *Delftia*. Similarly *Actinobacteria phylum* appeared to be more represented among the rhizosphere isolates than in the matrix and, conversely *Firmicutes* resulted to be less abundant in the 5 rhizosphere soils.

It could be hypothesized that the preponderance of *Bacillus* spp. strains at the highest soil arsenic levels might be related to enduring the toxicity of arsenic in sporulated form or the structure itself of the bacterial cell wall of Gram positive species. Concerning this, the nature of M sample must be considered. Scarlino's plan it's indeed characterized by Mediterranean climate, with scarce precipitation and high temperature from June to September; moreover, the fact that pyrite ashes are black can exacerbate these conditions. Whereas, for what concerns the rhizosphere isolates, it's attested that Gamma-proteobacteria *phylum* includes species able to establish a symbiontic relationship with the rhizosphere environment (Khan, 2005; Sun *et al.*, 2009). Besides many of these strains belong to species known for their Plant Growth Promoting (PGP) traits. Just to cite some of them, *Pseudomonas putida* has been reported to be an IAA producer (Ahmad *et al.*, 2006); *Bacillus cereus* and *Achromobacter* sp. are ACC deaminase producers (Govindasamy *et al.*, 2008; Lugtenberg and Kamilova, 2009); *Flavobacterium* sp., *Rhodococcus* sp., *Arthrobacter* sp., *Microbacterium* sp. are siderofore producers (Rajkumar *et al.*, 2010); *Pseudomonas putida*,

Delftia sp., *Arthrobacter* sp., *Bacillus megaterium* are able to solubilize phosphorus (Chen *et al*., 2006; Malboobi *et al*., 2009; Jeong *et al*., 2012). Therefore the recovery of these *genera* is totally explicable in a context of heavy metals contaminated rhizosphere.

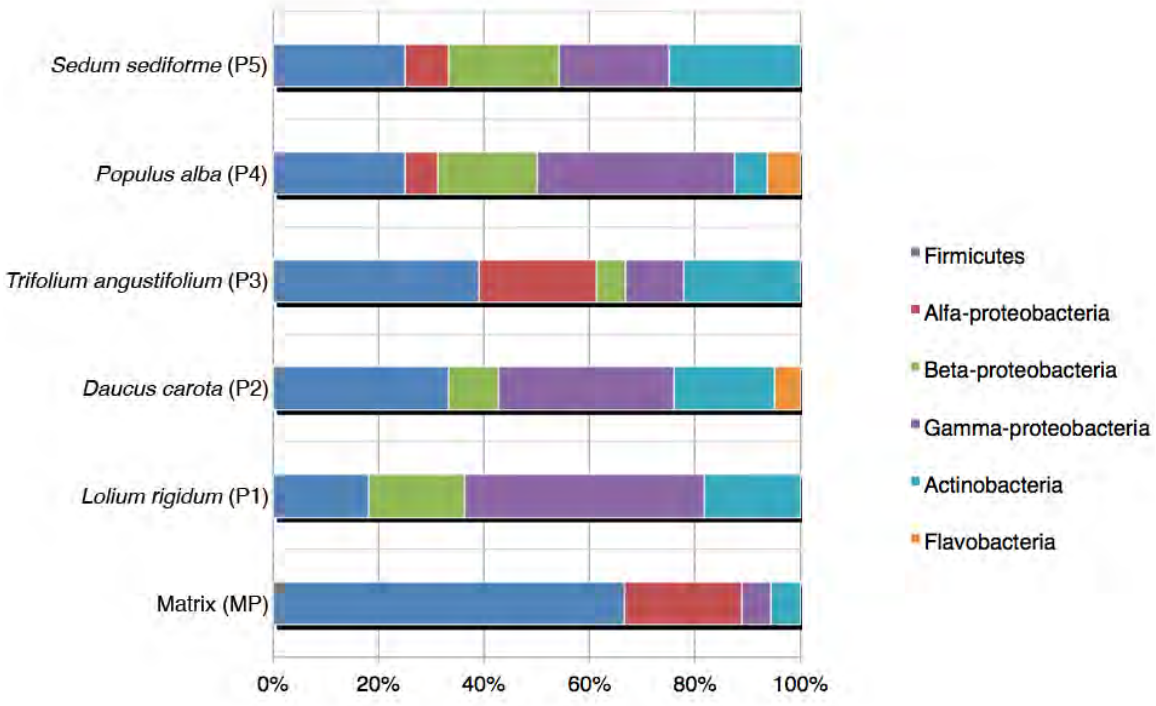


Fig. 3.8 *– Distribution of the bacterial phyla among the isolates of the different samples.*

3.2.1 Diversity index, coverage values and rarefaction analysis of the analyzed microbial community.

Table 2.2 shows Shannon-Wiener and Simpson diversity (Dominance) indexes, coverage and evenness calculated for each enrichment culture of the different sampling points.

The Shannon's diversity index is defined as the proportional abundance of species in a community and in substance it calculates the rarity and commonness of species in a community (Lefauconnier *et al*, 1994). Having that the S-W index values can range of 0 to 4.6, these analyzed samples, considering the presence of toxic contaminants, showed a discrete biodiversity in particular for what concerns P3 (2,71), P5 (2,65) and P2 (2,59) samples. Anyway no substantial differences were shown by analyzed communities.

Evenness and Simpson Dominance indexes are measures that make the values comparable between communities by controlling for the number of species found within the communities; they are useful to describe the extent of dominance by individual OTUs or singletons (Magurran, 1996). In this study all samples approximated the maximum possible values (1) as some sequence types were recovered only once and the majority of sequence clusters comprised only two individuals, thus suggesting a high biodiversity.

Sampling point	Species richness	Coverage	Shannon-Wiener	Evenness	Simpson dominance D
MP	12	88	2.362	0.8165	0.1166
P1	9	90	2.095	0.9025	0.135
P2	15	90	2.528	0.8354	0.09297
P3	16	88	2.651	0.8853	0.08173
P4	12	89	2.327	0.8542	0.113
P5	17	90	2.575	0.7728	0.1012

Tab. 2.2 *– Diversity indices calculated for the enrichment cultures from the matrix and the five rhizosphere samples in Scarlino's industrial area.*

To estimate diversity coverage and to determine whether a sufficient number of bacterial isolates from each enrichments had been identified, rarefaction analysis was performed as illustrated in Fig. 3.9 (Brewer and Williamson, 1994; Good, 1953; Mullins *et al.*, 1995). The generated rarefaction curves were near saturation for all the samples, indicating that full coverage of the microbial diversity was obtained. These data were also calculated to determine how efficiently the enrichment cultures described the complexity of a theoretical community like an original bacterial community and are consistent with a good sampling of the examined soil samples.

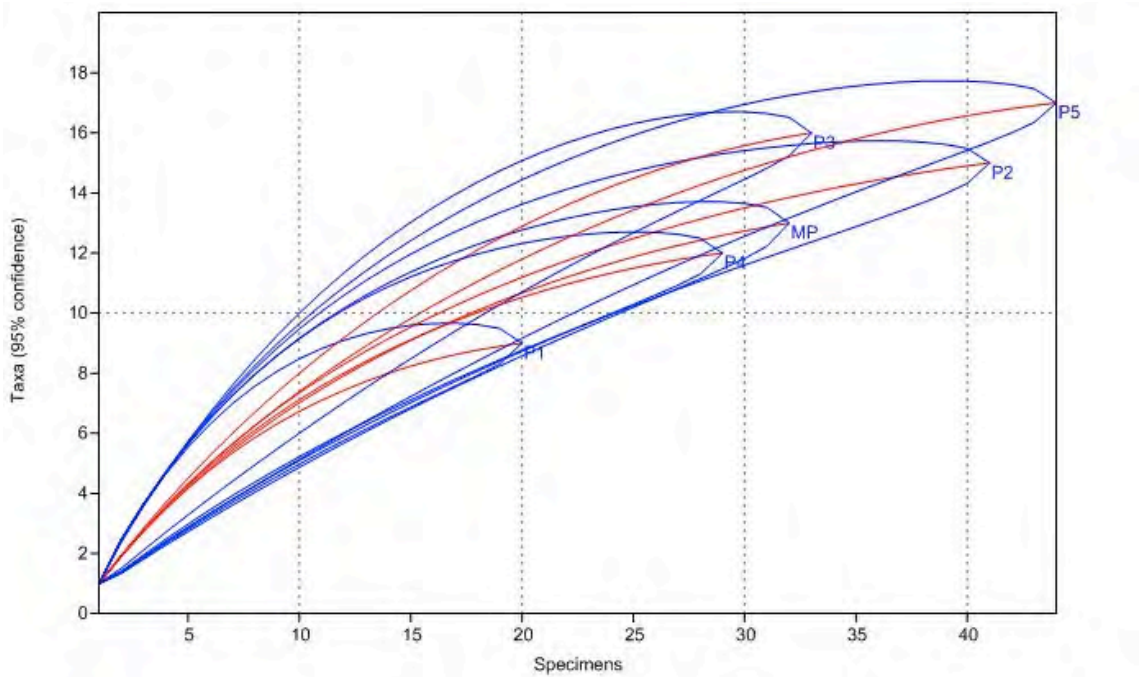


Fig 3.9 *– Rarefaction curves for the different isolates obtained from the enrichments cultures (M, P1, P2, P3, P4 and P5). The expected number of bacterial OTUs is plotted vs. the number of isolates. The faint lines represent 95% confidence intervals for each curve.*

3.3 Analysis of bacterial community structure of the matrix by PCR-DGGE

Traditional microbiological techniques may underestimate the diversity of microorganisms in environmental samples and could potentially provide unrealistic descriptions of microbial community structure. Molecular methods based on the analysis of *16S rDNA* sequences can provide information about the complexity of prokaryotic communities in natural environments and have revealed entirely new phylogenetic lineages (Pace, 1997).

Soil microbial communities are typically very diverse, therefore DGGE produces a complex pattern of amplification products revealing the principal components and the most dominant species of the bacterial community, comprehensive of the uncultivable fraction (Torsvik *et al*., 2002; Joynt *et al*., 2006). This procedure allows the direct discrimination of species into a bacterial community and to infer their relative abundance by bands intensity. Moreover major bands can be cut from the gel, cloned and sequenced allowing the identification of the corresponding microorganisms.

In order to reach a comprehensive evaluation of the microbial community structure at the matrix sampling point, PCR-DGGE analyses were carried out on *V3-16S rDNA* PCR products, amplified from soil total DNA extracted in duplicate. Arsenopyrite ashes samples were collected from 1m, 2m, 5m, 13m and 17m depth but DNA extraction was successful only for the sample 2m deep (Fig. 3.10 panel (B)). Unfortunately, problems in DNA extraction and/or in the following polymerase chain reactions are very common in the analysis of this metal rich samples. High concentration of iron and other metals could have indeed inhibited both some step of the extraction protocols and of the PCR reactions (Jackson *et al*., 2001).

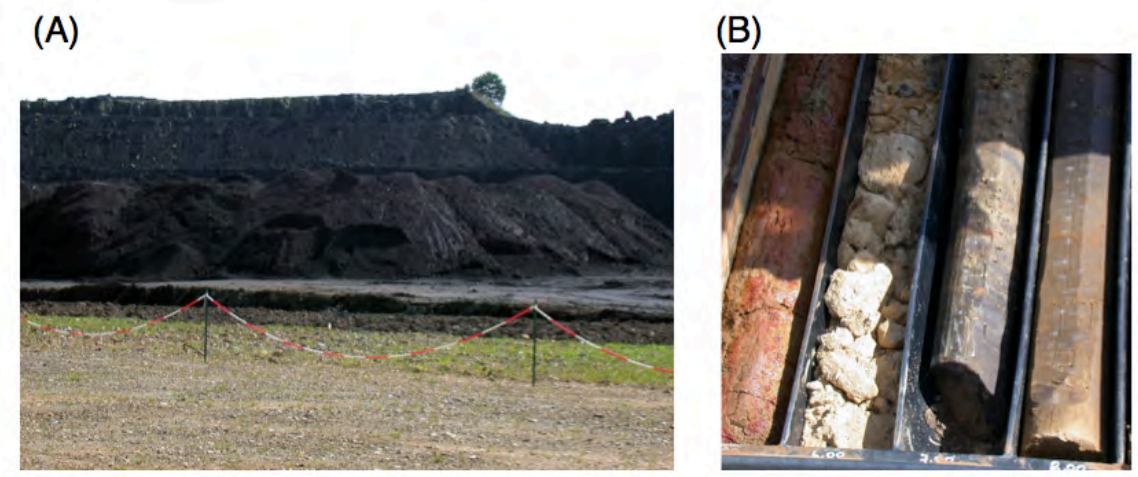


Fig. 3.10 *– Matrix sampling area: (A) the contaminated ashes cumulus and (B) some of the relative coring samples.*

As illustrated in Fig. 3.11 duplicate samples were found to match closely showing identic profile complexity and band composition, thus indicating that the procedure is reproducible and reliable. 9 bands of major intensity, corresponding to dominant components of the microbial community,

were detected indicating the selection imposed by the contaminant towards a resistant population. These bands were all excised sequenced.

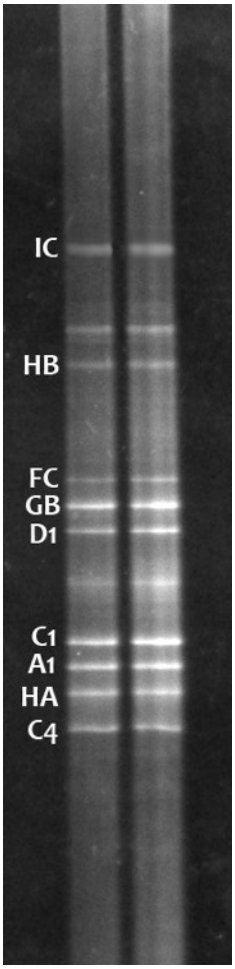


Fig. 3.11 *– DGGE analysis performed on the matrix targeting bacterial V3 16S rDNA region.*

In Tab. 3.3 the results representative of the alignment in data banks for the retrieved bacterial sequences are reported.

Band	Taxonomic Reference ID	Phylogenetic group	Homology (%)
IC	*Unc. Firmicutes* FM253001	*Firmicutes*	99%
HB	*Lactobacillus kimchii* AB626059	*Firmicutes*	98.46%
D1	*Achromobacter* sp. HE613447	*Beta-proteobacteria*	100%
GB	*Unc. Sphingobacteriales* FN594671	*Bacteroidetes*	98%
FC	*Unc. Sphingobacteriales* AM936464	*Bacteroidetes*	99%
A1	*Unc. Comamonadaceae* AM934917	*Beta-proteobacteria*	98%

HA	*Microbacterium hydrocarbonoxydans* AJ698726	*Actinobacteria*	98.87%
C1	*Unc. Burkholderiaceae* AM935801	*Beta-proteobacteria*	99%
C4	*Amycolicicoccus subflavus* CP002786	*Actinobacteria*	97%

Tab. 3.3 *– Percentage homology to EzTaxon-e Database and GenBank relatives for V3 16S rDNA gene sequences of the cloned DGGE bands.*

Before giving taxonomic information about the retrieved sequence of this study (illustrated also in Fig. 3.12), it has to be pointed out that the majority of the crosscheck in data bank was not precise to specie or *genus* level. This could be due to the fact that DNA extraction was a difficult step in the analysis, many contaminants (like fulvic and humid acid but also high concentrations of different metals) were present and could have impaired the PCR reactions or fragment the template. Moreover chimeras could have formed and altered the results (Wintzingerode *et al*., 1997; Tebbe and Vahjen, 1993).

Firmicutes

From the sequencing data emerged that four of the nine bands correspond to Gram positive bacterial species, 2 belonging to *Firmicutes* and 2 to *Actinobacteria*, in agreement with the major representation of these *phyla* detected by the culture study previously described (Par. 3.2).

As far as *Firmicutes phylum* is concerned, two bands were retrieved. The first doesn't identify a specific *genus* and it can be possibly related to *Bacillus* or *Clostridium genera*. The related and known arsenic respiring microorganisms from the *Firmicutes phylum* are *Alkaliphilus oremlandii*, *Caloramator* sp., *Bacillus selenatarsenatis* and *Bacillus arseniciselenatis*. *Alkaliphilus oremlandii* is able to use arsenate and thiosulfate as terminal electron acceptors, and it has the *arrA* gen, involved in anaerobic-arsenate-reducing metabolism (Fisher *et al*. 2008). *Caloramator* sp. metabolizes arsenic and produces beta-realgar (arsenic sulfide), a mineral that has been recently observed as a by-product of arsenic microbial metabolism (Ledbetter *et al*. 2007). Moreover *ars* operon is also spread among *Bacillus genus* members. Arsenite transporters gene (both the forms *arsB* and *ACR3*), arsenate reductase gene and the arsenic repressor *arsR*, arranged in a variety of configurations, have been often found in different *Bacillus* species (Achour *et al*., 2007; Silver and Phung, 2005a; Macur *et al*., 2004; Sato and Kobayashi, 1998). For instance in the case of *Acidithiobacillus ferroxidans*, an acidophilic, chemolithoautotrophic, bio-mining bacterium the *ars* genes are arranged in two divergently transcribed operons, *arsRC* and *arsBH* (Butcher *et al*., 2000).

The other identified *Firmicutes* in the gel belong to *Lactobacillus kimchii* specie. It represents a novel bacterium, isolated from a traditional fermented food, kimchi (*Kim et al*., 2012). To the best

of author knowledge this specie has never been found in association with matrixes contaminated by arsenic or heavy metals or organic compounds of any kind.

Actinobacteria

In relation to As polluted environment, *Actinobacteria* are quite ubiquitous; species related to this *phylum* were found in contaminated soils and aquifers, in mine tailings and acid mine drainage and in geothermal springs (Bruneel *et al*., 2011; Valverde *et al*., 2011; Sheik *et al.,* 2012).

In the present study two species were detected. One sequence matched with several *Microbacterium* species and among them with *Microbacterium hydrocarbonoxydans* that, as the name itself underlines, was isolated from oil contaminated environment and is able to degrade hydrocarbons (Schippers *et al*., 2005). The strain was also isolated from Pb polluted soils (Ferrari, 2012) and from As contaminated soils, showing to be able to reduce As(V) with high efficiency (Bachate *et al*., 2009).

The other band corresponded to different bacterial *genera* of *Actinobacteria phylum* and among them to *Amycolicicoccus subflavus*, a newly published novel species in the novel *genus Amycolicicoccus*. The organism has been isolated for the first time from two crude oil-polluted soils in the Daqing and Shengli oilfields in China (Cai *et al*., 2011). Genes involved in hydrocarbon degradation pathways were found in its genome but evidences of mechanism related to As tolerance or transformation hasn't been investigated yet. It has to be pointed out that for this sequence the homology research reached at top a value of 97% and it's not sufficient to affiliate this sample to any bacterial specie.

Beta-proteobacteria

Three other bands matched with Beta-proteobacteria taxa.

One band showed 100% homology with a specie belonging to *Achromobacter genus*, also recovered from the enrichment cultures (amended with As) and known in literature for Plant Growth Promoting activity and for its ability to oxidate arsenite (Cai *et al*., 2009; Quéméneur *et al*., 2010).

A band identified a member of *Burkholderiaceae*, to which important environmental isolates belonging to *Burkholderia, Cupriavidus* and *Ralstonia genera* are comprised. Actually the sequencing data don't allow a precise taxonomic collocation of the retrieved bands but it can hypothesized that one of these cited *genera* may correspond.

Burkholderia genus is widely distributed in the environment and is an important component of the soil microbial community (Dalmastri *et al*., 1999). *Burkholderia* distribution in the environment is an important issue due to its pathogenic status. However, it is also important since some species are involved in plant growth promotion, biological control and bioremediation (Compant *et al*., 2008). *Cupriavidus* and *Ralsonia* species have also been isolated from soil, and volcanic mudflow deposits (Coenye *et al*., 2003; Sato *et al*., 2006). More important, *Burkholderia*, *Cupriavidus*, and *Ralstonia* are involved in the biodegradation of toxic compounds and have been described as potential agents for bioremedation. For example, *R. pickettii* can degrade benzene (Bucheli-

Witschel *et al*., 2008), *Ralstonia* sp. can degrade thiocyanate in consortia with *Klebsiella pneumonia* (Chaudhari and Kodam, 2010) and other *Ralstonia* sp. are chemoattracted to p-nitrophenol in soil (Debarati *et al*., 2006). Some *Cupriavidus* species are able to grow on media containing phenol or trichloroethylene (TCE) and they can utilize different chlorophenols as a sole carbon source (Zilouei *et al*., 2006). The *Burkholderia genus* is well known for its ability to degrade toxic compounds. *B. fungorum* and members of the *Burkholderia cepacia* complex (BCC) are examples of species that are able to degrade different toxic organic compounds (Seo *et al*., 2007; Andreolli *et al*., 2011). Furthermore, some *Cupriavidus* species have the ability to grow in the presence of heavy metals, *C. basilensis* and *C. campinensis* were isolated from a zinc desert in Belgium (Goris *et al*., 2001). *C. metallidurans* strain CH34T is remarkably resistant to heavy metals; it has gained increasing interest as a model organism for heavy metal detoxification and for biotechnological purposes. This bacterium harbors two plasmids that contain genes involved in resistance to copper, chromium, mercury, nickel, silver, cadmium, cobalt, lead, and zinc (Monchy *et al*., 2007). This strain also carries arsenite/arsenate-resistance genes (Zhang *et al*., 2009); to date, this bacterium carries the largest number of genes encoding resistance to heavy metals.

Comamonadaceae, to which another band correspond, is also a bacterial families of major interest for the degradation of organic contaminants, bioremediation protocols and also detected in the rhizospheric microbial communities associated with plants grown in metal rich soils (Kamaludeen and Ramasamy, 2008). It includes *genera* like *Acidovorax, Comamonas, Delftia* and *Variovorax*, all characterized by high average arsenic resistance, and able to reduce As(V) to As(III) or vice versa thanks to the presence of both the *ars* and the *aox* operon in their genome (Achour *et al*., 2007; Cai *et al*., 2009; Bruneel *et al*., 2011; Bahar *et al*., 2012). The presence of this bacterial family is indeed ubiquitous in As or heavy metals contaminated matrix and one *Comamonas* isolate, one *Variovorax* and two *Delftia* were also detected in the culture study performed at Scarlino sampling points (Par. 3.2). To strains of these *genera* are reported both resistance to heavy metals (Abou-Shanab *et al*., 2007) and PAH degrading capacities (Singleton *et al*., 2009) and in the analysis of the bacterial community in Cambodian Sediments *Comamonadaceae* accounted even for the 79% of identified microorganisms (Lear *et al*., 2007).

Bacteroidetes

Finally two bands matched with bacteria belonging to the *Sphingobacteriales* family. *Bacteroidetes* are common inhabitants of soils but have rarely been observed in arsenic-rich environments except for a few *Flavobacteria* able to methylate As (Honschopp *et al*., 1996) and only recently, the *aoxB* gene has been amplified from environmental *Flavobacterium* strain isolated in Sainte-Marie-aux-Mines valley (France) (Heinrich-Salmeron *et al*., 2011).

On the other hand, *Bacteroidetes* have been implicated as major utilizers of high-molecular-mass dissolved organic matter in particular in marine ecosystems, however are under-represented in culture collections when compared with other abundant *phyla* such as the Proteobacteria (O'Sullivan *et al*., 2006). In the culture study described in Par. 3.2, two OTUs of this *phylum*,

belonging to the *Flavobacteria* class, were obtained in pure culture and isolated from As(V) enrichment cultures.

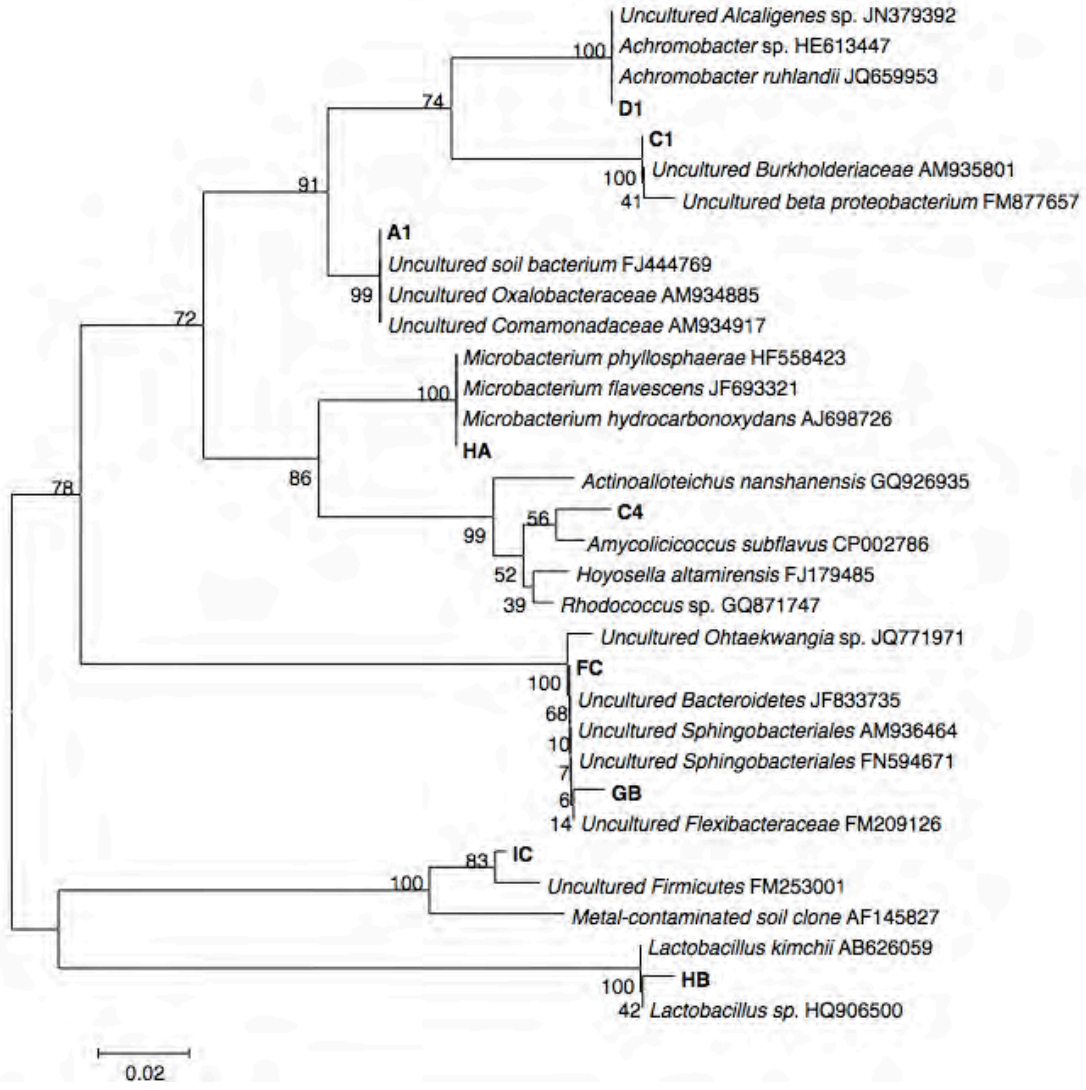


Fig. 3.12 *– Phylogenetic neighbour-joining tree of the V3 sequences obtained from excised bands from DGGE analysis and their closest database relatives with Species Name and GenBank accession numbers. Bootstrap values (n_1,000) above 50% are indicated at the nodes. The scale bar represents genetic distance (nucleotide substitutions per site).*

Comparing culture-dependent and culture-independent study at Scarlino site here presented, it was evident that no bands in DGGE profiles couldn't be accounted for sequenced OTUs from enrichment cultures, thus these bands may represent sequences that were not detected in the bacterial isolation approach. Fig. 3.13 shows a DGGE gel on which V3 sequences of all the isolated OTUs and the profiles obtained from genomic DNA extraction were loaded. From this comparative gel it's evident that there are no corresponding bands obtained from the same samples with the two different technique. Actually only in in the case of OTU E fragment there is a correspondent one migrated at the same height (band C1) but from the sequencing results emerged that the two bands belongs to two different species.

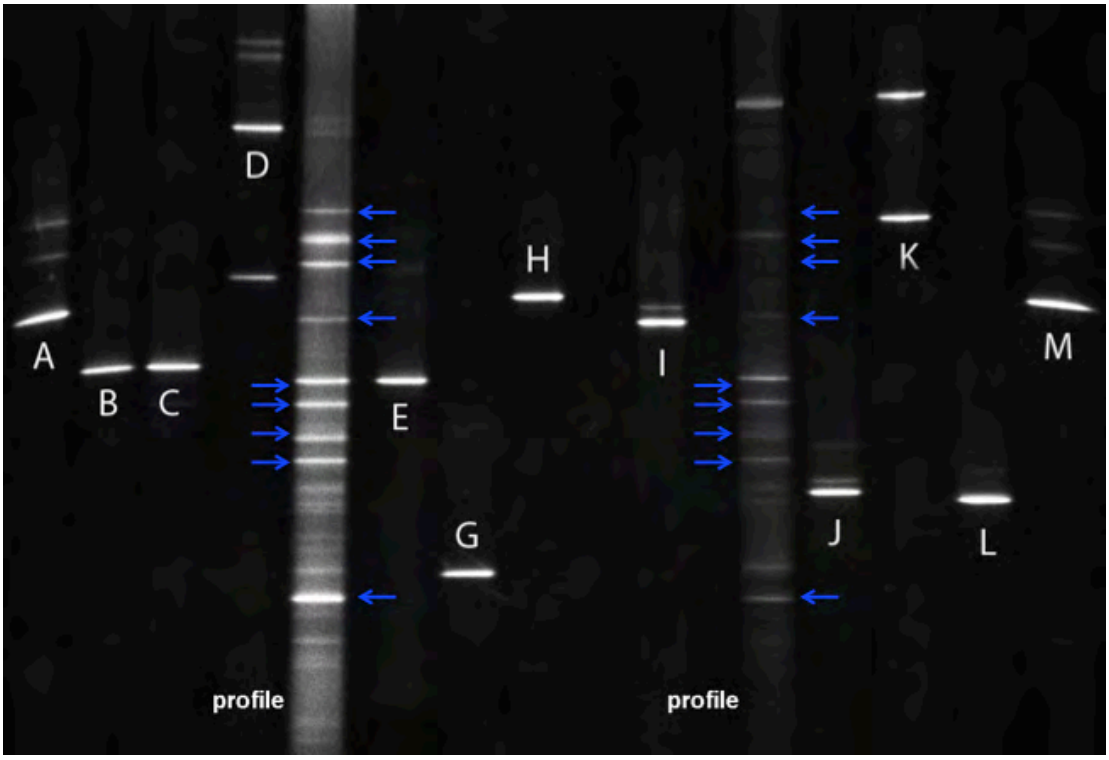


Fig. 3.13 *– Comparative DGGE gel with V3 sequences of all the isolated OTUs and the profiles obtained from genomic DNA extraction. Blue arrows indicate cloned and sequenced bands.*

In Tab. 3.4 the results of the alignment in data banks from the sequencing of *16S rDNA* from the isolates obtained from enrichment cultures from the same ashes samples are reported. Comparing these two sets of data, some evaluations can be assumed.

OTU	Taxonomic Reference ID	Phylogenetic group	Homology %
A	*B. weihenstephanensis* FN796835	*Firmicutes*	100%
B	*Stenotrophomonas rhizophila* AJ293463	Gamma-proteobacteria	99.35%
C	*Brevundimonas olei* GQ250440	Alfa-proteobacteria	100%
D	*Lysinibacillus fusiformis* AB271743	*Firmicutes*	100%
E	*Ochrobactrum cytisi* AY776289	Alfa-proteobacteria	99.01%
G	*Microbacterium oxidans* Y17227	*Actinobacteria*	98.37%
H	*Brevibacterium simplex* AB363738	*Firmicutes*	100%
I	*Brevibacterium frigoritolerans* KC355256	*Firmicutes*	100%
J	*Rhizobium mesosinicum* DQ100063	Alfa-proteobacteria	99.68%

K	*Bacillus flexus* AB021185	*Firmicutes*	100%
L	*Ochrobactrum cytisi* AY776289	Alfa-proteobacteria	100%
M	*Bacillus thuringiensis* ACNF01000156	*Firmicutes*	100%

Tab. 3.4 *– Percentage homology to EzTaxon-e Database and GenBank relatives for 16S rDNA gene sequences of representative strains for the matrix sample.*

In both cases at least one half of the sequences belong to Gram positive bacteria, this result obtained by the molecular analysis - therefore independent from the microorganisms cultivability - supported the prevalence of the *Firmicutes phylum* previously reported in the culture study. Besides Gram positive *phyla*, by isolation from enrichments Alpha-proteobacteria were detected and by the DGGE approach Beta-proteobacteria and *Bacteroidetes* were recovered.

The discrepancies must be due to the fact that the two techniques basically have different targets. The isolation of strains from enrichment cultures allows identifying only that part of cultivable bacteria under tightening conditions (in this case, the presence of inorganic As). On the other hand, molecular analysis allows to target also the so called viable but not cultivable bacteria present in a population but its limitations are obviously associated to molecular techniques, connected to inefficiency in DNA extraction from the complex soil matrix as well as biases implied in PCR amplifications. Actually poor DNA recovery, as it happened in this case, may have caused an increasing of detection limits achievable through PCR. Conversely overestimates of diversity and/or dominant populations may come from the formation of heteroduplex or chimera molecules during PCR, or due to sequence heterogeneities in multiple copies of the *16S rRNA* gene. Only a short portion of the *16S rRNA* gene (200 bp) was amplified by PCR for DGGE analysis in order to minimize chimera formation. Conversely, populations may be underestimated because all sequences are not amplified equally or because gel resolution is low (Joynt *et al*., 2006).

These considerations support therefore the choice of combining both culture and molecular approaches trying to complement respective biases and limits, to reach an overall more complete and reliable view on the bacterial community under study.

The molecular analysis also allowed identifying *genera* not detected within the culture study, further confirming the value of a combined approach integrating molecular and culture techniques. Anyway in both approaches were detected *genera* characterized by heavy metal resistances, reporting transforming potential of As and other recalcitrant compounds and PAH, used in bioremediation protocols and exerting plant growth promotion.

These results point out therefore the selection in the Scarlino area towards a soil autochthonous microbial community with high resistance to As and bioremediation potential even in the perspective of a bioremediation/phytoremediation approach.

CHAPTER 2:
Screening of the bacterial isolates

This part of the study basically aimed to the selection of interesting strains to be used in a following bioaugmentation experiment in association with the hyperaccumulator fern *Pteris vittata*, grown on Scarlino contaminated soil. Bacterial isolates were screened relatively to:

- As resistance (through MIC determination),
- The presence of Plant Growth Promoting traits (IAA and siderophores production and ACC deaminase activity),
- The genotypic study of the mechanisms of arsenic transformation,
- The ability to reduce arsenate to more mobile chemical species.

3.4 MIC (Minimum inhibitory concentration) determination for inorganic As(III) and As(V)

The minimum inhibitory concentration (MIC) of inorganic As(III) and As(V) (the lowest concentration of As(III) and As(V) that prevented bacterial growth) was determined for all the 55 OTUs obtained from enrichment cultures.

As synthetized in the Tab. 3.5, arsenic resistance varies greatly among the isolates. The most resistant bacterial strains were isolated both from the highly polluted matrix and the plants rhizosphere but even if *Bacillus* sp. isolates was the most represented at the highest contamination level, their MIC values weren't very high. In general it can stated that toward the trivalent form (As(III)), 9 of the bacteria exhibited high resistance (> 20 mM). In detail the highest MIC values were displayed by *Stenotrophomonas rhizophila* strain B with a MIC of 31 mM, *Brevundimonas olei* strain C (37 mM), *Microbacterium liquefaciens* strain AX (38 mM) and *Arthrobacter nitroguajacolicus* strain AY (35 mM). The last two cited strains belonging to *Actinobacteria phylum* are known in literature for their high MIC values and confirmed this feature in the present work (Achour-Rokbani *et al.*, 2010; Bachate *et al.*, 2009). Although Gamma-proteobacteria like *Stenotrophomonas* sp. and Alfa-protepbacteria *Brevundimonas* sp. are often associated with As contaminated soils, such high MIC values have never been reported for these cited strains. In general MIC values ranked above average, probably because high resistant phenotypes have been selected by the huge level and the long period of contamination. Higher resistance levels might be due to the presence of multiple resistances mechanisms, of multiple copies of the same resistance determinants or even connected to a higher expression of the same detoxification/resistance system (Cavalca *et al.*, 2010). At the best of our knowledge indeed only few bacterial species were found more resistant to As(III) then these cited above (Bachate *et*

al., 2009; Cavalca *et al*., 2010). In many similar works indeed reported MIC values are lower than these (Achour *et al.,* 2007; Pepi *et al*., 2007; Butt and Rehman, 2011).

As far as MIC for As(V) is concerned, microbial resistance to concentrations of As(V) exceeding 100 mM is considered very high (Jackson *et al*., 2005). Notably, under aerobic growth conditions, more than one half of the bacterial isolates exhibited high resistance to As(V) with MIC values exceeding 100 mM. But it's noteworthy that several factors, such as the method of determining the resistance and the medium composition, can affect arsenic bioavailability and toxicity, resulting in discrepancies in MIC values (Achour *et al*., 2007).

It's also interesting the fact that isolates belonging to the same specie or *genus* showed very different MIC values as in the case of *Microbacterium* W and *Microbacterium* AX with a respective MIC for As(III) of 1 mM and 38 mM. This probably deals with the different configuration of As resistance genes inside a single genome or with other mechanisms involved in As resistance or (i.e. intracellular sequestration, chelation or extracellular precipitation) (Slyemi and Bonnefoy, 2012).

Nevertheless the results obtained point out the selection in the soil of Scarlino industrial area towards a resistant microbial community, with members of particular high resistance belonging to the most represented gram-negative Proteobacteria classes but also to the gram-positive *Actinobacteria taxa* identified. The results therefore indicate that these populations had been under constant long-term metalloid stress and that the As in the soil could have exerted a selective pressure upon soil bacteria that would have developed a particular way to deal with the two As species.

SAMPLING POINT	OTU	Taxonomic Reference ID	Phylogenetic group	MIC	
MATRIX				AsV	AsIII
	A	*B. weihenstephanensis* FN796835	*Firmicutes*	50 mM	7 mM
	B	*Stenotrophomonas rhizophila* AJ293463	Gamma-proteobacteria	>100 mM	31 mM
	C	*Brevundimonas olei* GQ250440	Alfa-proteobacteria	>100 mM	37 mM
	D	*Lysinibacillus fusiformis* AB271743	*Firmicutes*	100 mM	11 mM
	E	*Ochrobactrum cytisi* AY776289	Alfa-proteobacteria	>100 mM	5 mM
	G	*Microbacterium oxidans* Y17227	*Actinobacteria*	50 mM	8 mM
	H	*Brevibacterium simplex* AB363738	*Firmicutes*	50 mM	4 mM
	I	*Brevibacterium frigoritolerans* KC355256	*Firmicutes*	25 mM	4 mM
	J	*Rhizobium mesosinicum*	Alfa-proteobacteria	25 mM	1 mM

		DQ100063			
	K	*Bacillus flexus* AB021185	*Firmicutes*	100 mM	4 mM
	L	*Ochrobactrum cytisi* AY776289	Alfa-proteobacteria	75 mM	12 mM
	M	*Bacillus thuringiensis* ACNF01000156	*Firmicutes*	>100 mM	11 mM
PLANT 1 (*Lolium rigidum*)	N	*Pseudomonas putida* Z76667	Gamma-proteobacteria	>100 mM	25 mM
	O	*Pseudomonas fuscovaginae* AB021381	Gamma-proteobacteria	>100 mM	28 mM
	P	*Achromobacter marplatensis* EU150134	Beta-proteobacteria	>100 mM	25 mM
	Q	*Arthrobacter ureafaciens* X80744	*Actinobacteria*	50 mM	5 mM
	R	*Arthrobacter nitroguajacolicus* AJ512504	*Actinobacteria*	50 mM	1 mM
	S	*Pseudoxanthomonas japonensis* AB008507	Gamma-proteobacteria	>100 mM	1 mM
PLANT 2 (*Daucus carota)*	T	*Stenotrophomonas chelatiphaga* EU573216	Gamma-proteobacteria	>100 mM	12 mM
	U	*Delftia lacustris* EU888308	Beta-proteobacteria	>100 mM	10 mM
	V	*Brevundimonas bullata* D12785	Alfa-proteobacteria	>100 mM	2 mM
	W	*Microbacterium yannici* FN547412	*Actinobacteria*	50 mM	1 mM
	X	*Bacillus megaterium* HQ285923	*Firmicutes*	100 mM	5 mM
	Y	*Microbacterium arabinogalactanolyticum* AB004715	*Actinobacteria*	>100 mM	10 mM
	Z	*Flavobacterium chungbukense* HM627539	*Flavobacteria*	40 mM	2 mM
	AA	*Bacillus niacini* AB021194	*Firmicutes*	60 mM	1 mM
	AB	*Microbacterium arabinogalactanolyticum* AB004715	*Actinobacteria*	40 mM	1 mM
PLANT 3 (*Trifolium angustifoliu m* L.)	AC	*Rhodococcus ruber* X80625	*Actinobacteria*	>100 mM	7 mM
	AD	*Devosia insulae* EF114313	Alfa-proteobacteria	>100 mM	7 mM
	AE	*Massilia oculi*	Beta-proteobacteria	>100	7 mM

		FR773700		mM	
	AF	*Xanthomonas vasicola* Y10755	Gamma-proteobacteria	>100 mM	10 mM
	AG	*Bacillus aryabhattai* HQ009875	*Firmicutes*	>100 mM	8 mM
	AH	*Brevundimonas alba* AJ227785	Alfa-proteobacteria	50 mM	8 mM
	AI	*Microbacterium niemengense* JN408293	*Actinobacteria*	50 mM	2 mM
	AJ	*Agromyces aurantiacus* AF3894342	*Actinobacteria*	50 mM	1 mM
	AK	*Arthrobacter humicola* AB279890	*Actinobacteria*	50 mM	1 mM
	AL	*Brevundimonas olei* GQ250440	Alfa-proteobacteria	60 mM	1 mM
	AM	*Bacillus bataviensis* AJ542508	*Firmicutes*	40 mM	1 mM
PLANT 4 *(Populus alba*)	AN	*Comamonas testosteroni* AHIL01000001	Beta-proteobacteria	>100 mM	18 mM
	AO	*Delftia acidovorans* NR074691	Beta-proteobacteria	>100 mM	9 mM
	AP	*Variovorax paradoxus* HQ231964	Beta-proteobacteria	>100 mM	5 mM
	AQ	*Microbacterum invictum* AM949677	*Actinobacteria*	10 mM	1 mM
	AR	*Bacillus drententis* AJ542506	*Firmicutes*	10 mM	1 mM
	AS	*Pseudoxanthomonas mexicana* GU908488	Gamma-proteobacteria	>100 mM	5 mM
	AT	*Pseudomonas plecoglossicida* AB009457	Gamma-proteobacteria	55 mM	11 mM
	AU	*Flavobacterium cauense* EU5211691	*Flavobacteria*	50 mM	1 mM
PLANT 5 *(Sedum sediforme)*	AV	*Bacillus nealsonii* EU656111	*Firmicutes*	>100 mM	20 mM
	AW	*Stenotrophomonas maltophilia* AB008509	Gamma-proteobacteria	>100 mM	20 mM
	AX	*Microbacterium liquefaciens* HM104368	*Actinobacteria*	>100 mM	38 mM
	AY	*Arthrobacter nitroguajacolicus* AJ512504	*Actinobacteria*	>100 mM	35 mM
	AZ	*Pseudoxanthomonas mexicana* AF273082	Gamma-proteobacteria	>100 mM	11 mM

	BA	*Brevibacillus brevis* AP008955	*Firmicutes*	20 mM	2 mM
	BB	*Lysobacter yangpyeongensis* DQ191179	Gamma-proteobacteria	>100 mM	4 mM
	BC	*Microbacterium suwonense* GQ246683	*Actinobacteria*	>100 mM	2 mM
	BD	*Sphingopyxis alaskensis* CP000356	Alfa-proteobacteria	>100 mM	5 mM

Tab. 3.5 *– Determination of the MIC of the isolates for As(III) and As(V).*

3.5 Genotypic study of the mechanisms of arsenic transformation

A molecular study in relation to arsenic resistances and to reducing/oxidating capabilities of As toxic oxides (As(III) and As(V)) has been performed to evaluate the resistance and degrading potential within the indigenous micro flora. Arsenic microbial metabolism is very complex and in this study the attention was focused on two different reaction mechanisms (illustrated in Fig. 3.14). Actually, the presence of either the As(III) efflux pump and the arsenate reductase genes (*ars* operon) or arsenite oxidase (*aox* operon) was investigated. Each isolate was tested by PCR reactions using group-specific primer pairs targeting the following genes: the *arsB* and *ACR(3)* gene families of arsenite transporters, the arsenate reductase and the arsenite oxidase.

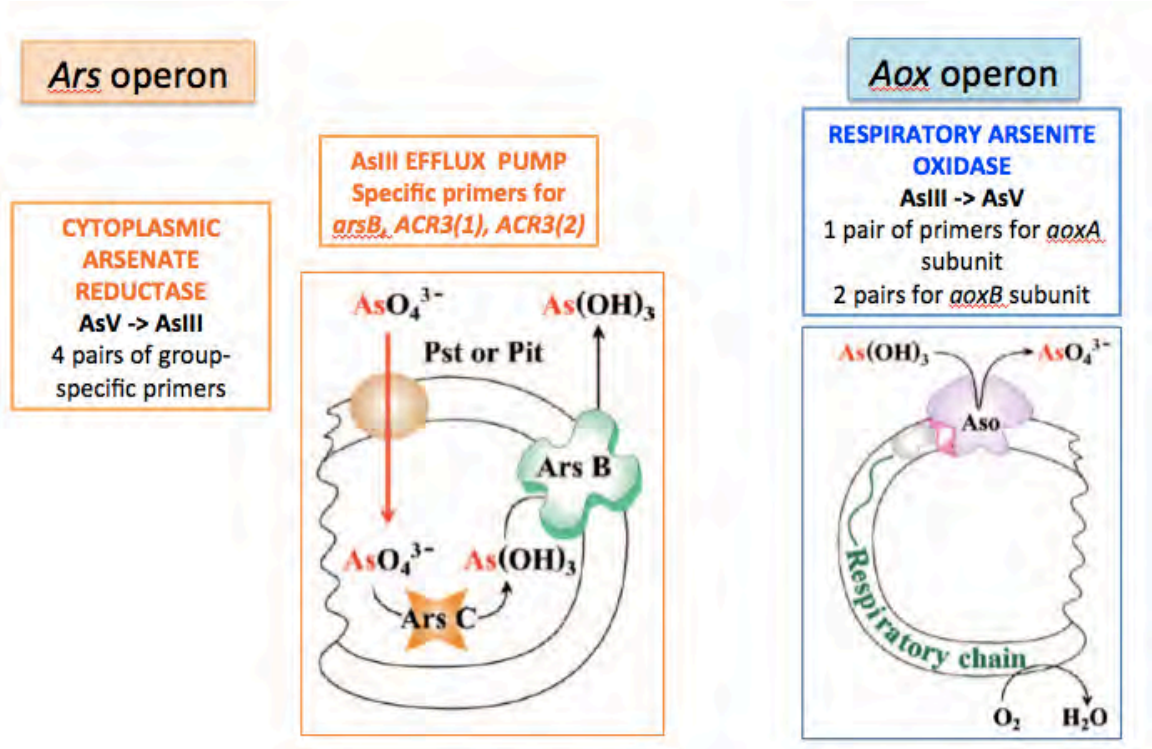


Fig. 3.14 *– Target genes of the present molecular study in relation to arsenic transformation determinants.*

The results of this screening and the occurrence of these genes among the isolates are summarized in Tab. 3.6. In general terms, of the 55 representative isolates, 21 showed successful amplifications of the arsenite transporter genes, 22 of arsenate reductase and 13 of the arsenite oxidase.

SAMPLING POINT	OTU	Taxonomic Reference ID	*ars* operon		*aox* operon
			AsIII efflux pump	Arsenate reductase	Arsenite oxidase
MATRIX	A	*B. weihenstephanensis* FN796835	-	+	+
	B	*Stenotrophomonas*	ACR3(1)	-	-

		rhizophila AJ293463			
	C	*Brevundimonas olei* GQ250440	ArsB	-	-
	D	*Lysinibacillus fusiformis* AB271743	ArsB	+	-
	E	*Ochrobactrum cytisi* AY776289	ACR3(2)	+	-
	G	*Microbacterium oxidans* Y17227	-	+	-
	H	*Brevibacterium simplex* AB363738	ArsB	+	-
	I	*Brevibacterium frigoritolerans* KC355256	ArsB	+	-
	J	*Rhizobium mesosinicum* DQ100063	ACR3(2)	-	+
	K	*Bacillus flexus* AB021185	-	+	+
	L	*Ochrobactrum cytisi* AY776289	ACR3(2)	-	+
	M	*Bacillus thuringiensis* ACNF01000156	-	+	+
PLANT 1 *(Lolium rigidum)*	N	*Pseudomonas putida* Z76667	ArsB	+	-
	O	*Pseudomonas fuscovaginae* AB021381	-	-	-
	P	*Achromobacter marplatensis* EU150134	ArsB ACR3(2)	-	-
	Q	*Arthrobacter ureafaciens* X80744	-	-	-
	R	*Arthrobacter nitroguajacolicus* AJ512504	-	-	-
	S	*Pseudoxanthomonas japonensis* AB008507	ACR3(2)	-	-
PLANT 2 *(Daucus carota)*	T	*Stenotrophomonas chelatiphaga* EU573216	-	-	-
	U	*Delftia lacustris* EU888308	ArsB	-	-
	V	*Brevundimonas bullata* D12785	ACR3(1)	-	+
	W	*Microbacterium yannici* FN547412	-	-	-
	X	*Bacillus megaterium* HQ285923	ArsB	+	-

	Y	*Microbacterium arabinogalactanolyticum* AB004715	-	+	-
	Z	*Flavobacterium chungbukense* HM627539	-	-	+
	AA	*Bacillus niacini* AB021194	-	+	-
	AB	*Microbacterium arabinogalactanolyticum* AB004715	-	+	-
PLANT 3 (*Trifolium angustifoliu m* L.)	AC	*Rhodococcus ruber* X80625	-	-	-
	AD	*Devosia insulae* EF114313	-	-	-
	AE	*Massilia oculi* FR773700	ArsB	-	-
	AF	*Xanthomonas vasicola* Y10755	ArsB	-	+
	AG	*Bacillus aryabhattai* HQ009875	-	+	+
	AH	*Brevundimonas alba* AJ227785	-	-	-
	AI	*Microbacterium niemengense* JN408293	-	-	-
	AJ	*Agromyces aurantiacus* AF3894342	-	-	-
	AK	*Arthrobacter humicola* AB279890	-	-	-
	AL	*Brevundimonas olei* GQ250440	-	+	+
	AM	*Bacillus bataviensis* AJ542508	-	-	-
PLANT 4 (*Populus alba*)	AN	*Comamonas testosteroni* AHIL01000001	ArsB	+	-
	AO	*Delftia acidovorans* NR074691	ArsB	-	-
	AP	*Variovorax paradoxus* HQ231964	ACR3(2)	-	-
	AQ	*Microbacterum invictum* AM949677	-	-	-
	AR	*Bacillus drententis* AJ542506	-	+	-
	AS	*Pseudoxanthomonas mexicana* GU908488	ACR3(2)	+	-
	AT	*Pseudomonas plecoglossicida* AB009457	ArsB	+	-

	AU	*Flavobacterium cauense* EU5211691	-	+	-
PLANT 5 (*Sedum sediforme*)	AV	*Bacillus nealsonii* EU656111	-	-	-
	AW	*Stenotrophomonas maltophilia* AB008509	ACR3(2)	-	-
	AX	*Microbacterium liquefaciens* HM104368	ArsB	-	+
	AY	*Arthrobacter nitroguajacolicus* AJ512504	-	-	-
	AZ	*Pseudoxanthomonas mexicana* AF273082	-	-	-
	BA	*Brevibacillus brevis* AP008955	-	-	-
	BB	*Lysobacter yangpyeongensis* DQ191179	-	+	-
	BC	*Microbacterium suwonense* GQ246683	-	-	-
	BD	*Sphingopyxis alaskensis* CP000356	-	-	-

Tab. 3.6 *– ars and aox genotypes of each bacterial isolate.*

As(III) transporters genes

Three sets of degenerate primers targeting the *arsB* and *ACR3* gene families of arsenite transporters were used to detect the presence of these genes in the bacterial isolates genome. All PCR-positive isolates produced a single amplicon with an approximate length of 750 bp.

As shown in Fig. 3.15 *arsB* sequences were prevalently detected in Beta-proteobacteria (36%), *Firmicutes* (29%) and Alfa-proteobacteria (21%) while *ACR3* were detected only on Proteobacteria belonging to Gamma (50%), Alfa (30%) and Beta (20%) classes. In *Achromobacter marplatensis*, a highly resistant strain, both forms were found, probably indicating the presence of multiple operons.

If it's hypothesize that the *arsB/ACR3* genes are the primary determinants in arsenite resistance, their absence in arsenite-sensitive strains and their presence in resistant isolates should be expected. Anyway this situation didn't occurred in the present study, just to make an example *arsB/ACR3* gene was absent in the hyper resistant *Arthrobacter nitroguajacolicus* (characterized by a MIC value for As(III) of 35 mM) but *ACR3* was present in the sensitive strain of *Pseudoxanthomonas japonensis* S (with a MIC for As(III) of 1 mM) and *Brevibacillus brevis* (MIC of 4 mM) was positive for *arsB*. In the case of isolates that exhibited an arsenite resistance phenotype, unsuccessful amplification may be due to the presence of an arsenite transporter

gene homologue with a highly divergent DNA sequences or alternative mechanisms could be used by these isolates to cope with arsenite toxicity. Such mechanisms could include arsenite methylation that results in volatile products that escape from the cells (Qin *et al.,* 2006) or arsenite oxidation to produce the less toxic arsenate (Silver and Phung, 2005a). On the other hand, the presence of these genes in As sensitive isolates could be explained with the fact that many organisms may have acquired the determinants from a horizontal gene exchange, a situation observed also in other studies (Cai *et al.*, 2009; Valverde *et al.*, 2011).

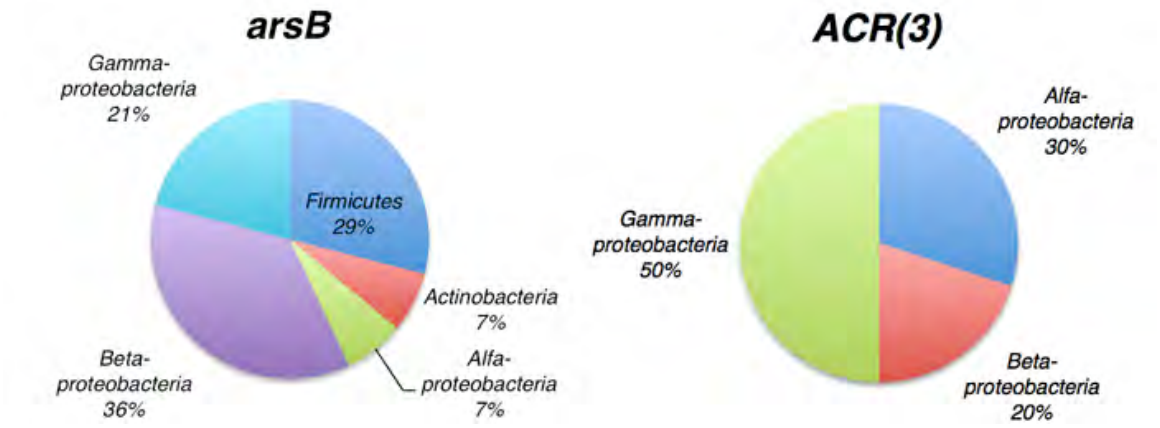


Fig. 3.15 *– As(III) transporter genes and their taxonomic distribution.*

Arsenate reductase genes

In this case four sets of degenerate primers targeting the *arsC* families of arsenate reductase were used to detect its presence in the bacterial isolates genome. All PCR-positive isolates produced a single amplicon with an approximate length of 500 bp.

As shown in Fig. 3.16 *arsC* sequences were amplified with an absolute prevalence in *Firmicutes* isolates (in *B. thuringiensis, B. megaterium, B. niacini* species for instance); but they were also found in *Actinobacteria* (*Microbacterium*) Alfa, Beta and Gamma proteobacteria (*Ochrobactrum*, *P. putida*, *Brevundimonas*) and in *Flavobacterium* isolates. This observation confirmed the wide distributions *arsC* genes among environmental isolates. Homologues of the *ars* operon have indeed been identified in diverse bacteria including *Pseudomonas, Bacillus, Klebsiella, Staphylococcus, Salmonella, Acidithiobacillus, Yersinia*, and *Escherichia* (Diorio *et al.*, 1995; Butcher *et al.*, 2000; Neyt *et al.*, 1997; Sato and Kobayashi, 1998). In addition, putative *ars* homologues have been detected in many of the *Eubacteria* and *Archaea* whose genomes have been sequenced, suggesting that *ars* genes are relatively common among prokaryotes (Jackson and Dugas, 2003).

The reduction of As(V) via detoxification may contribute to apparent non equilibrium conditions where As(III) has been observed in oxic soils and surface waters (Cullen and Reimer, 1989). For example, several As(V)-reducing bacteria have been found to mediate the reduction of As(V) under highly aerobic conditions resulting in enhanced mobilization of As from limed mine tailings

(Macur *et al.*, 2001). The characterization of several aerobic heterotrophs isolated from these tailings suggested that the probable mechanism of As(V) reduction was As(V) detoxification.

The activity of As(V) reducing microorganisms in soils and natural waters has significant implications for the behavior of As because the reaction product As(III) exhibit higher in solubility, mobility, bioavailability, and toxicity (Ferguson and Gavis, 1972) and, in a phytoextraction context, these microorganisms are fundamental. They could indeed solubilize arsenic and make it more bioavailable for the uptake by the vegetal species involved in bioremediation process.

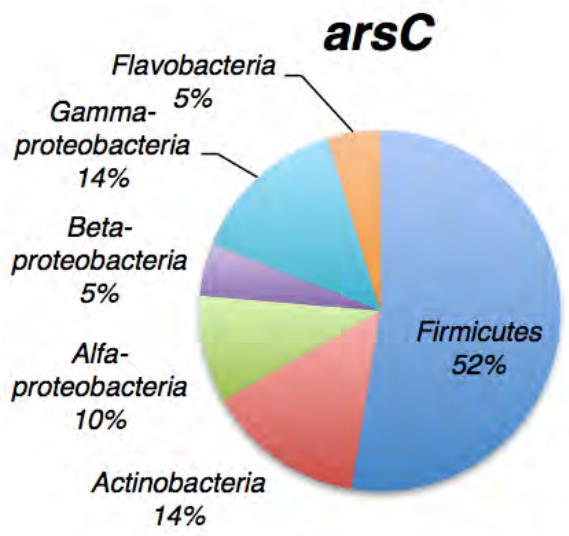


Fig. 3.16 *– As(V) reductase genes and their taxonomic distribution.*

Arsenite oxidase genes

For the amplification of As(III) oxidase three sets of degenerate primers were used to detect the presence of this gene in the bacterial isolates genome. All PCR-positive isolates produced a single amplicon with an approximate length of 400 bp.

The *aox* operon too appeared in very different taxonomic group, it was indeed detected in all the isolated bacterial *phyla* (*Flavobacteria* included), with a preponderance in *Firmicutes* (36%) and Alfa-Proteobacteria (22%). In detail *aox* sequences were found in the following *genera*/species; *B. thuringiensis, B. weihenstephanensis, B. megaterium, Microbacterium, Brevundimonas, Stenotrophomonas, Flavobacterium, Ochrobactrum, Shinella*.

According to literature data, *aox* genes have been identified in 25 bacterial and archaeal *genera* isolated from various arsenic-rich environments, most of which belong to the Alpha, Beta, or Gammaproteobacteria class but the number is constantly increasing. In a recent work *aox* gene were detected in *genera Achromobacter, Pseudomonas, Agromyces, Rhodococcus, Flavobacterium, Bosea, Acinetobacter*, and *Bacillus* representing four bacterial *phyla* (*Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and Proteobacteria) (Heinrich-Salmeron *et al.*, 2001). Other studies based on environmental DNA extracted from soils, sediments, and geothermal mats with different chemical characteristics and various levels of arsenic contamination have suggested that the

distribution and the diversity of arsenite-oxidizing microorganisms may be greater than previously suggested (Cai *et al.*, 2009, Chang *et al.*, 2010; Escalante *et al.*, 2009, Quéméneur *et al.*, 2010). From an ecological point of view, As(III) oxidizing bacteria seem to be responsible for arsenite oxidation as a detoxification potential rather than as an energy source for chemolithoautotrophic growth. As(III)-oxidizing bacteria can contribute to a natural attenuation of As pollution by decreasing its bioavailability and can help remove As from mine wastewaters through bioprocessing (Battaglia-Brunet *et al.*, 2002).

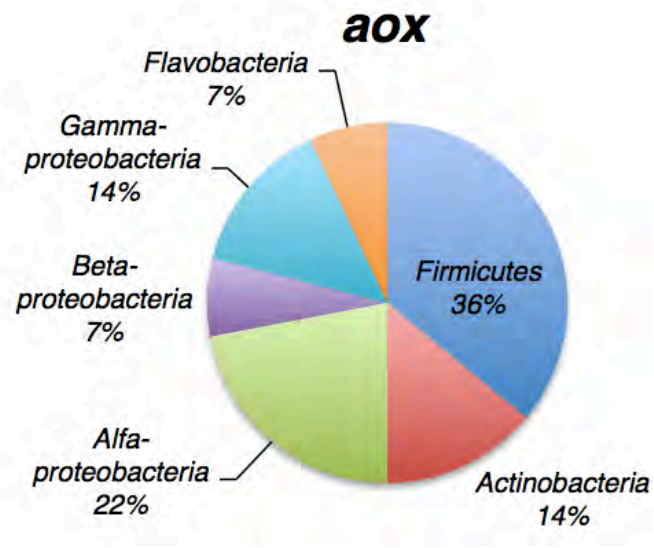


Fig. 3.17 *– As(III) oxidase genes and their taxonomic distribution.*

In general the fact that all genes, believed to belong to a specific taxonomic group, were found in phylogenetically different lineages support the idea of horizontal gene transfer events (HGT). It's been reported that HGT represents the most likely hypothesis to explain the presence of identical (or nearly identical) sequences in some distant lineages and this was described for *arsC* genes (Saltikov and Olson, 2002; Jackson and Dugas, 2003) for arsenite efflux genes (Cai *et al.*, 2009; Valverde *et al.*, 2011) and for *aox* genes (Oremland *et al.*, 2005) under high arsenic pressures.
Other evaluations must be considered. In general in this genotypic screening it has to be pointed out that various PCR negative results in some of the arsenic resistant isolates may be due to sequence differences between genes and primers used in this study. Thus this screening could have underestimated the real frequency of these genotypes. For instance, strains showing positive result only for *arsB* or *arsC* gene in the experiment must have also the rest of the operon although the amplification was negative because the minimal content of *ars* operon is composed by three genes: *arsB*, *arsC* and *arsR* (Paéz-Espino *et al.*, 2009). That being stated it's evident how the *ars* operon was detected in all strains isolated from the matrix sample while it was less represented in the strains isolated from the different plants' rhizosphere (Fig. 3.18). This is probably due to the high level of contamination and prohibitive conditions in the M sample that exerted a selective pressure on the bacterial community. Moreover, *ars* operon appears to be

more diffused than *aox* one (none of the strains from P4 sample – *Populus* sp. rhizosphere – for instance has the *aox* operon) (Fig. 3.18).

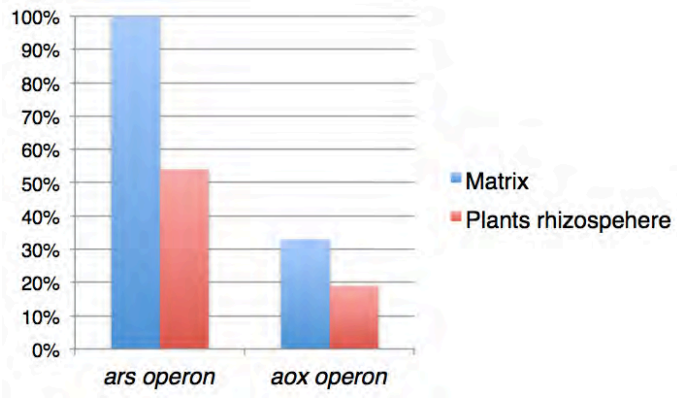


Fig. 3.18 *– Occurrence of ars and aox genotypes in the examined samples.*

Another limitation in the PCR approach used lies in its inability to directly show the presence of multiple alleles of a gene in single strain, as it happened in other studied bacterial species such as *C. glutamicum* (Ordonez *et al*., 2005) or *Pseudomonas putida* KT2440 (Canovas *et al*., 2003). Moreover, looking for a correlation between MIC values and the genotype of arsenic transformation here considered it was observed that: i) independently on the As resistance level strains can have both the operon or don't have none or have only the *ars* one, ii) different strains belonging to the same *genus*/specie can have very different MIC values (like in the case of *Microbacterium* sp. strain AQ and strain AX), iii) different strains belonging to the same *genus* can have different genotypes (*Bacillus flexus* and *B. megaterium* X). In other words, in the conditions tested in this study, the distribution of As-related genes appears to be quite random. This observation is not unprecedented and has been reported in other studies dealing with bacterial arsenic resistance in the environment; the works of Ford *et al*. (2005) and Achour *et al.* (2007) also failed to correlate the level of arsenate resistance with the prevalence of *ars/aox* genes in a set of aerobic and anaerobic bacteria. This may due to the fact that besides the resistance mechanisms here analyzed, others can be present and can explain these observed discrepancies. In fact As(III) can be detoxified by complexation with Cys-rich peptides, inorganic arsenic can be transformed into organic species in a methylation cascade and volatilized, As(V) can be used as the ultimate electron acceptor during respiration, it can also be adsorbed on the bacterial cell wall and As(III) can be oxidated by membrane lipids (Abdrashitova *et al*., 1986; Frankenberger and Arshad, 2001; Saltikov and Newman, 2003; Paéz-Espino *et al*., 2009).

All these results and observations clearly have remarkable implications for As cycling in soils. The coexistence of both As(III)-oxidizing and As(V)-reducing aerobic populations in the same soil suggests that the relative numerical and or metabolic dominance of these populations will influence the predominant As valence state (Macur *et al*., 2004). The ability to either oxidize

As(III) or reduce As(V) is variable even among strains that proliferate under the same environmental conditions. Because of such variation in phenotype within similar organisms, phylogenetic identification of microorganisms based on *16S rRNA* sequence analysis is not sufficient to predict the As-transforming capabilities of specific bacterial populations. Further, As contamination in oxic environments may not select for microorganisms capable of utilizing As in energy metabolism but rather may shift the microbial community structure to favor organisms capable of detoxification either via As oxidation or reduction. Specifically, the broad phylogenetic distribution of *arsC* genes suggests that the importance of non-dissimilatory As(V) reduction may be underestimated as a mechanism of As redox cycling in natural systems. Results from the current study as well as other examples (Macur *et al*., 2001) support the hypothesis that the oxidization and reduction of As occurs in phylogenetically diverse soil bacteria via mechanisms that are not directly associated with respiration or chemolithotrophic metabolism.

3.6 Arsenate reduction by isolates

As(V)-reducing bacteria have been found to mediate the reduction of As(V) under highly aerobic conditions resulting in enhanced mobilization of arsenic from limed mine tailings (Macur *et al*. 2001). In this particular study case the ability to reduce As(V) to As(III) represents a very interesting advantage for bioremediation purpose, As(III) is indeed more mobile and bioavailable and can be easily absorbed by the fern roots and so removed from contaminated soils and aquifers.

Ten different bacterial strains were tested for their ability to reduce As(V) to the more mobile specie As(III). The best performances were exhibited by *Delftia lacustris* strain U that was capable of completely reducing 5 mM As(V) in 48 h of incubation and *Pseudomonas putida* strain N, able to totally reduce As(V) 5 mM in 72 hours of incubation under aerobic condition (Tab. 3.7). Complete reduction of As(V) occurred when strains attained maximum cell density and did not occur in controls without cells indicating that As(V) reduction was a microbial process. To the best of authors' knowledge, the complete reduction of such a high As(V) concentration has never been reported for these species.

STRAINS	0 h	6 h	24 h	30 h	48 h	72 h	96 h
Pseudomonas putida strain N	100% ± 5,5%	82,5% ± 3%	62,9% ±0,8%	63,6% ± 3,2%	9,2% ±3%	0% ±2,5%	0% ±2%
Delftia lacustris strain U	100% ± 2,5%	89% ± 2%	78% ± 1,5%	67% ± 3%	0% ± 1,5%	0% ± 2%	0% ± 0%

Tab. 3.7 – As(V) reduction rate by Pseudomonas putida and Delftia lacustris.

The mechanism of As(V) reduction by isolates can be related to a detoxification purpose as in aerobic conditions the arsenate reductase, which is encoded by *arsC* gene, is involved in As(V) reduction. Further, the amplification of *ars* operon determinants for both the isolates confirmed the presence of detoxification mechanism for the reduction of As(V).
The fact that on ten tested strains only these two were found able to efficiently reduce As(V) may also suggest that As oxidizing bacteria (not investigated in this study) could play a possible protective role towards the plants populating the pollutes area. Arsenite is generally considered more toxic than As(V) for most plants. Rhizobacteria encounter As(V) and As(III) in soil solutions before they enter the root, and oxidizing As(III) from soil solutions might help the plant to grow on As-contaminated soils and thus lower As(III) toxicity (Cavalca *et al*., 2010).

Although direct role of these bacteria in arsenic mobilization was not studied, the ability of the isolates to reduce As(V) suggests that these bacteria can potentially mediate arsenic transformations in these soils and their role in the arsenic cycling may become relevant in a phytoextraction context. As(III) removal efficiency could be indeed increased by synergic function of microbial As(V) reduction in the rhizosphere followed by more rapid root uptake of As(III).

3.7 Identification of As-resistant plant growth promoting rhizobacteria

How successful phytoremediation will be at any site depends on the extent of soil contamination, bioavailability of the metal contaminant involved, and the ability of the plant used to absorb and accumulate metals as biomass. Generally, plants with exceptionally high metal accumulating capacity often grow slowly and produce limited biomass, particularly when the metal concentration in the soil is high. However, there is a way to maximize the chances of success of phytoremediation by utilizing Plant Growth Promoting Rhizobacteria (PGPR), which are soil microbes that inhabit the rhizosphere. When PGPR are introduced to a contaminated site, they increase the potential for plants that grow there to sequester heavy metals and to recycle nutrients, maintain soil structure, detoxify chemicals, and control diseases and pests; PGPR also decrease the toxicity of metals by changing their bioavailability in plants. The plants, in turn, provide the microorganisms with root exudates such as free amino acids, proteins, carbohydrates, alcohols, vitamins, and hormones, which are important sources of their nutrition. The rhizosphere has high concentrations of root-exuded nutrients and attracts more bacteria than does bulk soil. These bacteria of the rhizosphere, therefore, facilitate plant growth. (Han *et al*. 2005; Babalola, 2010).

Thus, the strains that showed the highest resistance both to inorganic As species were then tested for some PGPR characteristics for their potential exploitation in two different bioaugmentation experiments. The OTUs in examination were qualitatively screened for ability to produce the auxin indoleacetic acid (IAA), for the ability to produce siderophores and to utilize 1-aminocyclopropane-1-carboxylic acid (ACC) as the sole N source.

3.7.1 IAA production test

As the most studied phytohormones, IAA produced in the plant shoot and transported to the root tips is associated with cell elongation and cell division (Rashotte *et al*., 2000) and contributes to plant growth and plant defense system development (Navarro *et al.,* 2006). The ability to produce IAA was tested adding the Salkowski's reagent to the bacterial culture grown in presence of tryptophan, precursor of IAA; as shown in Fig 3.19 the development of a pink color is indicative of IAA production and the intensity of the color is proportional to the amount of IAA in solution (Ahmad *et al*., 2006).

Of the 32 As resistant strains tested 24 resulted positive for this PGP trait, and 7 of these produced a very intense color (*Ochrobactrum cytisi* OTU E and OTU L, *Pseudomonas putida* OTU N*, Delftia lacustris* OTU U*, Stenotrophomonas maltophilia* OTU AW*, Microbacterium liquefaciens* OTU AX*, Arthrobacter nitroguajacolicus* OTU AY) (Tab. 3.7). These *genera* were previously identified as IAA producers in other works and in the present study, the fact that they are also resistant to very high concentrations of As(III) and As(V) make them promising

candidates to use in a bioaugmentation experiments in association with the hyperaccumulator fern *Pteris vittata* (Glick, 2012; Rajkumar *et al*., 2012; Ma *et al*., 2011a).

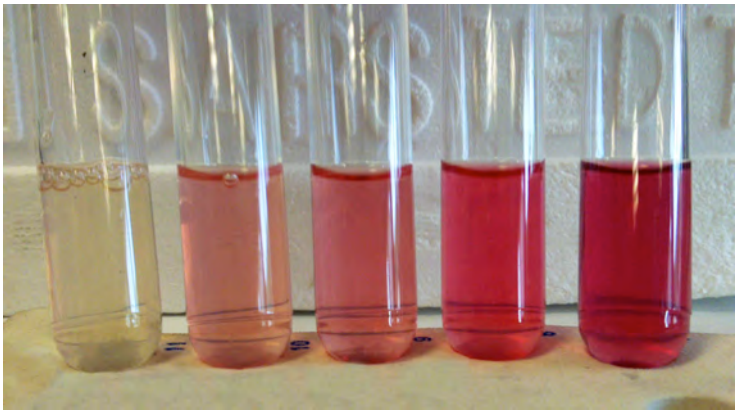


Fig. 3.19 *– Some of the results of the test for IAA production, the development of pinkish color is indicative of IAA production. The intensity of the color is representative of the amount of IAA produced.*

3.7.2 Siderophores production test

Most plant-associated bacteria and fungi can produce iron chelators called siderophores in response to low iron levels in the rhizosphere. Siderophores are low-molecular mass (400–1,000 Daltons) compounds with high association constants for complexing iron, but they can also form stable complexes with other metals, such as Al, Cd, Cu, Ga, In, Pb and Zn (Glick and Bashan, 1997; Schalk *et al*., 2011). Moreover, since siderophores solubilize unavailable forms of heavy metal bearing minerals by complexation reaction, siderophores producing microbes that inhabit the rhizosphere soils are believed to play an important role in heavy metal phytoextraction (Braud *et al.,* 2009b; Dimkpa *et al*., 2009; Rajkumar *et al.,* 2010). Several examples of increased Fe uptake in plants with concurrent stimulation of plant growth as a result of PGPR inoculations have been reported (Burd *et al*., 2000; Carrillo-Castañeda *et al*., 2003; Barzanti *et al*., 2007). Siderophores also promote bacterial IAA synthesis by reducing the detrimental effects of heavy metals through chelation reaction (Dimkpa *et al*., 2008). Besides, as already mentioned, siderophores play a significant role in metal mobilization and accumulation (Dimkpa, *et al*., 2009; Rajkumar *et al*., 2010), as these compounds produced by PGPR solubilize unavailable forms of heavy metal-bearing Fe but also form complexes with bivalent heavy metal ions that can be assimilated by root mediated processes (Carrillo-Castañeda *et al*., 2003).

As for IAA, also in this case the majority of the tested isolates exhibited the ability to synthetize siderophores (an example is shown in Fig. 3.20), 25 on a total of 32 strains resulted indeed positive. The most promosing isolates in this case were *Bacillus thuringiensis* OTU M*, Stenotrophomonas maltophilia* OTU AW*, Microbacterium arabinogalactanolyticum* OTU Y*, Pseudomonas plecoglossicida* OTU AT*, Pseudoxanthomonas Mexicana* OTU AS*, Variovorax paradoxus* OTU AP and *Bacillus megaterium* OTU X. These OTU are therefore of particular interest in the context of a microbe enhanced phytoextraction protocol. To these species is

reported an extraordinary range of activities including both resistance to toxic metals as arsenic and association to various plants as PGPR (Tripathi *et al*., 2005; Chakraborty *et al*., 2006; Kang *et al*., 2006; Lugtenberg and Kamilova, 2009; Rajkumar *et al*., 2012). Despite the still limited understanding of PGPR-plant interactions, a number of these bacteria are nevertheless used commercially as adjuncts to agricultural practice. Commercialized PGPB strains include the aforementioned species as *B. megaterium, P. putida* and *Delftia* sp (Banerjee *et al*., 2006).

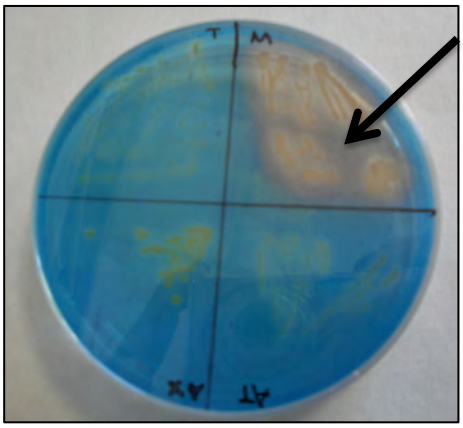


Fig. 3.20 *- Some of the results of the test for IAA production, the development of yellow halos around colonies is indicative of siderophores production. The intensity of the color is representative of the amount of siderophores produced.*

In Tab. 3.8 the results of the described tests are reported.

SAMPLING POINT	OTU	Taxonomic Reference ID	IAA production	Siderophores production
MATRIX	B	*Stenotrophomonas rhizophila* AJ293463	+	+
	C	*Brevundimonas olei* GQ250440	++	+
	D	*Lysinibacillus fusiformis* AB271743	++	+
	E	*Ochrobactrum cytisi* AY776289	+++	-
	L	*Ochrobactrum cytisi* AY776289	+++	-
	M	*Bacillus thuringiensis* ACNF01000156	1/2	+++
PLANT 1 (*Lolium rigidum*)	N	*Pseudomonas putida* Z76667	++	+
	O	*Pseudomonas fuscovaginae* AB021381	+++	+
	P	*Achromobacter marplatensis* EU150134	-	+
	S	*Pseudoxanthomonas japonensis* AB008507	+ 1/2	+

	T	*Stenotrophomonas chelatiphaga* EU573216	++	+
PLANT2 (*Daucus carota*)	U	*Delftia lacustris* EU888308	++++	++
	V	*Brevundimonas bullata* D12785.	-	-
	X	*Bacillus megaterium* HQ285923	-	++
	Y	*Microbacterium arabinogalactanolyticum* AB004715	1/2	++
PLANT 3 (*Trifolium angustifoliu m* L.)	AC	*Rhodococcus ruber* X80625	-	-
	AD	*Devosia insulae* EF114313	++	++
	AF	*Xanthomonas vasicola* Y10755	1/2	+
	AG	*Bacillus aryabhattai* HQ009875	++	+
PLANT 4 (*Populus alba*)	AN	*Comamonas testosteroni* AHIL01000001	-	-
	AO	*Delftia acidovorans* NR074691	+ 1/2	-
	AP	*Variovorax paradoxus* HQ231964	-	++
	AS	*Pseudoxanthomonas mexicana* GU908488	-	++
	AT	*Pseudomonas plecoglossicida* AB009457	+	++
PLANT 5 (*Sedum sediforme*)	AV	*Bacillus nealsonii* EU656111	-	-
	AW	*Stenotrophomonas maltophilia* AB008509	+++	+++
	AX	*Microbacterium liquefaciens* HM104368	+++	++
	AY	*Arthrobacter nitroguajacolicus* AJ512504	+++	+
	AZ	*Pseudoxanthomonas mexicana* AF273082	++	+
	BB	*Lysobacter yangpyeongensis* DQ191179	+	++
	BC	*Microbacterium suwonense* GQ246683	++	++
	BD	*Sphingopyxis alaskensis* CP000356	1/2	+

Tab. 3.8 *– Results of the PGP traits study for OTUs reporting high As resistance.*

Although Rhizobacteria with Plant Growth Promoting activity were detected in members of all *taxa* identified by enrichment cultures (except *Flavobacteria*) it's interesting to notice how Gamma-

proteobacteria represent the majority of PGPR both for what concerns IAA and siderophores production (Fig. 3.21). This draws the attention on the genera *Pseudomonas* and *Stenotrophomonas* which are, as previously mentioned, important members of the autochthonous bacterial cenosis and reported in literature for PGP activities (Bloemberg and Lugtenberg, 2004; Pages *et al.*, 2008).

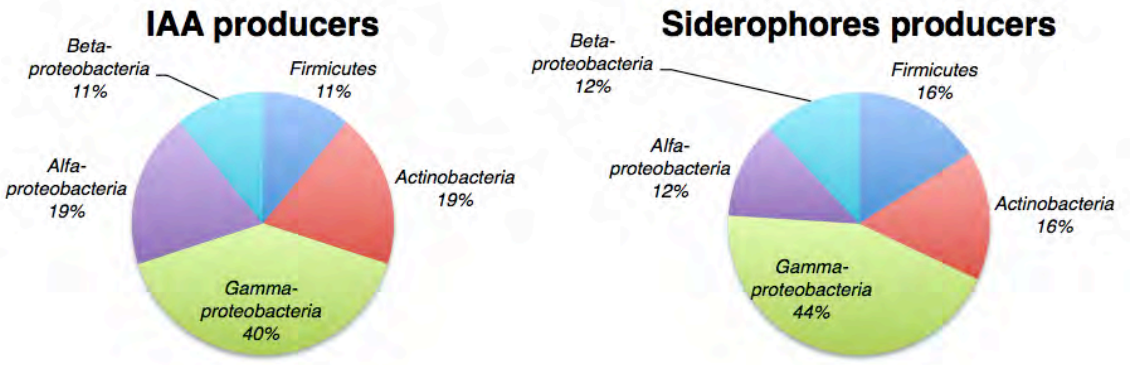


Fig. 3.21 *– Taxa distribution of tested OTUs positive to IAA and siderophores tests.*

3.7.3 ACC deaminase test

Finally the ACC deaminase activity was determined by comparing the strain growth in DF salts minimal medium, DF added with Ammonium Sulfate and DF with ACC as sole nitrogen source (Par 2.3.4.1). Monitoring the strain growth in DF supplied with ammonium sulfate as a positive control allowed to check for cells viability, and the absence of growth in the negative control DF with no N source allowed to verify the ability of the strain to utilize ACC as a source on nitrogen and of not being a diazotrophic strain (Fig 3.22).

In this case 11 strains were tested and the only positive results is represented by *Achromobacter marplatensis* OTU P, a Beta-proteobacteria isolated from the rhizosphere which is known for its high arsenic resistance and this peculiar PGPR characteristic (Cavalca *et al.*, 2010). Moreover in a recent example of bacterially assisted phytoremediation of nickel-contaminated soils, a strain of *Achromobacter* sp. increased root and shoot length, fresh and dry weight of *Brassica juncea* thanks to its activity ACC deaminase activity, IAA production and phosphorus solubilization (Ma *et al.*, 2009).

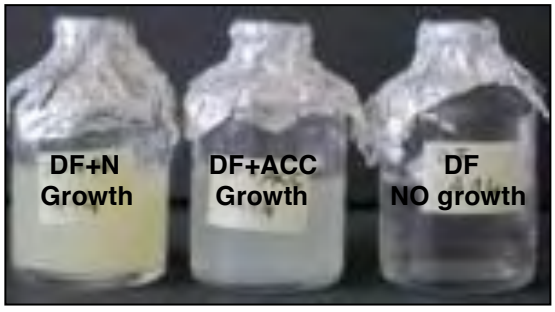


Fig. 3.22 *– Positive ACC deaminase test.*

CHAPTER 3: Microbe-enhanced phytoextraction experiments

3.8 1st phytoextraction experiment

3.8.1 Selection of a consortium of autochthonous strains for a phytoremediation study in a bioaugmentation protocol

The work of screening described in Chapter 2 led to the 3rd part of the work: the identification of possible candidates to be used in a microbe-enhanced phytoextraction experiment. The study has been performed in a bioaugmentation protocol with components selected within the autochthonous community in analysis.

In stressed environments, rhizosphere bacteria with PGP characteristics could in fact play an important role in plant growth. However to be able to actually exert their promoting effect, strains reporting PGP traits must be rhizospheric competent, able to survive and colonize in the rhizospheric soil (Ahmad *et al.,* 2008). Their tolerance to the concentration of toxic metal is in fact reported to be the most important limiting factor for the application of PGPR, limiting their efficient use to slight and moderately contaminated sites (Wu *et al*., 2006). Selection of strains characterized by both PGP characteristics and metal resistances is therefore interesting in the perspective of a phytoremediation approach, increasing biomass production and therefore remediation efficiency through their addiction in bioaugmentation protocols. Selecting microorganisms that are both metal-resistant and able to produce plant growth-promoting compounds could in fact prove useful as *inocula* in phytoremediation processes (Cavalca *et al*., 2010). Moreover not only PGPR are known to be able to promote plant growth in heavy metal contaminated soils, but in addition a variety of bacteria (mainly PGPR) such as *Pseudomonas* spp. and *Delftia* sp, are able to act as phytoextraction assistant promoting the phytoextraction process (Koo and Kyung-Suk, 2009; Rajkumar *et al*., 2012).

On the basis of the study previously performed (Chapter 2), OTUs reporting heavy metals resistances and PGPR traits were chosen among the isolated components of the culturable indigenous micro flora to be used in an experiment of bioaugmentation with the fern *Pteris vittata*. Therefore a first consortium of bacterial isolates was composed as reported in Tab. 3.9. The selected strains are not only of particular interest as characterized by PGP traits, in the mean time they are well adapted to this particular soil and contamination, from which they have been isolated. These isolates are all characterized by high resistance to both the toxic species As(V) and As(III), by the presence of the *ars* operon, involved in As solubilization, and by the presence of different PGP traits.

Taxonomic Reference	MIC		As genes	PGP traits	As(V) reduction
	As(V) mM	As(III) mM			
Ochrobactrum cytisi OTU E	>100	5	*ars* operon	IAA	-
Pseudomonas putida OTU N	>100	25	*ars* operon	IAA Siderophores	+
Achromobacter marplatensis OTU P	>100	25	*ars* operon	ACC deaminase	-

Tab. 3.9 *– List of As resistant isolates with interesting Plant Growth Promoting traits selected for the first bioaugmentation experiment.*

3.8.2 Experimental setup

Cultivation experiments were carried out in 6 kg pots in a temperature-controlled glasshouse (24/28 ˚C) for six months. Plants were grown on unpolluted soil (control) and on contaminated soil collected at the Scarlino area spiked with compost (30%) in which an average As concentration was of 270 mg/kg. For each soil, four different trials were set up in triplicate (Fig. 3.23):

n.i. - non inoculated, and plants inoculated with:

A - *Achromobacter marplatensis* (ACC deaminase producer),

B - *Ochrobactrum cytisi* + *Pseudomonas putida* strain (IAA-producers),

C - *inoculum* A + B.

The experiment lasted six months and the different *inocula* were applied every 2 months at a final concentration of 10^8 CFU/g of soil.

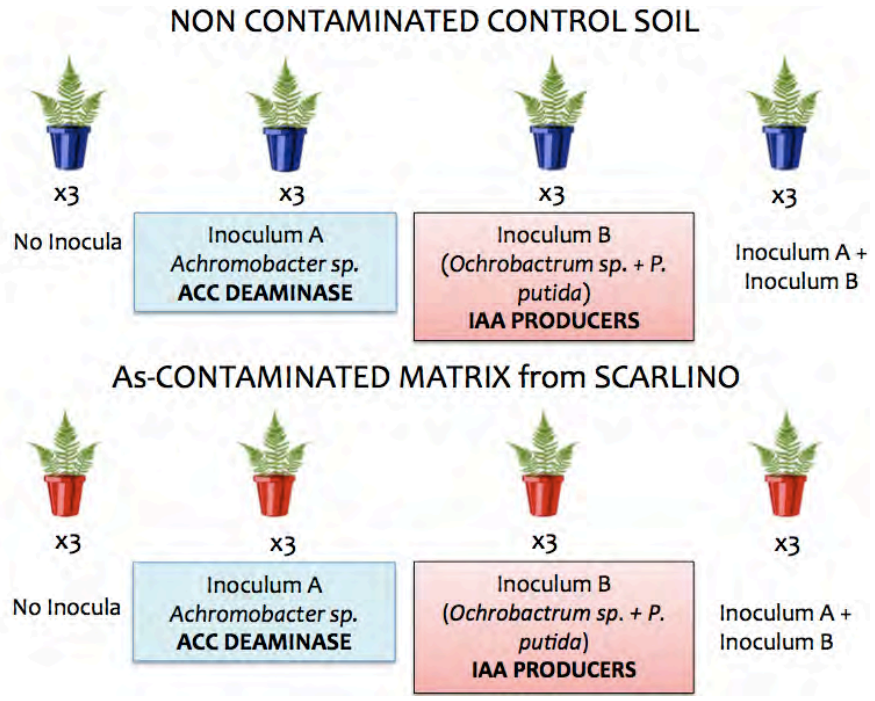


Fig. 3.23 *– Experimental set up for the 1st phytoextraction trial in lab scale.*

3.8.3 Evaluation of the phytoextraction process

P. vittata established successfully in the experimental plots, and showed no obvious plant toxicity symptoms during the 6-month experimental period (Fig. 3.24).

At the end of the trial, the plants were harvested and some parameters in relation to the phytoextraction process were measured.

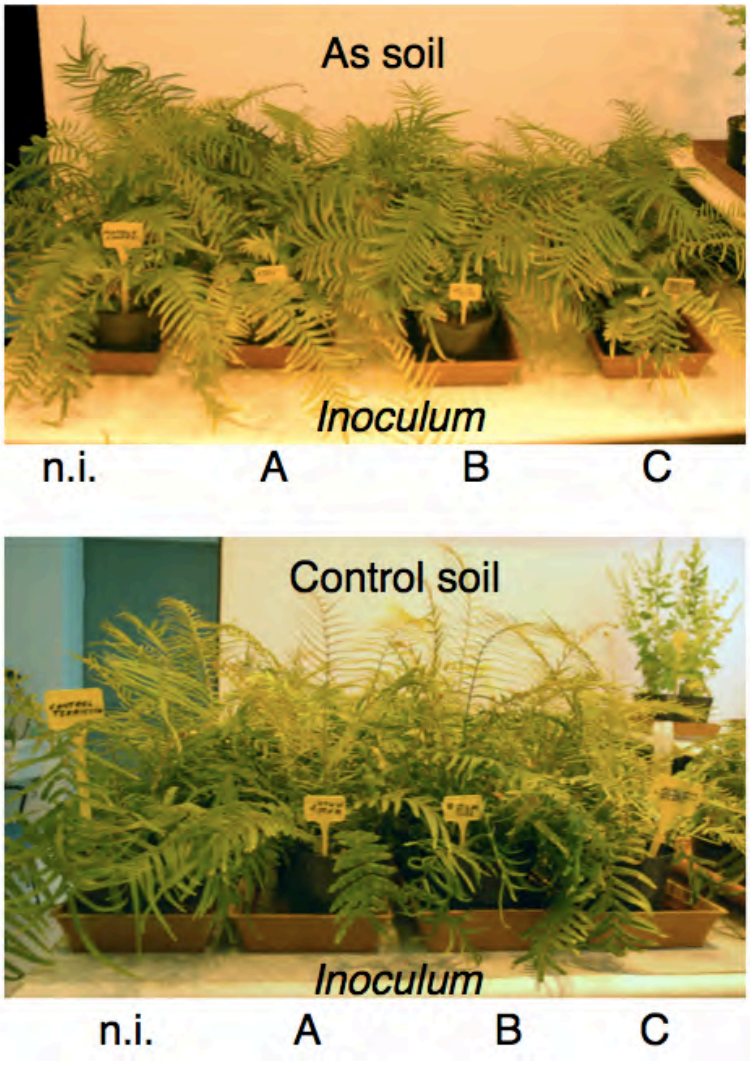


Fig. 3.24 *– Mesocosms of P. vittata at the end of the phytoextraction trial.*

Biomass production

As far as the biomass production is concerned the first evident result is how all the plants grown on As-contaminate soils from Scarlino have higher values even without any kind of *inocula* (Fig. 3.25 and Fig. 3.26). This founding is curios but not surprising, also other authors indeed observed that plant biomass increase after exposure to As and hypothesized that As induce an increase in P uptake *P. vittata* (Ghosh *et al.*, 2011). In a hydroponic experiment, Luongo and Ma (2005) observed that plant biomass increase after exposure to 1 mg/l As for 2 weeks was associated with a substantial increase in P uptake by *P. vittata*. Compared to the control, P concentrations increased from 2.33 to 5.19 g/kg in the fronds, and from 0.91 to 5.76 g/kg in the roots (Luongo

and Ma, 2005) and similar results were obtained by Tu *et al*. (2004).

The most interesting observation was the evident plant growth promotion effect exerted by the bacterial strains. Biomass production was clearly increased in all inoculated plants and in particular in plants inoculated with *Pseudomonas* and *Ochrobactrum* (IAA producers) and *Pseudomonas, Ochrobactrum* and *Achromobacter* C, possessing ACC deaminase enzyme. This increase was totally expected since bacterial IAA can affect plant cell division, extension, and differentiation, can increase the rate of xylem and root development, control processes of vegetative growth, initiates lateral and adventitious root formation, biosynthesis of various metabolites, and resistance to stressful conditions (Spaepen and Vanderleyden, 2011; Tsavkelova *et al*., 2006). Overall, bacterial IAA increases root surface area and length, and thereby provides the plant has greater access to soil nutrients. In addition, bacterial IAA loosens plant cell walls and as a result facilitates an increasing amount of root exudation that provides additional nutrients to support the growth of rhizosphere bacteria (Patten and Glick, 2002; Xie *et al*., 1996). On the other hand many recent studies have revealed that plants inoculated with rhizosphere bacteria containing ACC deaminase were better able to thrive in metal polluted soils (Rodriguez *et al*., 2008; Madhaiyan *et al*., 2007). Zhang *et al*. (2011) have also confirmed that Pb-resistant and ACC deaminase-producing endophytic bacteria conferred metal tolerance onto plants by lowering the synthesis of metal-induced stress ethylene and promoted the growth of rape.

Moreover since biomass is proportional to the total amount of pollutant accumulation, biomass increase is thus a positive effect of addition of microbes on phytoremediation efficiency (Chen *et al.* 2002).

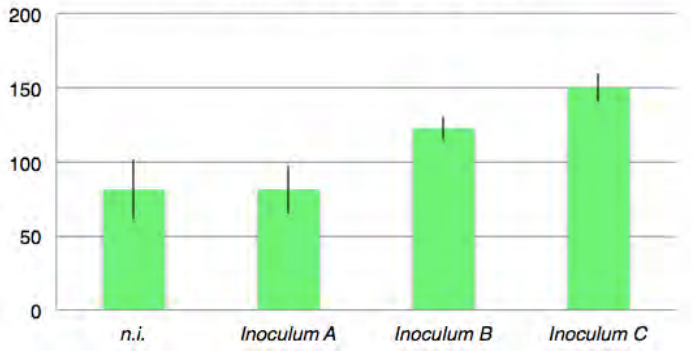


Fig. 3.25 *– Determination and plants biomass (dry weight) of ferns grown on control soils.*

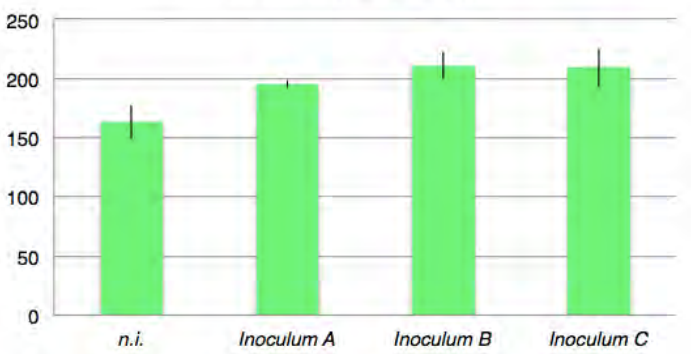


Fig. 3.26 *– Determination and plants biomass (dry weight) of ferns grown on As polluted soils.*

Arsenic content in the ferns' fronds

The arsenic content has been determined in the ferns' fronds harvested at the end of the experiment by ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry). As illustrated in Fig. 3.27 and 3.28 the brake ferns extracted arsenic efficiently from the soils into the fronds: plants growing in the contaminated soil contained 1200 – 3300 ppm arsenic and those from the uncontaminated control soil contained 7 – 24 ppm. These values are much higher than those typical for plants growing in normal soil, which contain less than 3.6 ppm of arsenic (Kabata-Pendias and Pendias, 1991). Measured concentrations actually are not highest reported for the fern object of study, since it's been found that *P. vittata* can accumulate up to 23.000 ppm of As in the areal tissues (Ma *et al.*, 2001) but it must be also said that such high concentrations were accumulated in ferns' fronds grown on a soil containing about 1600 ppm of As while in this case As concentration in the soil used in the experiment were attested around 270 ppm. Moreover the greenhouse conditions do not reproduce exactly the ideal environment in which the fern usually grows.

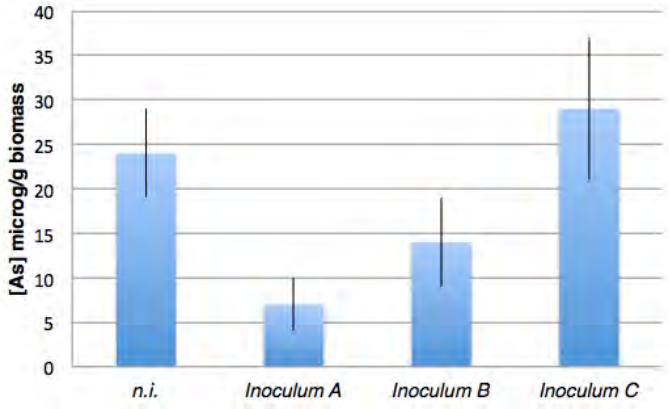


Fig. 3.27 *– As concentration in the ferns' grown on control soils.*

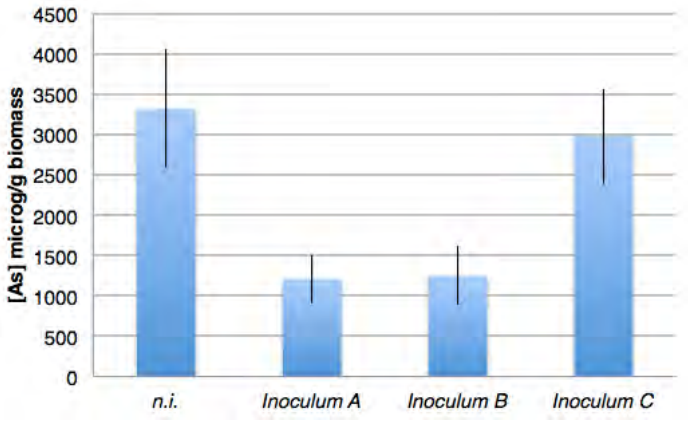


Fig. 3.28 *– As concentration in the ferns' grown on As polluted soils.*

It can also be observed how the not inoculated plants accumulated higher concentration of the metalloid in their aerial parts in comparison with the inoculated plants. In substance, the bacterial *inocula* decreased the As concentrations in the plants' fronds. The contrary was expected since different studies demonstrated the increase in As (and other toxic metals) uptake in ferns

inoculated with PGP bacteria (Yang *et al*., 2012; Kumar *et al*., 2008; Rajkumar and Freitas, 2008). The underlying mechanism for the observed phenomenon is not certain but some evaluations can be assumed. A possible explanation of these data is that the *inocula* exerted a sort of detoxification effect in the rhizosphere, for instance oxidating As(III) to As(V) that is less toxic but also less mobile and this resulted in a phytostabilization of As in the soils. A possible interpretation is that the EPS (the production of which has been reported to be stimulated in the presence of toxic substances, including heavy metals (Sheng *et al*., 2005)) produced by the inoculated cells were involved in metal ion chelation, thus rendering them unavailable to plants (Joshi and Juwarkar, 2009). However there is no direct evidence to claim these hypotheses.

Along with the previous considerations it has also to be mentioned that in Scarlino industrial area, metal contamination deals with other metals such as Pb, Fe, Zn and Cd, besides As (Par. 1.4) and the efficiency of arsenic accumulation by the fern depends also on the concentration of other metals in the soil (Fayiga *et al*., 2004; An *et al*., 2005). In the study of Fayiga *et al*. (2004), arsenic uptake decreased with increasing metal concentration for each metal, except in lead-treated soils. Though effective in taking up arsenic, *P. vittata* had limited capability to take up other metals. Caille *et al*. (2004) also reported that *P. vittata* grew poorly in a soil heavily contaminated with As, Cu, Pb, and Zn, probably as a result of Zn and Cu toxicity. Phytotoxicity of other heavy metals not only decreased plant growth, but also arsenic uptake, resulting in a negligible phytoextraction of As from the soil.

Thus contamination by multiple metals or metalloids, particularly at high concentrations, presents a difficult challenge for phytoremediation.

Evaluation of the phytoextraction efficiency

The Phytoextraction Efficiency (PE) of a system is defined as “mg of metal accumulated in plant’s tissue x dried biomass” and it gives a measure of the total amount of the contaminant extracted by the plants under exam. PE was calculated at the end of the experiment to have an idea of the amount of As concentrated into the harvestable part of the plant – only [As] in the fronds was indeed measured.

As far as phytoextraction efficiency is concerned, obviously negligible values were reached for plants grown in control soil (Fig. 3.29) while ferns grown on As polluted soil extracted the contaminant with a good efficiency (Fig. 3.30) in particular in the case of plants without bacterial *inocula* and plants amended with *inoculum* C. As earlier described *inocula* A and B exerted positive effects on plant biomass, nevertheless, the total phytoextraction efficiency was not to ameliorated by the inoculation of these PGPR, probably resulting in a phytostabilization of metal contaminated soils. This founding is not unprecedented, as applications of bacterial strains decreased Ni(II) and Cd(II) sequestration in tomato shoots and roots conferring protective effect in the presence of heavy metals (Madhaiyan *et al*., 2007) while the inoculation of a strain of *P. putida* decreased lead and cadmium content in *Phaseolus vulgaris* in another phytoextraction experiment (Tripathi *et al*., 2005). However these results pointed out the positive effect of the indigenous micro flora selected by and adapted to the As contamination in the Nuova Solmine

area.

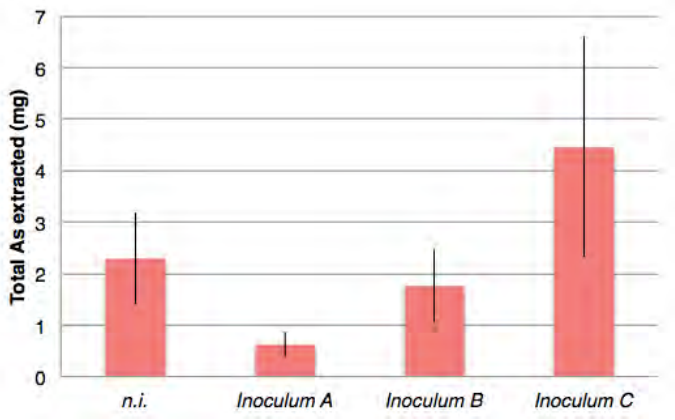


Fig. 3.29 *– Determination of total As accumulated in the fronds of the ferns grown on control soils.*

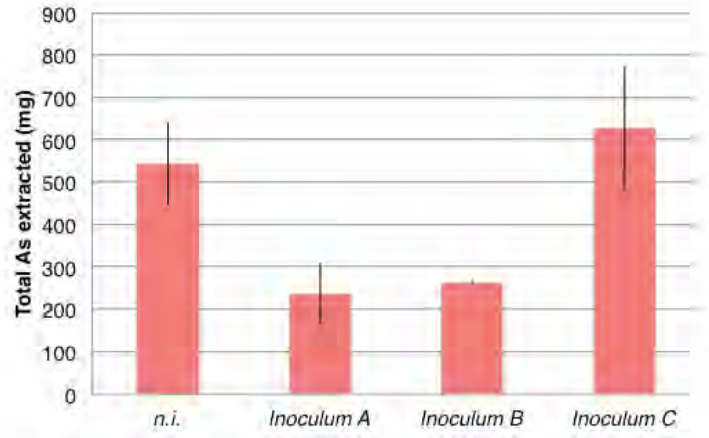


Fig. 3.30 *– Determination of total As accumulated in the fronds of the ferns.*

The Bioconcentration Factor (BF) that is the concentration of As in tissues divided by that in soil, defined as C_{TISSUE}/C_{SOIL}, measures the plant ability to bioconcentrate the toxic element into its tissues. BF is an interesting parameter to consider as it measures the plant capability to bioconcentrate the toxic element into its tissues, taking also in account the concentration level within the contaminated matrix. As summarized in Tab. 3.10, BF has particularly low values in ferns grown on control soil and, even if the values are a bit higher, they are still quite low for the examined vegetal specie under study. In earlier pot studies, *P. vittata* has been reported to possess BF generally >10 (Ma *et al.*, 2001; Tu *et al.*, 2002; Zhao *et al.* 2002; Xie *et al.* 2009), where soluble form of As was added to experimental soils and equilibrated (aged) for a relatively short period of time. Wei *et al.* (2006) reported that ageing of As in soil at an Au-mineralization site reduced its plant (fern) availability and decreased the mean BF value for P. vittata to 0.9.
Anyway it has to be considered that these values can be misleading, as they take into account total arsenic content in soil, whereas only a low part is mobile and bioavailable.

	n.i.	*Inoculum* A	*Inoculum* B	*Inoculum* C
P. vittata in control soil	0.022	0.054	0.021	0.069
P. vittata in As soil	0.109	0.273	0.116	0.346

Tab. 3.10 *– Bioconcentration Factor (BF) values for mesocosms of P. vittata at the final sampling.*

Moreover the translocation factor (TF), which refers to the concentration of heavy metal in shoots divided by that in roots – defined as C_{SHOOTS}/C_{ROOTS} – gives a quantification of the plant capability of translocating As taken up from roots to the harvestable aerial part. This particular criterion, with a desirable value higher than 1, is especially important in phytoextraction as a higher shoot/root ratio of heavy metal content in plant is important in practical phytoremediation of heavy metal-contaminated soils (Karami and Shamsuddin, 2010). Unfortunately, as only shoot As concentration data were available and TF could not be determined.

Finally As concentration in soils at the beginning of the trial and at the final time in the different mesocosms was measured. As it's clearly visible from Fig. 3.31 all the plants – with or without bacterial *inocula* – were able to efficiently lower As concentration in soils. Remarkably inoculated plants extracted a sufficient amount of As to remediate the polluted soil in exam and in some cases to bring the value under the guideline established by the low of 50 mg/kg (for industrial area). Plants amended with PGPR abated As concentration in soil even more effectively than uninoculated plants (seven times for *inoculum* A, three times for *inoculum* B, and four times for *inoculum* C).

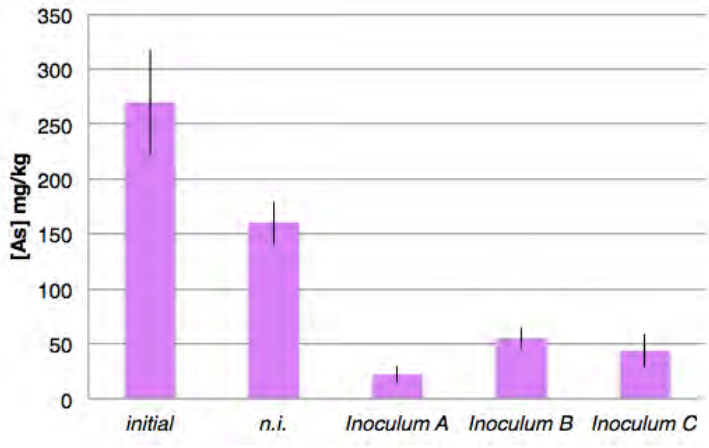


Fig. 3.31 *– As concentrations in soil at the beginning of the trial and at the final time in the different mesocosms.*

Since only data concerning As concentration in shoots were available, it can only be hypothesized that a non-negligible amount of the metalloid was accumulated also in fern roots or, although unusual, may have been methylated and volatilized by soil bacteria (Tsai *et al.*, 2009).

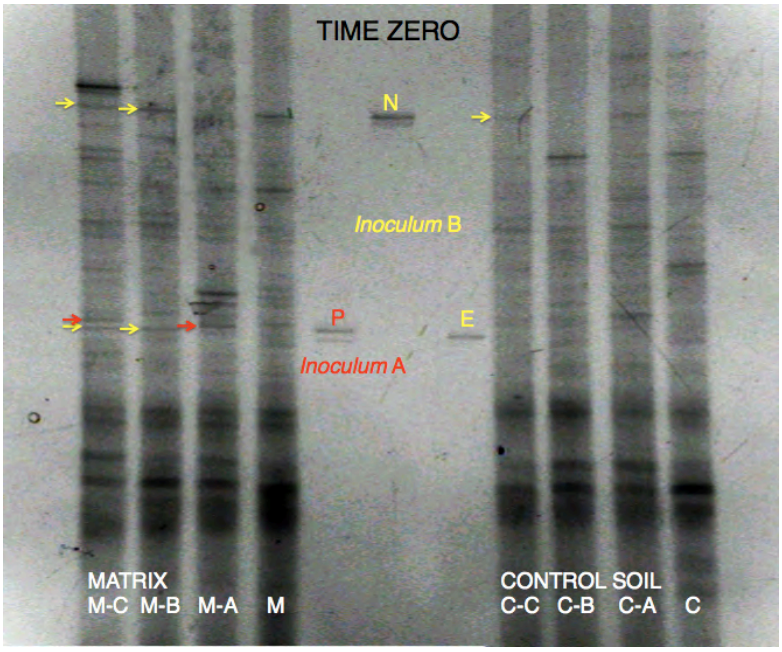
Conversely non inoculated ferns probably didn't accumulate high concentrations of arsenic in the roots as residual As concentration in rhizosphere of n.i. plants is higher that in the those of inoculated plants.

Nevertheless these data pointed out the positive influence exerted particularly by soil autochthonous bacterial strains on both plant growth and phytoremediation efficiency.

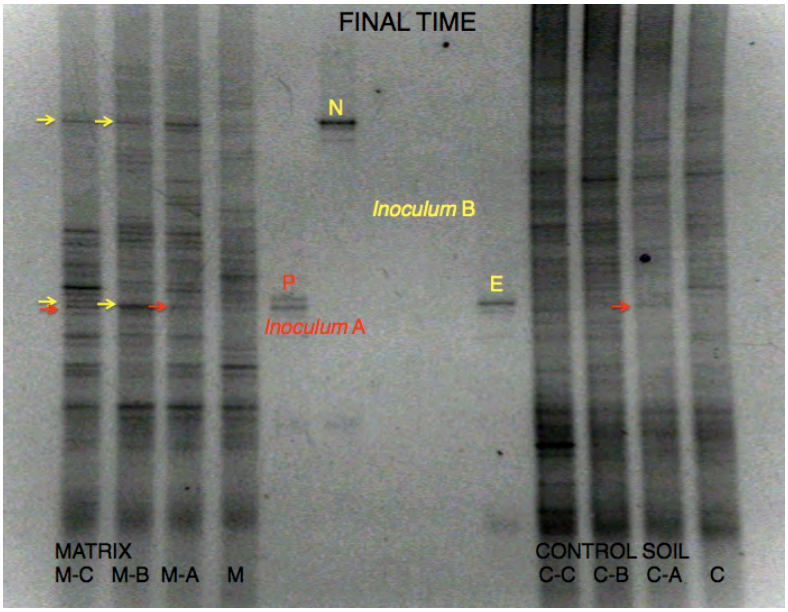
In conclusion this integrated system of plants and bacteria would be perfected as a reliable remediation tool to be applied in the Scarlino industrial area.

3.8.4 Monitoring microbial inocula

Bacterial *inocula* were applied to fern rhizosphere at a final concentration of 10^8 CFU/g of soil. During the experiment the presence and the persistence of inoculated strains were assessed in the different pots by means of PCR-DGGE analyses. Fig. 3.32 and 3.33 show the analysis performed on the rhizosphere soil at the beginning of the trial and at the final timing, indicating with yellow and red arrows the bands corresponding to the inoculated bacteria. All the inoculated bacterial species were retrieved from As contaminated pots. Conversely, some bands were missing in the controls. This might be explained by assuming that the inoculated strains autochthonous of the contaminated matrix (arsenopyrite ash), had probably difficulty in adapting to the control soil. Besides, since the bacterial communities in the samples were very complex, probably not all the bands were clearly visible on gels. This analysis however confirmed the presence of *Achromobacter marplatensis, Ochrobactrum cytisi*, and *Pseudomonas putida* in the rhizosphere of ferns grown on As contaminated soil, therefore confirming that the PGP effects observed on plants were associated with the bacterial *inocula*.



3.32 *– DGGE analysis performed on the rhizosphere soil at the beginning of the experiment. Blue and red arrows indicate bacterial inocula.*



3.33 *– DGGE analysis performed on the rhizosphere soil at the end of the experiment. Blue and red arrows indicate bacterial inocula.*

3.9 2nd phytoextraction experiment

3.9.1 Selection of a consortium of autochthonous strains for a phytoremediation study in a bioaugmentation protocol

More recently, a second group of bacterial strains to be used in a different experiment of bioaugmentation in presence of plant species *Pteris vittata* was selected (Tab. 3.11).
Like in the 1st trial, strains were selected on the basis of the study previously performed (Chapter 2). The selected strains presented different PGP traits (noteworthy all the isolates were able to produce siderophores) and in the mean time are characterized by high MIC values (especially for As(V)), by the presence of the *ars* operon, and two strains able to reduce As(V) *in vitro*.
Considered the results of the 1st phytoextraction experiment, in this selection particular attention was paid to siderophores production and As(V) reduction since they are the main mechanisms of As solubilization in soils and therefore should have a key role in its uptake by the hyperaccumulator ferns (Carrillo-Castañeda *et al.*, 2003; Macur *et al.*, 2001).

Taxonomic reference	MIC		As genes	PGP traits	As(V) reduction
	As(V) mM	As(III) mM			
Bacillus thuringiensis OTU M	>100	11	*ars* operon *aox* operon	IAA Siderophores	+/-
Pseudomonas putida OTU N	>100	25	*ars* operon	IAA Siderophores	+

Delftia lacustris OTU U	>100	10	*ars* operon	IAA Siderophores	+
Variovorax paradoxus OTU AP	>100	5	*ars* operon	Siderophores	-
Pseudoxanthomonas mexicana OTU AS	>100	5	*ars* operon	Siderophores	-

Tab. 3.11 *– List of As resistant isolates with interesting Plant Growth Promoting traits selected for the second bioaugmentation experiment.*

Analogously to the previous trial, cultivation experiments have being carried out in 1 kg pots in a temperature-controlled glasshouse (24/28 ˚C). Ferns were planted on unpolluted soil (control) and on contaminated soil collected at the Scarlino area spiked with compost (30%) in which an average As concentration was of 270 mg/kg. For each soil, four different trials were set up in quadruplicate (Fig. 3.34):

n.i. - non inoculated, and plants inoculated with:

A - *Pseudomonas putida* and *Delftia lacustris* (IAA producers and able to reduce As(V) to As(III) *in vitro*),

B - *Bacillus thuringiensis*, *Variovorax paradoxus* and *Pseudoxanthomonas mexicana* (siderophore producers),

C - *inoculum* A + B.

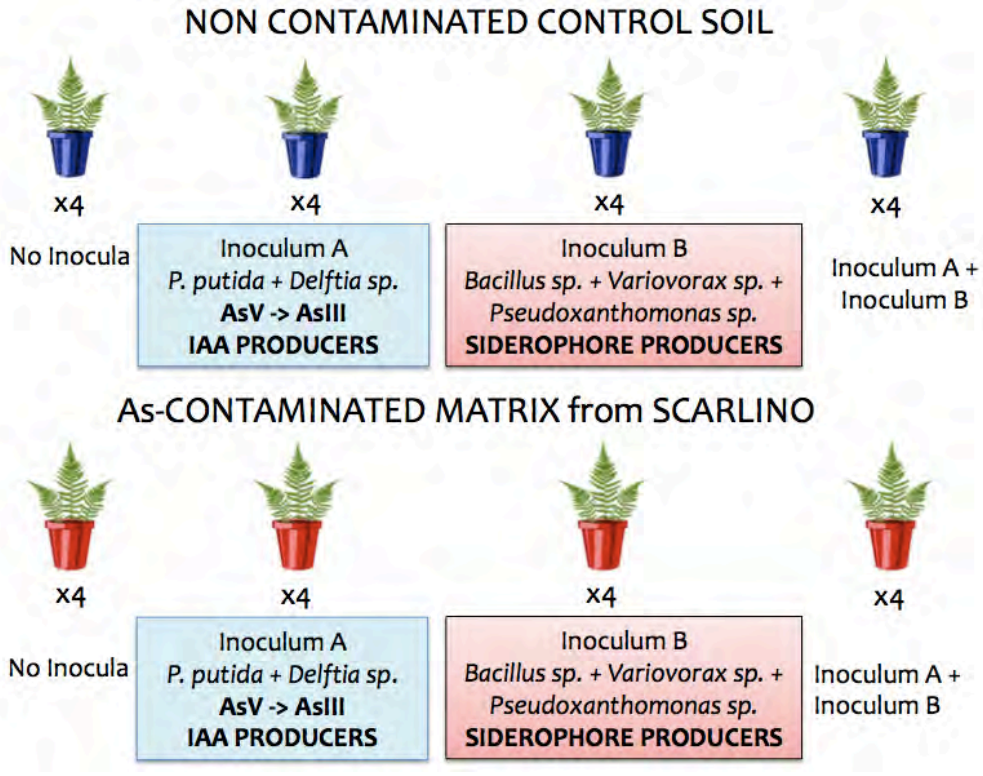


Fig. 3.34 *– Experimental set up for the 2nd phytoextraction trial in lab scale.*

A duration of 4 months and repeated *inocula* every 6 weeks were programmed. As the analyses are still in progress no data on this experiment are reported in this document.

These mesocosms experiments will allow to establish the efficiency of the selected strains to stimulate phytoextraction in perspective of the test in open field.

4. Concluding remarks

The present PhD deals with a real case of arsenic and heavy metal (Pb, Fe, Zn and Cd) contamination of an industrial site located in Scarlino (Tuscany, Italy). The contamination in this context has been caused for 40 years by roasted arseno-pyrite dumping as a consequence of sulfuric acid production at Nuova Solmine Chemicals industrial facilities plant. Natural arsenic background along with ash disposal has thus provoked a serious soil contamination and pollution of aquifers within the whole district where an average concentration of 140 mg/kg As can be found.

In this site physical-chemical remediation technologies can be effective and applicable even at the highest contaminant concentrations, but they are much expensive and invasive - disrupting both soil structure and biological activity – and are not applicable for extensive areas (Kirpichtchikova *et al.*, 2006). On the other hand bioremediation – which is the use of microorganisms and/or plant able to degrade, remove or detoxify the contaminant – offers an interesting alternative or complement to conventional technologies. In particular the phytoextraction approach enhanced by microorganisms – based on the use of plant in synergy with microorganisms – offers a low cost *in situ* applicable method to remediate and restore perturbed areas (McGuinness and Dowling, 2009; Shukla *et al.*, 2010).

The first aim of PhD research was to determine the structure of the autochthonous bacterial community established in a soil exposed for 50 years to inorganic arsenic at Nuova Solmine industrial site. The study was performed by culture-dependent techniques and examined distinct sampling points with different contamination levels chosen in the contaminated area (a sample from the ashes pile and 5 samples from the rhizosphere of 5 different autochthonous plants living in the area). The results obtained by the enrichment cultures analysis indicated the selection within the area of a tolerant soil bacterial community, characterized by a heterogenic structure and – for a perturbed soil – rich biodiversity. Actually almost all the isolated strains showed high homologies with bacteria diffused within different environmental niches including contaminated soils, capable of degrading recalcitrant pollutants and reporting high resistances to heavy metals (belonging to *Firmicutes*, *Actinobacteria.* Alfa-, Beta- Gamma-proteobacteria and *Flavobacteria*) (Cavalca *et al.*, 2010). Moreover new information about the extreme environment of the arsenopyrite ashes dumping site was gained by PCR-DGGE analysis. The molecular analysis performed further indicated the selection of dominant members within the autochthonous bacterial community exerted by the long-term contamination. The fact that *Firmicutes* and *Actinobacteria phyla* were highly represented it is indeed a novel founding.

The molecular analysis performed – along with the MIC determination – evidenced high arsenic resistance within the autochthonous microbial community with strains reporting genes for multiple determinants involved in arsenic transformation pathways. This research provided indeed valuable information of genes responsible for arsenate reduction and arsenite oxidation and resistance to the toxic oxides As(III) and As(V).

Afterwards 32 strains with the highest MIC values and possessing important genetic determinants for As transformation (i.e. the *ars* operon) were further tested in relation to Plant Growth Promoting traits (IAA and siderophores production and ACC deaminase activity). Actually tolerance to the concentration of heavy metal is in fact reported to be the most important limiting factor for the application of Plant Growth Promoting Rhizobacteria (PGPR), limiting their efficient use to slight and moderately contaminated sites (Wu *et al*., 2006). 90% of examined OTUs resulted positive for at least one potential PGP trait, suggesting a synergistic potential role in a phytoremediation perspective for the community components and the soil bacterial cenoses in exam.

Hence As-resistant isolates showing PGP traits and in some cases the ability to solubilize arsenic were chosen as *inocula* for a bioaugmentation experiment in presence of the hyperaccumulator fern *P. vittata*.
Three strains – namely *Ochrobactrum cytisi* OTU E, *Pseudomonas putida* OTU N and *Achromobacter marplatensis* OTU P – were chosen for the first phytoextraction trial and inoculated in three different ways: i) *inoculum* A: only strain P, ii) *inoculum* B: strains E + O, and iii) *inoculum* C: strains P + E + O. Plant biomass production and total As content in plant tissues were measured. Of particular interest was the promotion of plant growth elicited by the bacterial inoculants. In fact, all *inocula* exerted positive effects on plant biomass, with an increase up to roughly 20% when *inoculum* B (*Pseudomonas* + *Ochrobactrum*, IAA producers) and the *inoculum* C (*Pseudomonas* + *Ochrobactrum* + *Achromobacter*, IAA and ACC deaminase producers) were applied. On the other hand, uninoculated plants accumulated higher concentration of As in their epigeous portion in comparison with plants that had been treated with *inoculum* A and *inoculum* B. Thus, it might be hypothesized that the bacterial *inocula* used to treat *P. vittata* rhizosphere in this study lowered As uptake due to a potential role in the phytostabilization of the metalloid in the contaminated soil. However, by considering both the biomass production and the metalloid uptake, the *inoculum* C evidenced an appreciable enhancement of the phytoextraction process.

On the basis of the results obtained and discussed in this PhD thesis, in the context of the performed characterization of the soil autochthonous bacterial community it is therefore possible to make the following main considerations:

- The high arsenic and heavy metals contamination present in the Nuova Solmine industrial area has exerted a selection on the soil autochthonous bacterial cenosis towards a more tolerant and well adapted community, with a high biodiversity, resistance and degrading potential;
- The majority of strains examined within the area at the highest pollutant concentrations (M, the arsenopyrite dumping site) belonged to Gram positive *Firmicutes* and *Actinobacteria*, including strains possessing interesting genotypic traits involved in As transformation;

- Among the community members isolated in pure culture, strains belonging mostly to Gamma-proteobacteria reported particular PGP traits in a phytoremediation perspective with a bioaugmentation protocol;
- Isolates belonging to *Delftia lacustris*, *Pseudomonas putida* species showed the ability to reduce As(V) *in vitro* with high efficiency showing a potential ability to solubilize arsenic in soils;
- *Ochrobactrum cytisi* OTU E, *Pseudomonas putida* OTU N and *Achromobacter marplatensis* OTU P were selected and tested for a microbe-enhanced phytoextraction experiment in association with the hyperaccumulator fern *P. vittata*. They exerted positive effects on plant biomass and on the total phytoextraction efficiency, thus indicating that the integrated system of plants and bacteria would be a valid remediation tool to use on the experimental site of Scarlino.

The results of a second phytoextraction trial with another selection of As-resistant PGP strain (*P. putida, Delftia lacustris, B. thuringiensis, Variovorax paradoxus, Pseudoxanthomonas mexicana*) are attended. When completed, these preliminary estimations will allow to evaluate the phytoextraction potential of the bacterial strains under study in lab scale and to program a future trial a pilot scale in Scarlino contaminate area.

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